Purification and Characterisation of Amylolytic Enzymes from *Lipomyces starkeyi*

JYOTI PANDYA

A thesis submitted in partial fulfilment of the requirements of the University of Greenwich for the Degree of Doctor of Philosophy

April 2002

Acknowledgements

I would like to thank my supervisors Dr. Ivor Evans and Dr. Bob Yon for their knowledge, advice and guidance throughout the course of this study.

I would also like to thank GlaxoSmithKline for all the assistance and sponsorship I have received, and especially my friends and colleagues in Biopharmaceutical Product Development, Beckenham, for all their advice and encouragement.

I would also like to thank Dr. Jon Armstrong, Prof. Babur Chowdhry and the staff at the School of Chemical and Life Sciences, University of Greenwich, for their continued support.

Finally, but most importantly, I would like to thank my Parents, who have inspired and supported me throughout my education. And an especial thank you to my husband, Andrew Jolly, for all his assistance, encouragement, and patience. I couldn't have completed this project, without his staunch support. Thank you for everything.

Abstract

A purification scheme has been developed for the extracellular carbohydrases; α-glucosidase and α-amylase, secreted by the ascosporogenous soil yeast *Lipomyces starkeyi* NCYC 1436.

Growth and optimum enzyme yield conditions were determined with cultures of *L. starkeyi* grown at 30°C on a medium containing 2% soluble starch, 1% yeast extract and 1% Bactopeptone, appropriate enzyme assay procedures having been devised.

Both enzymes were initially precipitated from the cell free supernatant by an 85%(w/v) ammonium sulphate precipitation. Many different chromatographic media were then assessed, but the choice of a hydrophobic (Phenyl Sepharose CL-4B) column had the advantage of utilizing the ammonium sulphate precipitate with minimum sample preparation. The pellet was adjusted to 1M salt and adsorbed to the hydrophobic column. Elution of the two activities was carried out by a series of decreasing salt washes. Although fractionation of the two enzymes was not complete, the column was effective in eliminating a substantial quantity of inactive material, whilst maintaining good recoveries of approximately 59% for α-glucosidase and 72% for α-amylase.

Both enzymes were substantially purified using ion exchange chromatography (Q-Sepharose fast flow medium), with α-glucosidase being apparently close to electrophoretic homogeneity; elution of the two enzyme activities was carried out at 4°C, using a linear gradient with sodium acetate buffer, pH 5.0.

Individual HIC fractions purified by ion exchange showed between a 0.3 to 24.6-fold increase in purity for α-glucosidase, and between a 2.8 to 7.6 increase in purity for α-amylase. However, by selecting the most active α-glucosidase fraction isolated after running the 0.01M HIC fraction on ion exchange, the purification factor rose to 83.4.

Several properties of the purified α-glucosidase enzyme were investigated. The molecular weight of the α-glucosidase was determined by electrophoresis under denaturing and non-denaturing conditions, after ion exchange chromatography. A single major band was detected by SDS-PAGE with an estimated molecular weight of 93,000 ± 5,000 Daltons, and under native conditions, the molecular weight was estimated at 162,000 Daltons. Specific enzyme activity staining of the native gel confirmed that the single band was an α-glucosidase. However, isoelectric focusing of the purified *Lipomyces starkeyi* α-glucosidase, detected three bands - within the pI

range of 4.6-5.0. Further characterisation studies revealed the pH and temperature optimum of the purified *L. starkeyi* α-glucosidase at 4.5 and 55°C respectively.

*K*m and *V*max measurements using a range of substrates suggested that the α-glucosidase had a broad substrate specificity but showed a marked preference for substrates with α-1,4 linkages such as soluble starch, PNPG, and α-1,3 linkages, such as nigerose.

SDS and native gel electrophoresis (combined with specific activity staining) revealed 3 distinct α-amylase activities, and the molecular weights were determined as 82,000, 68,000 and 48,500 Daltons.

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Chapter 1: Introduction

1.1 Structure of Starch

Starch is a major, renewable biological resource, derived naturally from the plant kingdom, and is the primary storage polysaccharide in key crop species like potatoes, maize, sorghum, rice, wheat and barley. Thus, as well as being an essential energy source for many organisms, it is the principal source of metabolic energy in the human diet and the foundation of substantial food, drink and other industries: it is therefore of great economic importance.

Starch essentially consists of two high molecular weight polysaccharides, amylopectin and amylose (Figures 1.1a and 1.1b). Both components have different properties with regard to water solubility and iodine-binding capacities. Amylopectin is a highly branched polysaccharide, with linear chains of α-1,4 linked glucose residues crosslinked by α-1,6 glycosidic bonds. Amylopectin is structurally complex, with a bimodal distribution of chain lengths - peaking around 40-60 or 11-25 D-glucosyl residues (Wang *et al.*, 1993). The degree of branching within amylopectin and the ratio of amylopectin to amylose are dependent on the source of starch and age of starch concerned (Kennedy, 1988).

Amylose is the minor component, constituting 15-20% (w/v) - depending on its source. It is a flexible, unbranched polysaccharide composed of α-1,4 linked glucose residues, with an average chain length of 500-1000 glucose units. In its native state, amylose has a helical conformation that gives rise to the characteristic blue colouration of the amylose-iodine complexes, (Shannon and Garwood, 1984; Zobel, 1988; Yu *et al.*, 1996).

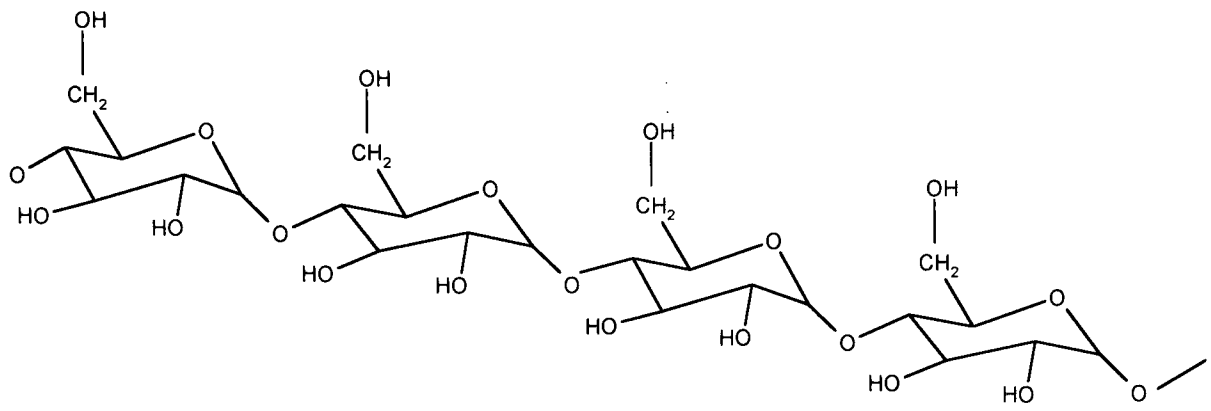


Figure 1.1a: Linear molecule of amylose

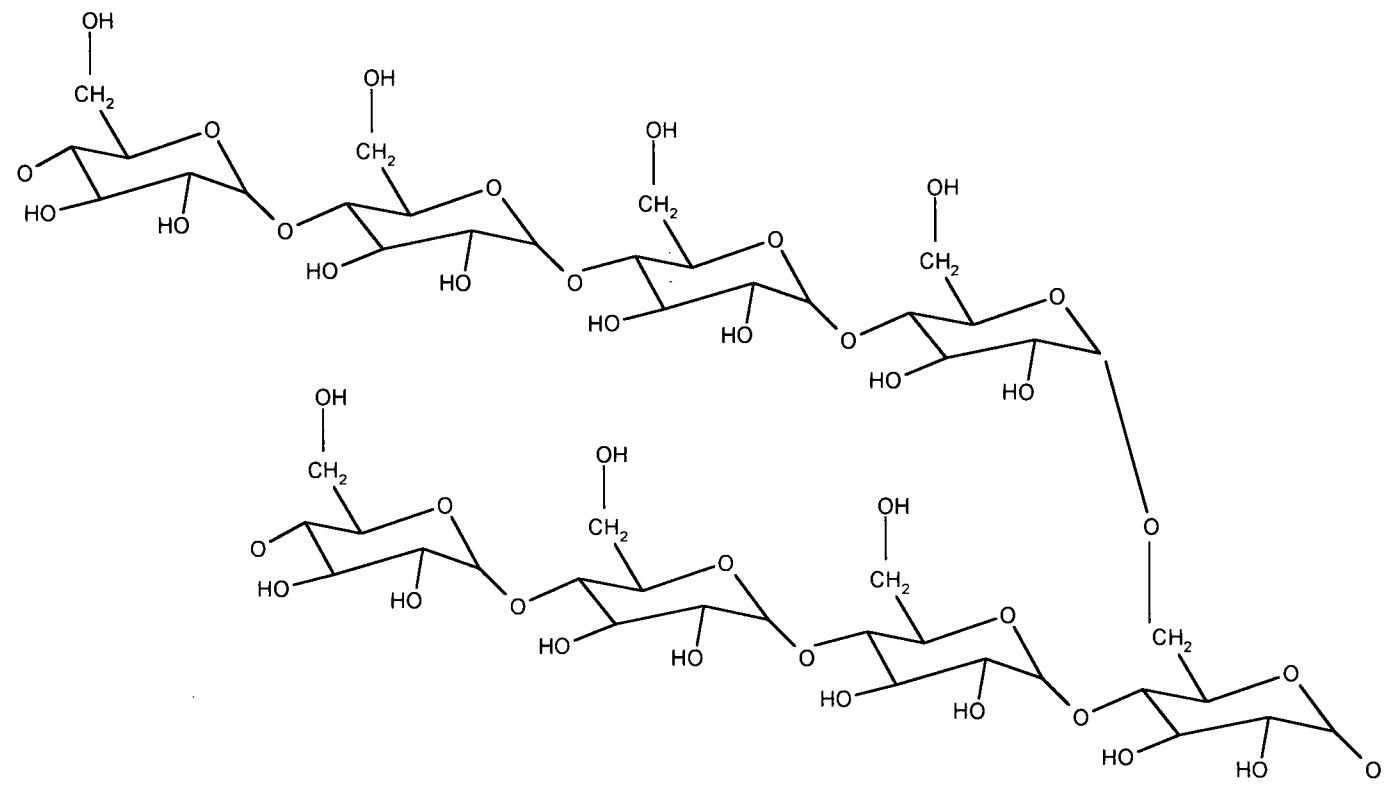


Figure 1.1b:Branched molecule of amylopectin

Starches have been found to contain a number of mineral components such as Ca^{2+}, K^{+}, Mg^{2+} and Zn^{2+} as well as bound phosphate ions. Recent methylation and hydrolysis analyses of waxy maize and waxy rice starches have discovered significant proportions of D-glucose-6-phosphate. Further analyses showed there to be an average of one mole of phosphate to six glucose residues in amylopectin in these starches (Ellis *et al.*, 1998).

Lipid material has also been found in certain starches, mainly due to the inclusion of the lipid molecules within the amylose helical structure. The presence of these non-

carbohydrate compounds influences starch quality and affects certain physical parameters such as swelling power and viscosity. Starch flavour, taste and smell also vary, depending on the fatty acid and protein content (Visser and Jacobsen, 1993).

All starch granules (Figure 1.2) contain detectable amounts of protein. The protein can be classified as either surface protein (readily extracted at temperatures below gelatinisation) or as integral protein (extractable at temperatures near or above the gelatinisation temperature). The amount and type of protein present can affect new uses of starch or its application to existing processes (Ellis *et al.*, 1998).

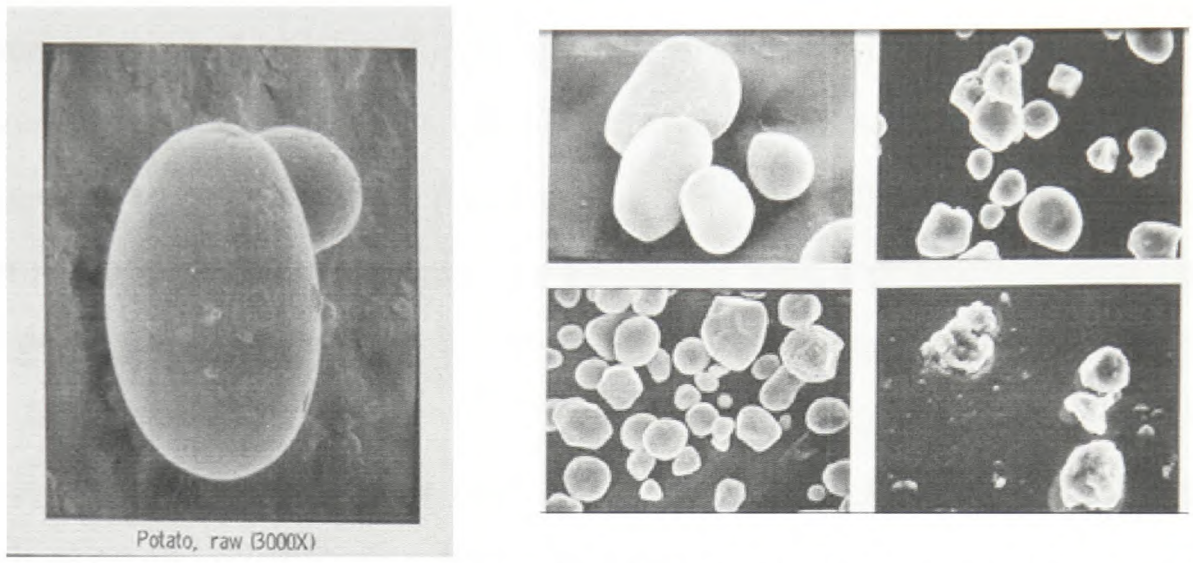


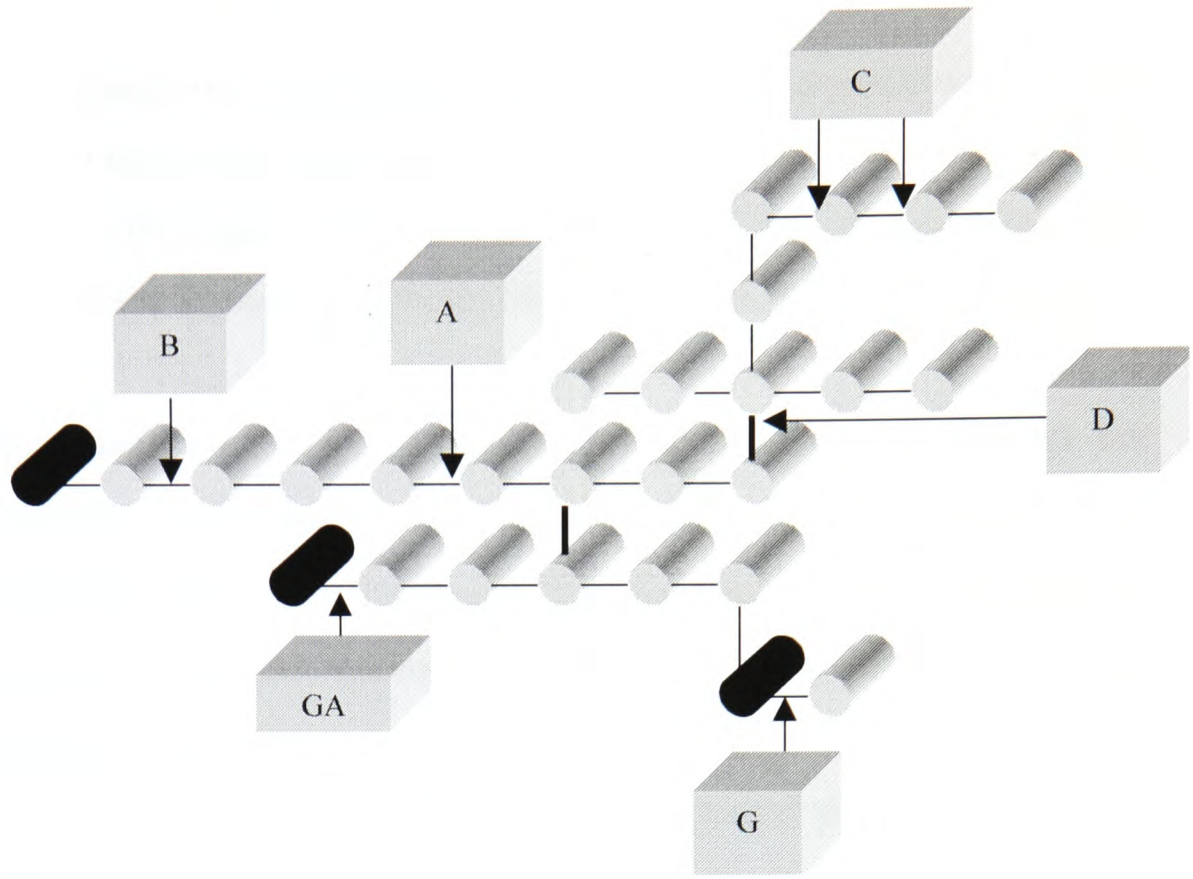
Figure 1.2: **Electron microscope photographs of different starch granules**
(Kossmann and Lloyd (2000). The potato starch granule (magnification x3000) appears to be large and lentical, whereas starch granules from various other sources, such as maize, cassava, and wheat, vary in size, shape and are typically species-specific.

1.2 Starch-Degrading Enzymes

The major starch-degrading enzymes have been classified by a number of different criteria, including their original source, substrate specificity, structure, and type of reaction catalysed. However, a generally accepted classification divides the enzymes involved in starch hydrolysis into four main groups (Tubb, 1986). Figure 1.3 illustrates the enzymic hydrolysis of starch.

Group one - which includes the endo-acting enzymes and group two, which includes exo-acting enzymes, act primarily on α-1,4 linkages in starch. Endo-enzymes act on bonds in the inner regions of the substrate, and the products of hydrolysis are oligosaccharides of varying chain length and have the α-configuration on the C_1 of the reducing glucose unit produced. Exo-enzymes act on the same substrates as endo-enzymes, but some enzymes can also cleave α-1, 6 bonds – though at a slower rate compared to α-1, 4 bonds. Exo-enzymes act externally on substrate bonds from the non-reducing end and produce low molecular weight products. Group three includes the debranching enzymes such as pullulanase and isoamylase, which act exclusively on α-1,6 linkages. The final group of starch-hydrolysing enzymes is composed of the cyclodextrin glycosyltransferases. These enzymes degrade starch by catalysing cyclisation and disproportionation reactions.

For the most part, the enzymes of interest to the starch hydrolysis industry are hydrolases such as α-amylases, glucoamylases and α-glucosidases derived from yeast, bacteria, fungi and plants (Guzman-Maldonado and Paredes-Lopez, 1995).



Key: : D-glucosyl residue, —— : α-1, 4-linkage, —— : α-1,6 linkage, : non-reducing end of chain, G: α-Glucosidase, A: α-Amylase, GA: Glucoamylase, B: β-Amylase, C: Cyclodextrin-forming enzymes, D: debranching enzymes (pullulanase and isoamylase)

Figure 1.3: Action of starch-hydrolysing enzymes

1.2.1 α-Glucosidases

Glycosides are sugar-containing compounds, which on hydrolysis yield one or more sugars. Glycosides contain two components in their molecule, a glycone and an aglycone. The glycone is the sugar component i.e. glucose and the aglycone is the non-sugar component. When the glycone is glucose, the glycoside is known as a glucoside.

α-Glucosidase, the systematic name of α-D-glucosidase glucohydrolase, catalyses the hydrolysis of terminal non-reducing, 1,4-linked α-D-glucose residues from oligosaccharides, with the release of α-D glucose. Various types of α-glucosidases are produced by a number of different microorganisms and possess different substrate specificities depending on their source.

α-Glucosidases have potential applications in the sugar industry; they can be used in conjunction with other carbohydrases such as α-amylases and glucoamylases, to effect the complete hydrolysis of starch. Used co-operatively with glucoamylase, α-glucosidase can assist in the industrial hydrolysis of maltodextrins and maltose, obtained by prior enzymatic hydrolysis by α-amylases, to produce high dextrose syrups or crystalline D-glucose. Again, if used in conjunction with glucoamylase, α-glucosidase could be used to resolve the problem of the reformation of glucosidic linkages that occurs when dextrose is produced in high concentrations, (reformation of linkages is catalysed by the enzyme - the reverse reaction of hydrolysis). This enzyme combination could also provide more efficient production of high glucose syrups. α-Glucosidases also form part of a group of important glycosidases with applications in the synthesis of novel oligosaccharides.

According to Phillips (1959), α-glucosidases possess a broad aglycone specificity, and yeast α-glucosidases are quite distinct in many properties from bacterial and mould enzymes; α-glucosidases can be classified into three main types:

1. Typical α-glucosidases, which hydrolyse heterogeneous substrates, e.g. phenyl α-glucoside and sucrose, more rapidly than maltose. Yeast α-glucosidases are typically found in this group.

2. The so-called maltases, which show a high specificity toward homogeneous substrates such as maltoligosaccharides, with little or no activity towards synthetic α-glucosides such as phenyl α-D-glucopyranoside, or to sucrose. True maltases possess the ability to transfer the α-D-glucosyl residue of maltose and α-D-glucosides, to suitable acceptors (Nisizara and Hashimoto, 1970). Typical bacterial and mould α-glucosidases fall into this category.

3. α-Glucosidases that possess glucoamylase-like activity and are capable of attacking glycogen and soluble starch in addition to the substrates attacked by enzymes in group 2. α-Glucosidases found in mammalian tissues are typically classified in this group. However, some yeast α-glucosidases from some *Candida species* (Chiba *et al.* 1973b), are also classed here.

α-Glucosidase's mechanism of action is based on its specificity towards the glycone-glucosidic residue i.e. the α-D-glucopyranosyl radical. Gottshalk (1950) defined the enzyme as one which hydrolyses the α-glucosidic linkage in oligosaccharides and α-D-glucosides, being highly specific for the glucose residue, but less so for the aglycone portion of the substrate.

α-Glucosidases possess at least two functional regions, a binding site and a catalytic site (Koshland, 1959). The binding site selects the carbohydrates that will be hydrolysed, (the specificity being determined by the aglycone component in the substrate). The catalytic site of the enzyme determines the nature of the glycone-aglycone linkage that can be hydrolysed.

A number of α-glucosidases have been previously characterised, and the physicochemical properties of some yeast, bacterial, and fungal α-glucosidases are presented in Table 1.1.

Table 1.1: Physicochemical Properties of some α-Glucosidases

Organism	Molecular weight	pH	Temperature optimum	Reference
Yeast α-glucosidases				
Lipomyces starkeyi CBS 1809	35,000	4.5	60	Kelly *et al.*, 1985
Torulaspora pretoriensis YK-1	69,000	6.8	35	Oda *et al.*, 1993
Saccharomyces carlsbergensis	63,000	6.7-6.8		Needleman *et al.*, 1978
S. cerevisiae	53,000			Legler and Lotz, 1973
S. cerevisiae 1412-4 D: I. Maltase II. α-Methyl glucosidase (isomaltose)	 68,500 +/ -1200 64,700+/- 2870	 7.0 - 7.5 7.0 - 7.5		Khan and Eaton, 1967
S. cerevisiae: Type 1 Type 2 Type 3		 6.3 - 7.1 6.3 - 7.1 6.3 - 7.1	 42 42 36	Matsusaka *et al.*, 1977
S. italicus Y1225	85,000 +/- 30000	6.6 - 6.8		Halvorson and Ellias, 1958
S. logos	270,000	4.6 – 5	40	Chiba *et al.*, 1973
Schizosaccharomyces pombe		4.0 - 4.4	45	Chiba and Shimomura, 1965
Bacterial α-glucosidase				
Bacillus brevis	52,000	6.5	48 - 50	McWethy and Hartman, 1979
Fungal α-glucosidase				
Aspergillus flavus	63,000	6	35	Olutiola, 1981.
Aspergillus niger	131,000		40	Brizova, *et al.*, 1992

1.2.2 α-Amylases

α-Amylases are widely distributed in bacteria, fungi, animals and certain plants. They are endo-acting enzymes capable of breaking down internal α-1,4 glycosidic bonds and giving rise to a variety of oligosaccharides of varying chain length, including maltose, maltotriose and α-dextrins. A characteristic of all α-amylase products is that the C_1 of the reducing glucose unit is in the α-D-configuration (the hydroxyl group of the anomeric carbon atom has the axial or α-position). These enzymes cannot break

down α-1,6 glycosidic bonds, but are capable of by-passing them to leave a percentage of unhydrolysed dextrins.

The ability of α-amylase to by-pass α-1,6 linked branch points allows the enzyme to release additional α-1,4-linked regions for subsequent glucoamylase attack. Glucoamylase appears to hydrolyse these additional α-1,4 regions faster than it does untreated α-1,6 linked material, a synergistic mechanism exploited in industry for the rapid production of glucose syrups (Schenck and Hebeda, 1992).

α-Amylases have diverse roles in industry, although starch hydrolysis with a view to dextrin, sugar and ethanol production are the main industrial uses for amylolytic enzymes.

They have been traditionally extracted from bacterial sources, with species of the gram-positive organism - *Bacillus* as one of the most favoured producers of α-amylase (Fogarty and Kelly, 1983). While α-amylases from some microorganisms, e.g. *Bacillus* and fungal (A*spergillus*) species are now actually used in various brewing and distilling processes, there is recognition that other amylolytic organisms (yeasts), could swell the sources of useful enzymes with specific properties valuable for starch/sugar bioconversions.

Most industrial applications of α-amylases are carried out at high temperatures and a major focus of research into these enzymes is to explore new sources of enzymes with increasingly better thermostability. Screening of new sources for thermostable carbohydrases has been carried out by a number of groups (De Mot and Verachtert, 1987; Spencer-Martins and Van Uden, 1977). Alternatively the thermostability properties of an existing enzyme might be improved by site-directed mutagenesis. Table 1.2 summarises some of the physicochemical properties and molecular weight determinations of various α-amylases activities purified from different species of the genus *Lipomyces.*

Table 1.2: Properties of α-amylases from *Lipomyces* species

Organism and Strain	Molecular weight (KDa)	pH optimum	Temperature optimum (°C)	Reference
L. starkeyi (CBS 1807)		3.5	50	Moulin and Galzy (1979)
L. kononenkoae	38	5.5	40	Spencer-Martins (1979)
L. kononenkoae (CBS 2514)	65	5.6	30	Spencer-Martins (1982)
L. starkeyi (CBS 1809)	76	4.0	70	Kelly *et al.*, (1985)
L. starkeyi (HN-606)	56	-	-	Punpeng *et al.*, (1992)
L. kononenkoae (CBS 5608)	76	4.5-5.0	70	Prieto *et al.*, (1995)
L. kononenkoae (IGC 4052)	-	-	33	Estrela *et al.*, (1982)
L. kononenkoae (IGC 4052B)	76	4	40	Steyn and Pretorius (1995)

1.2.3 Glucoamylases

Glucoamylase (amyloglucosidase, 1,4-α-D-glucan glucohydrolase) is an exo-acting enzyme capable of hydrolysing terminal α-1,4 linkages from the non-reducing ends of starch, dextrins and glycogen by consecutive bond hydrolysis, yielding β-D glucose as the end product. Although the enzyme exhibits highest affinity for α-1,4-linkages it also shows reduced activity on α-1,6 and α-1,3 glycosidic bonds (McCann and Barnett, 1984).

Glucoamylase is one of the most important industrial enzymes and is used in the production of high glucose and fructose syrups (Section 1.3.2). The rate of substrate hydrolysis by glucoamylase is affected by the molecular size and structure, and by the next bond in sequence (Abdullah *et al.*, 1963; Fleming, 1968). Thus the rate of hydrolysis of α-1,4- bonds increases linearly with the relative molecular mass of the substrate at least up to maltopentaose - for some glucoamylases (Yamasaki *et al.*, 1977*a, b*).

1.2.4 β-Amylases

β-Amylases are extracellular exo-acting enzymes producing β-maltose (the hydroxyl group of the anomeric carbon being in the β-position), from the non-reducing ends of starch or other polysaccharides. Amylose is converted almost completely to maltose, whereas amylopectin and other branched polymers are hydrolysed to various extents, depending on the degree of branching of the starch. This hydrolysis leads to about 50-60% conversion to maltose due to inability of β-amylase to hydrolyse α-1,6 linkages (Guzman-Maldonado and Paredes-Lopez, 1995).

The industrial use of β-amylase is in the production of high maltose syrups (80% (w/v) maltose) from barley, soyabean and wheat starches.

1.2.5 Debranching Enzymes

1.2.5.1 Pullulanases

Enzymes capable of effectively hydrolysing α-1,6 branches are of increasing interest to the modern starch industry as the efficiency of the traditional hydrolysis reaction can be obviously improved upon - (glucoamylase can only cleave α-1,6 bonds slowly and α- and β-amylases have no activity on these bonds).

Pullulanase is specific for the substrate, pullulan, which is an extracellular polysaccharide comprised of maltotriose units linked by α-1,6 bonds. On hydrolysis, maltotriose is released. Pullulan is commonly used as a substrate for the detection of enzymes with debranching activity - with the exception of isoamylase, which cannot degrade pullulan (Matsuzaki *et al.,* 1974). Pullulanase also differs from isoamylase, in that it does not degrade glycogen completely.

Pullulanases are produced by a number of microorganisms and are used in the production of maltose and maltose syrups, but only the enzymes produced from *Klebsiella pneumoniae* and *Bacillus cereus* var *mycoides* have been reported to be industrially important (Saha and Zeikus, 1989). Pullulanase is commonly used in conjunction with α-amylases in an industrial method to achieve a rapidly high yield of hydrolysis products from starch. Also, when pullulanase is used simultaneously with glucoamylase during saccharification of starch, then this can reduce the glucoamylase

requirement and also the hydrolysis time. To date, pullulanase has been more widely used than isoamylase due to its wider availability.

1.2.5.2 Isoamylases

Isoamylases are distinguished from pullulanases on the basis of substrate specificity. They do not degrade pullulan, and cannot hydrolyse two or three glucose-unit side-chains of β-limit dextrins and α-limit dextrins. Isoamylase is competitively inhibited by maltotriose and maltotetraose. However, isoamylase is the only enzyme to completely hydrolyse glycogen. At present, commercial isoamylase is derived from only one organism, *Pseudomonas amyloderamosa*.

1.2.6 Cyclodextrin-Forming Enzymes

Cyclodextrins are rings of α-1,4-linked glucopyranose residues. There are two recognised enzymes that produce cyclodextrins, cyclomaltodextrin glucotransferase and cyclodextrin glucanotransferase. Both are exo-acting enzymes which catalyse the conversion of starch to cyclodextrin by intramolecular transglycosylation (cyclisation) i.e. they detach short chains from starch and then link the two ends of each fragment to form a cyclic molecule. Since the enzymes do not detach specific chain lengths, the resulting cyclical dextrins may contain 6-12 glucopyranose residues per ring structure with the most common structure formed possessing, 6, 7 or 8 glucose units. They also catalyse the transfer of saccharide residues from α-1,4 glucans or cyclodextrins to water (hydrolysis) or to a suitable acceptor such as glucose or sucrose (disproportionation). These enzymes occur almost exclusively in bacteria particularly in species of *Bacillus* and *Klebsiella*, where they have been found to have calcium ion requirement to achieve maximum stability (Guzman-Maldonado and Paredes-Lopez, 1995).

Cyclodextrins are used in novel applications in the food, pharmaceutical, and fine chemicals industry due to possessing the ability to form inclusion complexes in aqueous solution in which "guest" molecules of suitable dimensions are included within the cavity of the "host" cyclodextrin without any covalent bonds being formed. Possible uses include the controlled release of biologically active compounds such as medicines or pesticides, and the protection of flavours or fragrances.

1.3 Industrial Applications of Starch

Worldwide, the vast bulk of agricultural starch production is consumed directly, as human or animal foods or feeds, or dry milled into flour for subsequent food use. However, starch is exploited in many other ways, which are of considerable economic significance, and which can be classed as "industrial applications". The oldest of these is the production of alcoholic beverages - in essence, the beer and spirits industry; here the product often depends on the plant tissue itself releasing the starch products (e.g. malting). In another group of industries, the first step in starch exploitation is the efficient release of starch granules from plant tissue, by a process known as "wet-milling"- hence the wet milling industry.

The development of the starch/wet milling industry can be traced back to the late 18th century, when the Napoleonic wars resulted in a continental blockade of goods and merchandise, and sugar became a scarce commodity in Europe. Rewards were offered for alternatives to imported cane sugar (Fullbrook, 1984), and it was Kirchoff, a German chemist, who discovered that starch, boiled with acid and neutralized, yielded sugar. Although this acid treatment process resulted in undesirable reversion products (and the syrup had a relatively high salt content), the results were promising, and research and development continued into the nineteenth century into new ways of using starch.

At present, nearly two thirds of all starch production by wet-milling is used in the manufacture of food and feed products, typically, after hydrolysis by enzymes. Enzymatic starch hydrolysates are used as sweeteners in confectionery and drinks, or as thickeners in semi-solid foods such as sauces, custards, desserts and pie-fillings. Glucose is the most important of the starch hydrolysis products, as it can be further transformed into a whole range of other products, for example, fructose, or via fermentation for biosynthetically produced antibiotics such as penicillin (Sukuki *et al.,* 1977; Kennedy *et al.,* 1988).

Approximately one third of total wet-milled starch is used for non-food purposes, with modified starches utilized in the paper, textile, soap, and laundry, cosmetic and pharmaceutical industries. Starch also has applications in fireproof preparations,

explosives and fuel-binding agents, as flocculating agents for water treatment, and in the building industry (Guilbot *et al.*, 1985; Koch *et al.*, 1988).

An innovative use of starch is as a biodegradable polymeric material, targeted at supplanting the plastic (non-biodegradable) market. An alternative to plastic for single-use, disposable packaging materials such as serviceware (forks, spoons, etc), and also non-food packaging has been a major objective for many of the industrialized nations (Nayak, 1999). In 1991, the US congress approved an initiative mandating exploration of starch-based technologies and potential applications. To successfully compete against other biodegradable polymers such as cellulose to become a thermoplastic material, starch must contain enough water so that it will melt below its decomposition temperature and yield a final product in which the polysaccharide forms a continuous polymeric phase. Novan technology, which has been patented by Warner-Lambert Co (U.S. Patent. 4,026,849, (1997), has been used to produce single-use items from potato starch, (Nayak, 1999).

The sizes of the different sectors of the wet-milled starch industry are indicated in Figure 1.4.

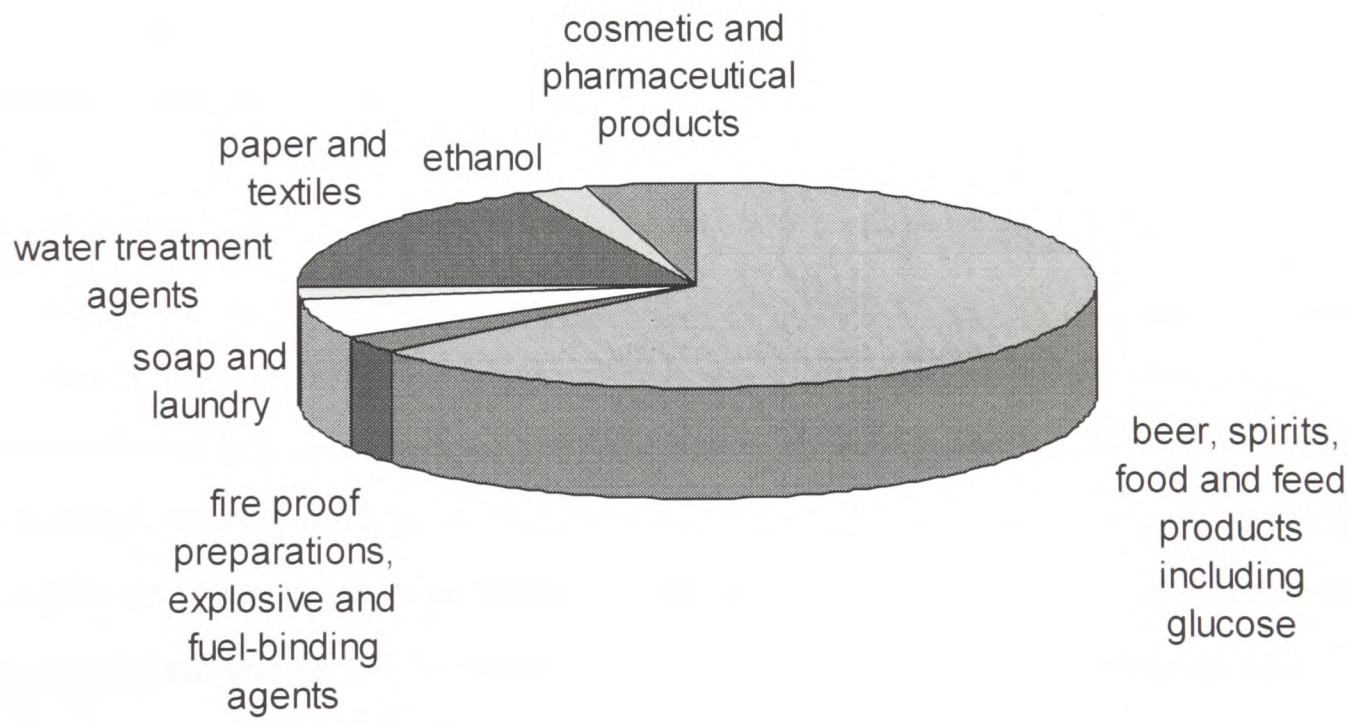


Figure 1.4: Existing Uses of Starch. Adapted from Guzman-Maldonado and Parades-Lopez, (1995).

Other interesting new developments in the starch industry and related areas include "tailor-made" starches, starch as a renewable energy source/biofuel and the synthesis of oligosaccharides using glycosidases.

1.3.1 Beer and Spirit Production

In the traditional brewing process, malted barley is usually the major source of both the starch and amylolytic enzymes, preferred on the basis of its contribution to the final flavour of the product. Malt, however, is an expensive source of starch - twice as expensive as unmalted grain and is therefore used as a minor component in the bulk manufacture of whisky.

The barley malt is allowed to germinate to an extent that ensures sufficient α- and β-amylases are produced for the hydrolytic steps not to be rate-limiting. Distillers' malt is lightly kilned to arrest development but retain amylolytic activity. During the next stage, known as "mashing", where the starch is gelatinised in hot water (65°C), the majority of α-1,4 glycosidic bonds are broken by the action of α- and β-amylases. A debranching pullulanase (limit dextrinase) is also present in the germinating grain, but only a small proportion of it remains after kilning. The majority of α-1,6 linkages survive the brewing process, leaving a high proportion (25%) of unfermentable dextrins not utilized by distilling strains of *S. cerevisiae* (Enevoldsen, 1978; Tubb, 1986).

Adding glucoamylase to cleave all available α-1,6 bonds during the mashing stage or to the ''wort" at the start of fermentation could result in a more complete degradation of starch into fermentable sugar. When used in brewing, this would lead to the production of low carbohydrate beer, since very little oligosaccharide would remain.

Therefore, there would be a considerable number of advantages to using an efficient amylolytic yeast strain capable of producing a combination of enzymes (α-amylase, glucoamylase and/or α-1,6 debranching activity) active during the fermentation stages of the brewing processes. There would be a reduced need to purchase (or produce in a separate process), expensive amylolytic enzymes or barley malt. There would be an automatic simplification of the processing stages, (which precede fermentation), with improved efficiencies of starch conversion, and the possibility of producing amylolytic enzymes - for use in syrup manufacture - as by-products of alcohol fermentations

(Tubb, 1986). However, the addition of hydrolytic enzymes prior to fermentation in brewing beer has not been practiced extensively. Commercial enzyme preparations of, for example, glucoamylase, are not always pure, and protease activity can lead to a distortion in flavour, increased beer foam, and reduced overall product quality.

In fact, enzyme supplementation has been used more widely in the production of neutral spirits i.e. vodka, gin, whisky, industrial spirits and fuel additives, where potatoes, wheat, rye or sorghum are the main starch sources. In neutral spirit production, starch is first gelatinised by cooking, rendering it more susceptible to enzyme attack, and then liquefied (i.e. dispersed into aqueous solution) by the action of microbial α-amylases.

At present, enzymes derived from three *Bacillus* species are highly favoured, due to being very thermostable. *B. subtilis* produces a heat-stable α-amylase, and the α-amylase from *B. licheniformis* has an optimal temperature of 92°C, and produces maltose, maltotriose and maltopentaose. *B. amyloliquefaciens* secretes an α-amylase, with an optimal of 70 °C, its end-product being maltohexaose. All these enzymes are in current use and are useful in speeding-up the initial part of the saccharifying process. To achieve complete saccharification of maltodextrins to glucose, a glucoamylase (again from *Aspergillus niger*), which is capable of cleaving α-1,4 and α-1,6 glycosidic bonds is also added.

Whilst amylases from bacterial (*Bacillus*) and filamentous, fungal (*Aspergillus*) species, are now actually used in various brewing and distilling processes, there is recognition that other amylolytic microorganisms could swell the sources of useful enzymes, with specific properties - valuable for particular starch and sugar bioconversions. Amylolytic yeasts have been especially neglected, and one of these, *Lipomyces starkeyi* (NCYC 1436), is the organism used in this study.

Again, as in the case of beer and whisky production, one possible development would be using an (engineered) amylolytic *S. cerevisiae* strain, which would allow the saccharification and fermentation processes to proceed concurrently, avoiding the additional step of adding-in enzyme.

1.3.2 Wet-Milling and the Production of Syrups

A fundamental factor in the continued expansion and diversification of the starch hydrolysis industry has been the development of efficient methods for isolation and degradation of raw starch.

In its native state, starch exists as granules, in a polycrystalline state, the shape of the granules being characteristic of the origin of the starch; the industrial isolation of starch from these granules is hindered by their insolubility in water and resistance to a variety chemical treatments. Historically, following Kirchoff, starch was hydrolyzed by mineral acid, but this has largely been superseded by gelatinsation by heating, followed by enzymatic hydrolysis. Acid treatments, although effective, were relatively unspecific, producing undesirable by-products and off-flavours, due to the harsh reaction conditions. Gelatinsation by heating in water enhances the chemical reactivity towards liquefying, and subsequently, saccharifying enzymes. In the future, the gelatinisation step, with its high heating costs, may be avoided by using enzymes capable of digesting raw starch (Guzman-Maldonado and Paredes-Lopez, 1995).

It was from the investigative work carried out in 1894, on digestive aids for Asian populations who had difficulty digesting rice (starch), that the chemist Takamine, developed the idea of using enzymes for starch hydrolysis - specifically, an enzyme mixture from *Aspergillus oryzae.* However, the real industrial breakthrough came in the mid- to late -1960s, when the early work that had identified amylases capable of partially hydrolysing starch, was consolidated with the identification of amyloglucosidase (glucoamylase). This enzyme was capable of completely hydrolyzing starch to glucose, either on its own or more effectively, in combination with other amylases.

Enzymatic hydrolysis has the immediate advantage over a chemical route of low energy and pollution costs. Enzymes are not consumed in the reaction, they catalyse the reaction under controlled, moderate conditions of temperature and pH, and are used in minute quantities. Furthermore, they make fewer by-products, and do not contribute to the level of unwanted material that must be removed from the final product (Teague, 1992).

Various combinations of microbial enzymes are now used to produce a wide range of syrups from starch sources as corn, maize and barley; these syrups may be used

directly in food manufacture or as a fermentation feedstock, to make high value products such as antibiotics (as presented in Table 1.3).

Table 1.3: Industrial Applications of Hydrolysed Starch Products (adapted from Kennedy *et al.,* 1988).

TYPE OF SYRUP	DE*	COMPOSITION (%)	PROPERTIES	APPLICATIONS
Glucose syrups	96-98	95-98 D-glucose 1-2 maltose 0.5-2 isomaltose	commercial liquid "dextrose"	soft drinks, baking, brewing and fermentation, raw material
Low DE maltodextrins	15-20	1-20 D-glucose 4-13 maltose 6-22 maltotriose 50-80 higher oligosaccharides	low osmolarity	raw material for enzymic saccharification; clinical feed formulations; glues; thickeners; glues; pastes; fillers
Maltose syrups	40-45	16-20 D-glucose 41-44 maltose 36-43 higher oligomers	high viscosity, reduced crystallization, moderately sweet	confectionery, soft drinks, jams, jellies, sauces, conserve, brewing and fermentation
High maltose syrups	48-55	2-9 D-glucose 48-55 maltose 15-16 maltotriose	increased maltose content	hard confectionery; brewing and fermentation
High DE syrups	56-68	25-35 D-glucose 40-48 maltose	increased sweetness, reduced content of higher sugars, reduced viscosity, increased moisture holding, higher fermentability	confectionery, jam, sauces, conserves, soft drinks, brewing and fermentation
Fructose Syrups	98	48 D-glucose 52 D-fructose	alternative industrial sweeteners to sucrose	soft drinks, sauces, conserves, canned fruits, yoghurts

* dextrose equivalents – (the extent of hydrolysis of starch, expressed as a percentage)

Although some manufacturers still use acid hydrolysis to produce glucose syrups up to 45 DE (dextrose equivalents), for higher DE syrups they use enzymes (glucoamylase and acid stable fungal α-amylase from *Aspergillus niger*) for the controlled saccharification of acid-thinned liquefact, as they are specific, and produce fewer by-products than traditional acid hydrolysis.

To produce high-fructose corn syrups (HFCS), containing between 42-52% fructose, wet millers enzymatically isomerise higher DE glucose syrups. The isomerization of

glucose to fructose is accomplished by passing the hydrolysate stream through a column of immobilized glucose isomerase.

Maltose-containing syrups can be classified into high maltose, extra-high maltose or high-conversion syrups, and are produced by batch saccharification of starch liquefact. α-Amylase from *Aspergillus oryzae,* or a plant-derived β-amylase, is used to produce a 40-45% (40-45 DE) maltose syrup with less than 5% glucose. A debranching enzyme (pullulanase or isoamylase), in conjunction with the amylases, can then be used to produce an extra-high maltose syrup (48-55 DE), typically containing 70-85% maltose. An overview of syrup production from starch is given in Figure 1.5.

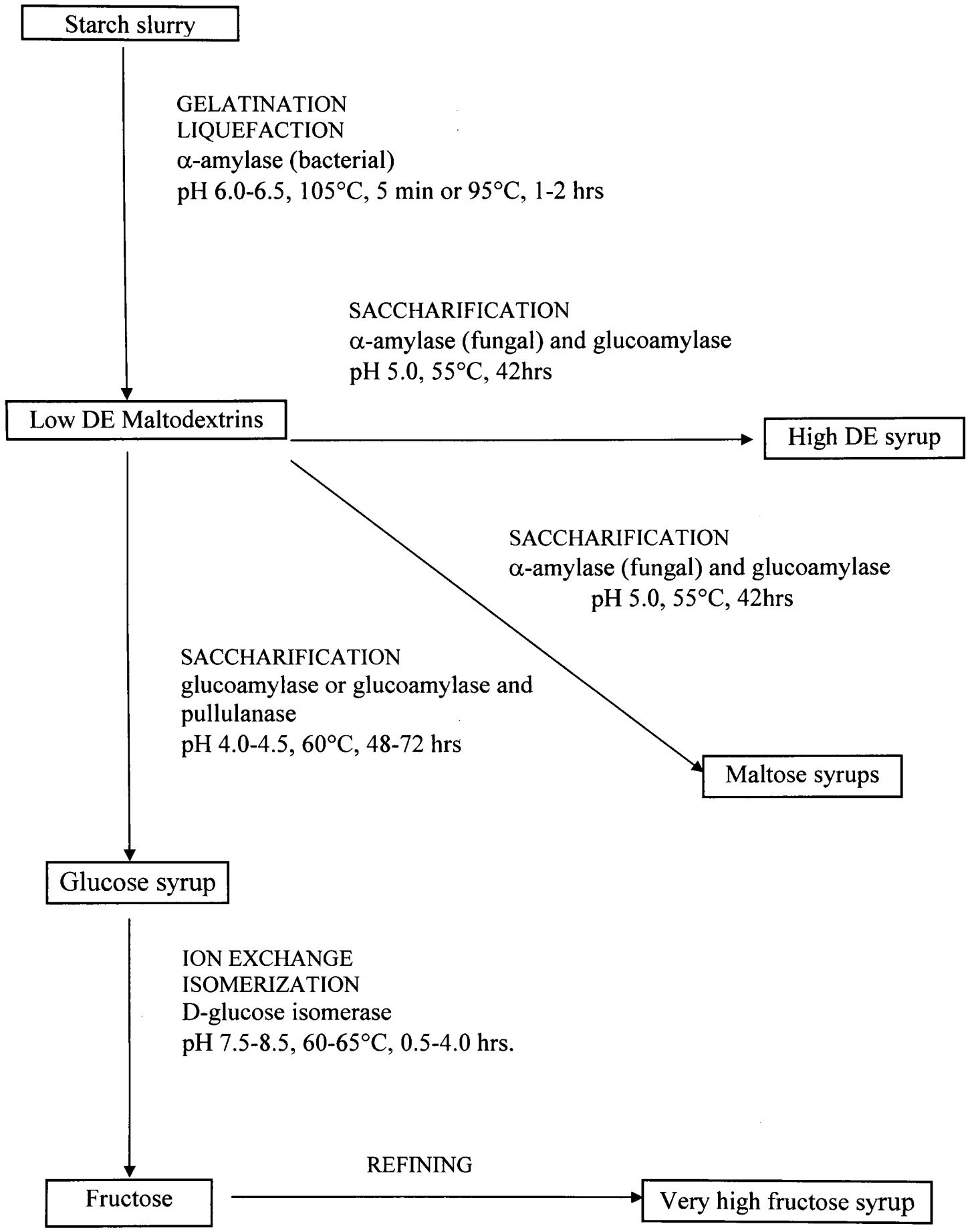


Figure 1.5 Starch processing using enzymes, (adapted from Kennedy *et al.*, 1988).

Recently, American workers Shaw *et al.,* (1999), have engineered an α-amylase from *Bacillus licheniformis* to perform at low pH, thereby effectively increasing the efficiency of the industrial-scale starch liquefaction process, which is currently constrained to pHs above 6.0.

1.3.3 "Tailor-made" Starches

The first efforts to "tailor" starches involved chemical modification - such as cross-linking and etherification – largely to inhibit/prevent "retrogradation" in which the linear amylose molecules associate and crystallise, causing viscosity changes and hence problems in industrial processes. Chemicals used in these treatments are named in Table 1.4, which lists major events in the history of the starch industry.

Table 1.4: Historic Events in the Starch Industry (adapted from Bruinenberg *et al.*, 1995).

Year	Event
1811	Kirchoff: discovery of starch sugars
1867	First clear patent on roasting of starch with acid (USP: 61,991)
1870-1880	Maltose syrups (malt extract)
1909	Discovery of waxy maize containing 100% amylopectin starch
1930-1940	Maltodextrins, glucose syrups and high-DE glucose syrups, (fungal α-amylases)
1896-1950	Chemical derivatisation: Cross-linking using formaldehyde, epichlorohydrin, adipic acid anhydride, sodium trimetaphosphate.
1920-1960	Chemical derivatisation: Etherification using monochloroacetic acid, ethylene oxide, propylene oxide, acrylonitrile, chlorohydroxypropyltrimethylammonium chloride.
1940-1960	Chemical derivatisation. Esterification; phosphoric acid + urea, acetic anhydride, octenylsuccinic anhydride, vinylacetate
1940-1945	Waxy maize to market
1940-1970	Discovery of varieties of maize containing varying amounts of amylose/amylopectin
1965-1970	High fructose with glucose-isomerase
1973	Starch hydrolysis with thermostable bacterial α-amylases
1986	*amf*-mutant of potato, 100% amylopectin
1990	Genetically modified 100% amylopectin potato

It can be seen that the chemical processing of starch can involve using a number of toxic and possibly carcinogenic chemicals. Starch modification procedures are typically carried out at high pH, and the reaction mixture is then neutralised with HCl. Large volumes of NaCl are found present in the effluent (Visser, 1993): industrially, the process is environmentally unfriendly, relying heavily on water, and producing a huge amount of polluted wastewater.

Avoidance of such chemical procedures is evidently appealing. In the case of the starch-hydrolysing and ethanol-producing industries, possible approaches include the use of enzymes active on raw starch and amylolytic yeasts. An alternative is to use genetic strategies to produce crop plants that contain little or no amylose in their starch: genetic "tailoring" of starch.

Waxy maize is a naturally occurring variant with 100% amylopectin starch; the starch is commercially available, requires little chemical modification, and has many industrial applications (Bruninenberg *et al.*, 1995). The starch from waxy maize resembles that of tapioca in many properties (e.g. gelatinization, clear pasting characteristics) and when, during the Second World War, tapioca was unobtainable from the Far East, waxy maize was used as a substitute. By genetically altering or tailor-making the starches of other crop plants, such as wheat, potato or cassava, the potential exists to not only reduce post-harvest modifications and reduce water pollution, but also endow the starch with novel properties for corresponding new commercial applications.

Much of the current research on genetic tailoring of starch has been carried out on the potato, a well-characterized crop plant with established genetic transformation techniques.

The potato starch industry has extended from the traditional areas of food bioprocessing to other technical applications such as paper, textiles and adhesives (Kraak, 1993; Koch and Roper, 1993). Potato starch is the preferred starch in the paper industry, due to its low protein and lipid content. However, potato starch used in the manufacturing process of coating paper must be pretreated - stabilized by chemical modification to reduce viscosity. An immediate advantage of using genetically modified potato starch with a high amylopectin content (which is less viscous and more stable), would be to reduce expensive and laborious chemical pre-treatments.

Progress has been made to produce potato plants with low amylose (0-23%), starch using techniques such as mutation induction, introducing heterologous genes, or inhibiting expression of endogenous genes by the antisense approach (Visser, 1993).

For example, an amylose-free potato mutant (*amf*) isolated in 1986 (Hovenkamp-Hermelink *et al.*, 1987) has been used in breeding programmes and the first commercially useful mutant varieties containing 100% amylopectin were available in 1997.

A transgenic approach to the generation of novel starches enables the specific modification of a single character in a high-yielding disease resistant variety to be carried out. For example, Calgene, a US based organisation, has produced a potato with a high degree of branching in the amylopectin fraction by introducing a glycogen synthase gene from *E.coli*. This did reduce the total starch content to half, but other manipulations carried out at Monsanto (Stark *et al.*, 1992) successfully increased the amount of starch in a consumer variety of potato by introducing a bacterial ADP-glucose pyrophosphorylase gene. Thus, restoration of normal starch levels in Calgene's recombinant potato should be possible.

Another genetic engineering approach is antisense technology; this introduces a gene which is transcribed to yield antisense RNA complementary to the target mRNA, so inhibiting translation of the target mRNA. An antisense gene acts as a dominant suppressor gene, regardless of ploidy level and the number of genes present, and it may therefore be possible to create a new commercial variety after only five years of breeding (Visser and Jacobsen, 1993). This approach has been used to suppress granule-bound starch-synthase (GBSS), a key enzyme in the formation of amylose. The inhibition of the GBSS gene has led to the production to amylose-free starch to market, and importantly without any apparent effect on the yield or dry matter of the starch (Visser *et al.*, 1991).

Table 1.5 summarises some of the current recombinant DNA approaches being explored for the modification of potato starch.

Table 1.5: Potential strategies for obtaining modified natural starches (reproduced from Visser, 1993).

Goal	Proposed strategy
Block and/or reduce starch synthesis	Antisense RNA to AGPase (ADP-glucose pyrophosphorylase)
Increase amount of starch and number of granules	Overexpressing (heterologous) AGPase
Increase amount of starch	In relation to AGPase activity change inorganic phosphate/ PGA (3-phospho-glycerate) affinities, decrease the amount of inorganic phosphate
Alteration of amylose: amylopectin ratio, depletion of amylose from starch	Antisense RNA to GBSS (granule bound starch-synthase)
Alteration of chain length and degree of branching	Overexpression of heterologous starch-degrading enzymes
Change amylopectin structure, quantitative variation in starch content, variation in starch granule size	Antisense RNA or overexpression of (heterologous) soluble starch-synthase

Wild-type potato starch is sensitive to salt (Ca^{2+} and Na^{+}), leading to a decreased viscosity in the presence of these ions. High amylopectin starch has approximately the same viscosity - in the presence of salt - as wild-type potato starch at a third of the concentration. Possible new future applications may therefore exist for modified potato starch as compared to normal wild type starch.

An increase in the starch content in potatoes can lead to lower calorie products, such as in crisps and chips, as less fat will be absorbed. Amylose-free potatoes might prove more easily digestible, as in the case of waxy rice (Kaushik and Khush, 1991). Also, the increasing popularity of microwaveable ready-made meals should create a sizeable market for amylose-free potato starch, because it yields clear pastes, which do not retrograde and so impair the look and texture of the sauces.

In conclusion, amylose-free or high amylopectin starches can and will be used to develop new products. The potential industrial applications of modifying the starch composition and content of plants are obvious, but a major hurdle exists - that of the general public's acceptance of genetically modified crops, especially when intended for food.

1.3.4 BioFuels

Starch has a number of advantages that might enable it to compete against crude oil, or other potential raw materials such as cellulose, as a "biofuel". The first consideration of any new material for energy production is its continued availability: while crude oil sources are continually shrinking, starch reserves – like those of cellulose - are abundant and renewable. The second consideration is the existence of a well-developed conversion technology by which the raw material can be safely, cheaply, and efficiently converted to energy-yielding products. This is where starch is currently superior to cellulose. There is of course the well established alcoholic drinks-derived technology for converting starch from various sources – barley, wheat, potatoes etc – to sugars fermentable to ethanol by *Saccharomyces cerevisiae* (section 1.3.1); fuel alcohol can then be recovered by distillation. Enzymatic hydrolysis of starch to fermentable sugars is the obvious route to fermentable sugar production, for energy and pollution cost reasons, and appropriate enzymes are well known and are commercially available (sections 1.3.1 and 1.3.2).

At present there are two main markets for fuel alcohol, Brazil produces Proalcohool, which is produced from sugar cane, and the USA produces Gasohol, ethanol produced from maize starch. Both are used either blended with petroleum or as 95% ethanol/water mixtures in purpose-built engines.

By contrast, conversion of cellulose to fermentable sugars requires several chemical and physical, as well as enzymatic pre-treatments. The degradation of cellulose requires at least three different enzymes (exo-and endo-β-1-4 glucanases and cellulobiase), which is expensive, and contributes to the cost of production of commercial quantities of ethanol.

1.3.5 Oligosaccharide Synthesis

Although starch hydrolysis – with a view to dextrin, sugar and ultimately ethanol production – is always likely to be the main industrial use for amylolytic enzymes, there is increasing interest in exploiting these enzymes in synthetic mode to convert sugars to oligosaccharides.

Oligosaccharides have a wide variety of roles within living cells, including recognition of plant host cells by phytopathogenic bacteria, and an involvement in cell-cell interactions e.g. binding to root hairs by nitrogen fixing bacteria (Lerouge *et al.*, 1990). Oligosaccharides also play an important role in cell-virus interactions – indeed, oligosaccharides are a virtually universal feature of exterior biological membranes, and are thought to positively influence the stability of glycoproteins (Wang *et al.*, 1996).

However, little is understood about the molecular mechanisms underlying the roles of the molecules, essentially because oligosaccharides are usually found in only small quantities, in complex biological matrices and therefore so difficult to purify and study. This problem can be circumvented by the synthesis of oligosaccharides, but chemical synthesis - for example Garegg's (1990) procedure - is not only expensive and complex, but most importantly, has a poor yield. An alternative is to use enzymes, such as glucosidases from starch-degrading microorganisms, in "reverse-synthetic mode" (Toone *et al.*, 1989; Bucke and Rastall, 1990; Ichikawa *et al.*, 1992).

There have been remarkable advances in oligosaccharide synthesis using enzyme-catalysed reactions (Crout and Vic, 1998). One of two approaches is typically used: the first uses a glycosyl transferase, where it is then possible to glycosylate carbohydrate substrates. A sugar nucleotide donor and acceptor are incubated with the appropriate glycosyl transferase which catalyses the transfer of glycosyl residue to the acceptor.

The second method is known as glycosidase-catalysed synthesis; here enzymes that normally catalyze the transfer of an enzyme-bound glycosyl residue to water are induced to transfer it to a different acceptor.

At present, most glycosidases used for the synthetic purposes are exo-glycosidases. Glycosyl transfer takes place only to the non-reducing terminal monosaccharide unit of substrates, (the glycosyl donor can be a monosaccharide or an oligosaccharides). Incubation with the glycosidase gives a glycosyl-enzyme intermediate that can be

intercepted either by water, to give a hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. A high concentration of monosaccharide is used to drive the reaction towards equilibrium. This is known as reverse hydrolysis.

Reverse hydrolysis has been found to be an effective way to produce a number of glycosides. For example, a group of Japanese researchers (Anindyawati *et al.*, 1995) successfully purified an α-glucosidase from a culture of *Aspergillus sp.* strain KT-11. This glucosidase was found to have a high enzyme transferring activity, when reactions were carried out in a high concentration of leucrose, at pH 4.5. Two novel oligosaccharides were synthesized by the α-glucosidase from this organism.
Other examples are summarized in Table 1.6.

Table 1.6: Glycosidase-catalysed synthesis of glycosides by reverse hydrolysis using different glycosidases. (Adapted from Crout and Vic, 1998).

Glycosidase	Glycoside	Yield (%)
β-Glucosidase (almond)	6′-hydroxyhexyl β-D-Glc	61
	pent-4-enyl β-D-Glc	50
	2-(trimethylsilyl)ethyl β-D-Gal	11
β-Galactosidase (*Aspergillus oryzae*)	6′-hydroxyhexyl β-D-Gal	48
	pent-4-enyl β-D-Gal	22
α-Galactosidase (*Aspergillus niger*)	6′-hydroxyhexyl β-D-Gal	47
	pent-4-enyl β-D-Gal	37
β-Mannosidase (*Helix pomata*)	6′-hydroxyhexyl β-D-Man	12

Many of the glycosides produced in the above table are expensive or difficult to produce - such as the α-Gal (1-4) Gal disaccharide which, is an important target in molecular recognition because it is the minimal ligand of the Shiga toxin, verotoxin1 and uropathogenic strains of *E.coli* (Crout and Vic, 1998).
The oligosaccharide synthesis field has been dominated by exoglycosidases but the use of endoglycosidases will increase in the future. Using endoglycosidases would lead to novel branched structures. A major advantage to using endoglycosidases is that

oligosaccharide fragments rather than a monosaccharide residue could be transferred (Crout and Vic, 1998).

However, a purified α-glucosidase preparation from *Lipomyces starkeyi* (the organism studied in the work described here) could prove to be a useful addition to the existing portfolio of enzymes suitable for use in oligosaccharide synthesis. One important consideration when selecting new glycosidases is to employ enzymes, which have a high temperature optimum. These enzymes can withstand the high temperatures essential for efficient reverse hydrolysis. Work published by Kelly *et al.* (1985) reported that an α-glucosidase from *L. starkeyi* was thermostable, making it a suitable candidate for further development.

At present, one of the major hurdles to using glucosidases for oligosaccharides synthesis is that most microbial enzyme preparations are usually impure mixtures and where there is any minor enzyme activity present, then it may be possible that this may catalyse the hydrolysis of the non-reducing terminal glycosidic bond in the substrate. A considerable advance to the field would be to provide highly purified glucosidase preparations for synthetic use, by developing robust enzyme purification methods.

1.3.6 Single Cell Protein

As the world's population exceeds 6 billion, conventional agriculture has been thought to be under pressure to meet the demand for protein. Single cell protein (SCP), which is the protein extract derived from microorganisms, can be produced at a faster rate than traditional agricultural products. Although SCP contains many essential amino acids, its main disadvantage to human consumption is the presence of nucleic acids, which will give rise to the accumulation of uric acid, which then leads to gout. Therefore SCP can be considered for animal feed and in fact there has been considerable interest in developing single cell protein (SCP), from raw materials to compete against the soybean and fishmeal feed supplement market (Boze *et al.*, 1992). Yeasts capable of utilizing renewable resources such as cellulose and starch are potential sources of single cell protein. Currently, only a few yeasts, such as *Kluyveromyces marxianus* and *Candida utilis*, have been identified as suitable SCP candidates for animal feed or human food (Saliceti-Piazza, *et al.*, 1992). However, amylolytic yeasts able to grow efficiently on cheap, starchy substrates and nitrogen sources could be potential candidates for SCP; amongst such yeasts is the species *Lipomyces starkeyi*, the organism used in the work described here.

Two SCP products have been successfully brought to market, the first product, Pruteen, was manufactured by ICI by using a genetically engineered strain of the bacterium, *Methylophilus metylotrophus* which, grows on methanol. However, high production costs made Pruteen more expensive than soybean and fishmeal and the plant was closed.

The second product - Mycoprotein ("Quorn") is a successful meat substitute that is produced from the mycelium of the fungus *Fusarium graminearum*.

1.4 The Genus *Lipomyces*

In 1946, during a study of nitrogen-fixing bacteria, Robert Starkey isolated a new species of yeast, which he subsequently named *Lipomyces starkeyi:* this was the first discovery of a *Lipomyces* yeast. Further investigation disclosed related species: most were capsulated, contained a fat globule and, after growth in a special medium, gave a positive starch reaction. In 1952, yeast taxonomists Lodder and Kreger-van Rij created a new genus, *Lipomyces,* to accommodate these species.

Different *Lipomyces* species have been isolated from various soil types - in cultivated and uncultivated fields, pastures, wasteland and woodland. The regularity with which the yeast was encountered suggested it was a common soil inhabitant, able to tolerate soils ranging in pH from 4.0-7.5. Sporulating yeasts e.g. species of *Lipomyces* and *Hansenula*, often favour adverse soil conditions; if also capsulated, as various species of *Cryptococcus*, *Rhodotorula* and *Lipomyces* are, they can survive in habitats poor in available nutrients, enduring much longer periods of desiccation than non-encapsulated yeasts (Golubev *et al.,* 1984).

The genus *Lipomyces* was identified as a typical ascosporogenous yeast, characterised by spherical ellipsoidal /globose budding cells, which are usually encapsulated by a mucous capsule. Older cells contain fat globules (and will shed their outer membrane). The cells reproduce by multilateral budding, with asci formed by a typical process from "active buds". An active bud on a cell may develop in a number of ways: (i) change into an ascus - a large pear-shaped structure with thin walls, typically containing eight spores (although this figure can rise up to sixteen), (ii) an active bud and a protuberance on another cell may conjugate, and the cell may turn into an ascus, - here, the ascus forms a solid-walled structure with four spores or fewer, (iii) the mother cell, after having produced an ascus through an active bud, may itself turn into an ascus - formed from the solid walls of the original mother cell.

The ascospores are oval, amber coloured and the spores may or may not be released. The ascospore walls are smooth, with irregular folds, or contain denticles (warty in appearance), or have longitudinal ridges (Slooff, 1970).

The genus *Lipomyces* originally encompassed the species: *starkeyi, kononenkoae, anomalus, lipofer* and *tetrasporus,* (Lodder and Kreiger-Van Rij, 1952). Since then, several studies have been carried out which have led to several revisions of the genus.

One of the earliest reports, Slodki and Wickerman (1966) showed that galactose was present in the constituents of the extracellular polysaccharides in *L. starkeyi,* but not in *L. lipofer,* and subsequently used this characteristic feature to differentiate between these two species. But no change was made to the inclusion of this species in the genus, *Lipomyces*, until 1985 when Yamada and Nakase, examined the nuclear DNA composition of the different species of *Lipomyces.* They reclassified *L. lipofer* in *Waltomyces.*

In 1995, *Lipomyces anomalus* was re-classified as *Babjeva anomalus,* as it forms non-encapsulated cells. The species also forms pseudophyphal cell-aggregates and pulvinate colonies, as opposed to viscous, confluent colonies of the type species, *L. starkeyi,* when grown on solid substrates. The ascospores are different from other *Lipomyces* species, which all have amber-coloured, ellipsoid-globose-shaped ascospores. *L. anomalus* ascospores are hyaline-shaped and are not amber in colour (Smith *et al.*, 1995).

In a separate study on rRNA base sequences, Yamada and Nogawa (1995) reported that *Lipomyces anomalus* was divergent from the rest of genus *Lipomyces,* because of substantial differences in the nucleotide sequence of its 18S, 25S and 26S ribosomal RNA species. In equivalent 18S rRNA regions (positions 1451-1618), *L. starkeyi, kononenkoae, lipofer* and *tetrasporus* were consistently characterised by the fingerprint sequence UUA, whereas *L. anomalus* had the sequence UAAUCUA

Van der Walt *et al.* in 1989 added another species, *japonicus* to the genus, *Lipomyces.* However, in 1995, Koch *et al,* assigned *L. japonicus* to the genus *Smithiozyma.* The species has a typical double-layered, ascomycetous ascosporal wall and the characteristic 18S rRNA "fingerprint" sequence of UUA.

Van der Walt *et al.*, (1997) raised two sub-specific taxa, to the rank of species: *L. mesembrius* and *L. spencer-martinisiae*. However, the most recent study by Van der Walt *et al.*, (1999) added a further four new species, *doorenjongli, kockii, yamadae,* and *yarrowii,* taking the total number of species in the genus *Lipomyces* to nine.

They then characterised the genus by four factors, common to all nine species.

1. The formation of globose to ellipsoid ascospores, which are not singly enclosed by a thin exosporal membrane.
2. The coenzyme Q9 system.
3. Independence of extraneous vitamins.

4. A DNA nucleotide composition of which the mol % guanine plus cytosine falls within the range 46.3 ± 0.5 to 49.1 ± 0.7.

Another important characteristic of the *Lipomyces* genus, as described by Barnett *et al.* (1990), is the ability to utilise a wide variety of compounds as a carbon source - besides glucose and its derivatives. Pentoses, oligosaccharides, polysaccharides (including starch), alcohols, and some organic acids can all be utilized. A strain of *L. starkeyi* was shown capable of degrading paraquat, a broad-spectrum herbicide (Carr *et al.,* 1985). Paraquat becomes immobolized on clay colloids and as a result it becomes unavailable for microbial degradation and can remain in the soil for many years. Using radiolabelled paraquat, Carr *et al.* showed that *L. starkeyi* was able to completely and efficiently degrade the herbicide when it was used as the sole nitrogen source.
The degradation of paraquat by *L. starkeyi* is associated with the integrity of the cell wall, and any disruption or removal of the cell wall resulted in a complete loss in degradative capability.

The genus *Lipomyces* is placed in the family Lipomycetaceae (also known as Lipomycetoideae), which also includes the genera *Waltomyces, Dipodascopsis and Zygozyma.* Anomorphs related to *Lipomyces* or *Zygozyma* have been placed in the genus *Myxozyma* (Van Uden, 1992).
The family incorporates some eighteen species, nine having been discovered within the last few years. Twelve of the eighteen species are characterised as teleomorphs and the remaining six as an anamorphs. The family is distinguished by possessing a whole cell monosaccharide structure, plus the ability for the large-scale production of amyloid polysaccharide, resulting in mucoid colonies in budding yeasts, and vitreous growth in hyphal taxa (Van Uden, 1992).
Another recognisable feature of the family lies in the ability to utilise a number of heterocyclic compounds and their derivatives (e.g. imidazole and pyrimidines) as the sole nitrogen source.
Research has continued into the evolution and phylogenetic characteristics of this diverse family. Lodolo *et al.,* (1990) examined the mitochondrial DNA, genome size and mitochondrial DNA hybridization for all the genera within the family. They concluded that the genera *Lipomyces* and *Dipodascopsis* were more closely related

than other members of the family - by examining similarities in size of the mitochondrial genome. They also concluded that the family Lipomycetaceae constituted a natural taxon distinct from the closely related Saccharomycetaceae family.

In 1990, two groups, Yamada and Nogawa, and Kurtzman and Lui, independently studied rRNA base sequences of a select number of genera to investigate evolutionary homology. Both groups' findings supported the hypothesis that the family Lipomycetaceae is distinct from the family Saccharomycetaceae.

A more complex and in-depth study into family characteristics was carried out by Cottrell and Kock (1990). Using pulsed field gel electrophoresis (PFGE) they studied the chromosomal band patterns from 29 different strains representing all the genera in the family. This particular study also compared the coenzyme Q system and carbon source utilization of different strains.

They showed that although different species within a genus produced bands that varied in size, they still produced the same number of chromosomal bands, the one exception being the genus *Myxozyma*. They found it was then possible to distinguish the different genera on the basis of the chromosomal band number, with *Dipodascopsis* containing one band, *Lipomyces* and *Waltomyces* having two bands, *Zygozyma* with three bands and *Myxozyma* with a range of five or more chromosomal bands. These results were consistent with the findings from the coenzyme Q system: strains from *Dipodascopsis* utilise coenzyme Q9 (CoQ9), *Lipomyces* strains use CoQ9, *Waltomyces* uses CoQ10, *Zygozyma* uses CoQ8, and *Myoxzyma*- which showed varied electrophoretic karyotypes - also possesses a variety of coenzyme Q systems.

1.5 Amylolytic Species of the Genus *Lipomyces*

1.5.1 Amylolytic System of *Lipomyces kononenkoae*

Several groups have investigated the amylolytic system of *L. kononenkoae*. Spencer-Martins and Van Uden (1977) described the species as being particularly suitable for industrial exploitation, due to the possession of a highly efficient amylolytic system, capable of producing a complement of amylases, including an α-amylase, a glucoamylase and a debranching transferase. They showed that this species had an efficient amylolytic system, utilising 99% of the starch-containing growth medium.

Horn *et al.,* (1988) compared the amylolytic ability of *Lipomyces* and *Schwanniomyces* yeast species. They evaluated the starch degrading ability of a number of strains on solid and in liquid media, and found that strains of *L. kononenkoae, L. starkeyi* and *S. occidentalis*, used virtually 100% of the starch supplied.

Of the 18 yeast species investigated, a derepressed mutant strain of *L. kononenkoae* (IGC 4052B) i.e. a strain insensitive to glucose repression of its amylases, was identified as having three times more α-amylase activity and five times more glucoamylase activity than any other of the yeasts included in their study. However, *Lipomyces starkeyi* (CSIR-Y158) exhibited the highest debranching activity.

Spencer-Martins and Van Uden (1977) explored the amylolytic system of a particular strain of *L. kononenkoae* (CBS 2514) and discovered the presence of an extracellular α-amylase and a glucoamylase. Optimum activity of the α-amylase, which had an estimated molecular weight of 38,000, occurred at pH 5.5 and at a temperature of 40°C. The molecular weight for the glucoamylase was estimated at 81,500, and was considered to possess a temperature optimum of 50°C and a pH optimum of 4.5.

In 1982, Spencer-Martins resolved the amylolytic system of *L. kononenkoae* (CBS 2514), into three species, a glucoamylase, an α-amylase and - a new finding - an isoamylase. For the purified isoamylase, the molecular weight was estimated at 65,000 Daltons and the pH and temperature optima were 5.6 and 30°C (Spencer-Martins, 1982). This complement of enzymes (an α-amylase, a glucoamylase and/or glucosidase/isoamylase), has been shown to be produced by the most amylolytically effective yeasts, including *Schwanniomyces alluvius, Schwanniomyces castellii* and *S.*

fibuliger (De Mot and Verachtert, 1987). So, the discovery of an isoamylase re-affirmed the potential commercial importance of the genus *Lipomyces,* as up until this time only bacteria had been known to be able to produce this enzyme.

Estrela *et al.* (1982) were one of the first groups to investigate the production of a glucoamylase and an α-amylase from a different strain of *L. kononenkoae* - IGC 4052. Using a 0.2% (w/v) starch-enriched mineral growth medium, they examined the effect of growth temperature on the production of amylases between 15°C and 36°C. They reported an optimum growth temperature of 33°C. Glucoamylase production did not follow any particular growth trend, and was produced between temperatures of 18°C to 25°C, but with highest glucoamylase production at the higher temperature of 36°C.
α-Amylase production had a temperature optimum, in the range 25-28°C. They were able to conclude that the growth temperature did not influence the yield of either enzyme. The enzymes were not characterised any further.

Prieto *et al.* (1995) purified and characterised an α-amylase of intermediate thermal stability from *Lipomyces kononenkoae* (CBS 5608). Cells were typically grown at 30°C in a 1% (w/v) yeast extract, 2% (w/v) peptone and 1% (w/v) starch or 2% (w/v) glucose. Other carbon sources were tried including YPDex (1% dextrin), MMS (minimal medium composed of 0.67% yeast nitrogen base without amino acids and supplemented with 1% starch), MMDex (supplemented with 1% dextrin), and YCS (1% yeast extract, casaminoacids, 1% starch). They found that cells grown on a rich medium secreted more α-amylase than cells grown on minimal medium or on YPG (yeast extract, peptone and glucose medium); the glucose present partially repressed the production of α-amylase, a phenomenon previously described in an earlier study by Sa-Correia and Van Uden (1981), who had examined the production of amylases produced by *Lipomyces kononenkoae* in starch-limited continuous culture.
The Prieto group harvested the cells after 72 hours and precipitated the protein from the cell-free supernatant by the addition of solid ammonium sulphate up to 60% saturation. The pellet was resuspended in 50mM Tris-HCl, dialysed against the same buffer, and applied to an affinity column packed with a cross-linked starch matrix. The enzyme-starch matrix column was washed with 200mM Tris-HCl to remove any proteins, which did not bind to the starch. The starch-enzyme pellet was adjusted to pH

5.5 by adding Tris-HCl buffer, and the matrix was then incubated at 30°C until it had been completely degraded. This resulted in the specific activity increasing from 0.043U/mg, as found in the supernatant, to 4.6U/mg - a 107-fold increase in purification, with a recovery of 64%.

The concentrated protein solution was then applied to a DEAE-Biogel A ion-exchange column equilibrated in 50mM phosphate buffer, pH 5.5. Inert protein was eluted in the void volume, and the α-amylase was eluted with 0.5M phosphate buffer. After ion exchange chromatography, the specific activity increased from 4.6 U/mg to 258 U/mg, equivalent to a 6000-fold increase in purification from the original cultural supernatant, and a recovery of 52%. Active fractions were pooled and lyophilised and characterisation studies performed on this material.

The purified α-amylase was electrophoretically homogeneous, a monomeric protein with an apparent molecular weight of 76,000, a pI of <3.5, and a pH optimum between 4.5 to 5.0. The α-amylase was found to be a glycoprotein, with both *N*- and *O*-linked sugars, but the total carbohydrate content - determined using the phenol-sulphuric acid method (Dubois *et al.*, 1956) - only amounted to 10% (μg sugar/μg protein). The enzyme exhibited intermediate thermostability, displaying a temperature optimum of 70°C. It was shown to have an endo-acting mechanism and released maltoligosaccharides with 2 to 6 residues on starch hydrolysis. Major end-products were maltotriose, followed by maltose and finally, maltotetraoase. Glucose was not detected as an end-product.

Prieto *et al.* concluded that the α-amylase purified from *L. kononenkoae* differed significantly both biochemically and enzymatically from the amylases produced by other isolates of *Lipomyces kononenkoae.* For example, the α-amylase purified by Spencer-Martins and Van Uden (1979) and Spencer-Martins (1982) had a molecular weight of 38,000, a pI of 7.1 and pH and temperature optima of 5.5 and 40°C respectively.

However, this enzyme showed some similarity to the α-amylase purified from *Lipomyces starkeyi* by Kelly *et al.* (1985), in both hydrolysis end-products and molecular weight estimates. The major difference between the two enzymes was in the thermostability of the enzyme: the enzyme purified by Prieto *et al.* denatured after 10 minutes at 70°C. However, the α-amylase purified by Kelly *et al.* was stable for a

prolonged period at this temperature, and only lost approximately half its activity after incubation at 80°C. The unique property of intermediate thermostability and acidic pH optima could make the α-amylase from *L. kononenkoae* (CBS 5608) suitable for certain industrial application, such as retarding the staling of baked foods (Hebeda *et al.*, 1990).

Also in 1995, a Korean group of researchers, Chun *et al.*, purified and characterised two glucoamylase isozymes from *Lipomyces kononenkoae* CBS 5608. Cells were grown on a yeast nitrogen base medium supplemented with 0.5% (w/v) starch at 30°C for 72 hours. Cells were removed by centrifugation and the supernatant was passed down a Sephacryl S-200 gel filtration followed by a DEAE Sephadex A-50 ionic exchange column. The molecular weight of the first isozyme (G1) was calculated as ca. 150 KDA, and ca. 128 KDa was the estimate for the second isozyme (G2). The pH and temperature optima for both isozymes were determined at 4.5 and 60°C respectively. Both isozymes were glycoproteins with isoelectric points of 5.6 (GI) and 5.4 (G2).

In 1995, Steyn and Pretorius purified a starch-degrading α-amylase from a mutant strain of *Lipomyces kononenkoae* (IGC 4052B) and went on to isolate the cognate α-amylase-encoding gene (LKA1), which they expressed in *Saccharomyces cerevisiae.* This strain of *Lipomyces kononenkoae* had been selected on the basis of the study by Horn *et al.*, (1988).

Cells were cultured in YNBS media containing 1% Lintner starch, 0.5% amylopectin, 0.5% peptone, 0.25% yeast extract, 0.67% yeast nitrogen base with amino acids and phosphate buffer and incubated at 28 °C for between 48 and 72 hours. The culture broth was then harvested by centrifugation at 6000 *g* for thirty minutes at 4°C. Protein was precipitated from the cell-free supernatant by the addition of solid ammonium sulphate up to 75% saturation. The pellet was dialysed against 30mM Tris-HCl (pH 8.0) and then this extract was applied to a Spectra/Gel Fast Flow DEAE column equilibrated in 30mM Tris-HCl. Elution was carried out with a linear gradient from 0-1M NaCl in 30mM Tris-HCl (pH 8) at a flow rate of 12ml/hour. Active fractions were pooled and concentrated by ultrafiltration.

The pI of the purified α-amylase was calculated at 4.17 by isoelectric focusing and the molecular weight of the α-amylase estimated at 76,250 by SDS-PAGE. This estimation was also close to the figure calculated by Kelly *et al.* (1985) for the α-amylase purified from *Lipomyces starkeyi*. The enzyme had a temperature optimum of 40°C, (lower than that of the α-amylase from Kelly *et al.*), but fell within the range reported for other yeast amylases (Vihinen and Mantsala, 1989). The optimum pH was determined as pH 4, but the enzyme was stable in the pH range of 3 to 8 - again comparable to other yeast α-amylases, but different from the α-amylase of the parent *L. kononenkoae* IGC4052 strain.

The mode of action of the α-amylase on Lintner starch was determined: acting by endo-hydrolysis, the α-amylase produced maltose, maltotriose and maltotetraose.

The enzyme was able to hydrolyse both α-1,4 and α-1,6 glycosidic linkages exhibiting high specificity towards highly branched molecules such as amylopectin, glycogen and linear substrates such as dextrin. Short-chain sugars such as pullulan were hydrolysed slowly as α-amylases typically require at least a linear chain of five α-1,4-glucose residues to exhibit hydrolysis (Manners, 1989).

The *L. kononenkoae* α-amylase was successfully expressed from a cDNA fragment in an *S. cerevisiae* host strain. The cultures were harvested after 48 hours, with no intracellular activity detected, all activity being extracellular. Only one difference in activity between the two enzymes was observed. The α-amylase produced from *L. kononenkoae* cells was heavily glycosylated. After de-glycosylation the carbohydrate moiety was identified as having a role in the secretion of the protein. The enzyme expressed from *S. cerevisiae* was also found to be a glycosylated protein but once de-glycosylated, was found to be more active than the glycosylated form, indicating a possible negative effect of *S. cerevisiae* glycosylation on enzyme activity.

1.5.2 Amylolytic System of *Lipomyces tetrasporus*

Gallagher *et al.* (1991) investigated the amylolytic system of a species of the genus *Lipomyces*, which produced a clearly identifiable α-amylase and a novel extracellular carbohydrase. This group assessed the growth of *L. tetrasporus* (CBS 5910) on a range of substrates, and showed that it was active on several substrates, including soluble starch, maltose, dextran, and isomaltose, amongst many others. The amylolytic system of this yeast was later resolved into two components; an α-amylase and a glucosidase-type activity.

Using a growth medium containing starch, yeast extract and malt extract they found that maximal growth was achieved after seven days. Replacing the soluble starch in the growth medium with either dextran or maltose gave yields of 17.7 and 21.3 units/ml respectively, compared to a yield of 9.8 units/ml with starch. In this study, Gallagher *et al.* defined one unit as the amount of enzyme releasing 1μg glucose (or glucose equivalent) from the substrate per 30 min at 40°C. In each case, cells were removed by centrifugation and the protein was precipitated from the cell-free supernatant using ammonium sulphate (40-70% saturation). This resulted in a 10.6-fold increase in purification where starch was the carbon source, approximately 90% of the extraneous protein being removed. The protein precipitate was then applied to DEAE Bio-gel A ion-exchange column, previously equilibrated with 0.05M phosphate buffer. Using a linear gradient of 0.05M to 0.15M phosphate buffer, the major enzyme peak containing the glucosidase eluted as a single peak entirely distinct from the minor α-amylase activity. A further ultrafiltration step followed by size exclusion FPLC using a Superose 12 column, did not improve the resolution between the two enzyme activities.

The purified glucosidase was homogeneous on SDS-PAGE electrophoresis and isoelectric focusing, with an approximate molecular weight estimate of 150,000 Daltons via SDS-PAGE and 183,000 Daltons by FPLC. The enzyme had a pH optimum of 4.5 and a temperature optimum of 50°C.

Yeast glucosidases are known to possess a broad aglycone specificity (Chiba *et al*, 1973b), and the carbohydrase from this strain of *L. tetrasporus* produced an unusual

glucosidase-type activity, hydrolysing a range of substrates and displaying properties related to α-glucosidases, glucoamylases and glucodextranases. The enzyme preferentially hydrolysed maltose, and α-1,3-(nigerose) and α-1,6-linked saccharides, indicating a glucosidase-type activity as opposed to an amylase-type activity. The purified enzyme could also hydrolyse polymeric substrates (dextran), using an exo-lytic glucoamylase-like mode of action, exclusively releasing α-D-glucose, as opposed to β-D-glucose.

Overall, these results indicated that the carbohydrase was more comparable to an α-glucosidase, as the release of β-D-glucose would have indicated the presence of a glucoamylase (Matsusaka 1977).

1.5.3 Amylolytic System of *Lipomyces starkeyi*

In 1977, Spencer-Martins and Van Uden carried out an extensive survey of starch degradation by a variety of amylolytic organisms. Of the 81 assorted assimilating yeast strains investigated, they discovered that most of the starch-converting yeasts hydrolysed only part of the starch supplied in the growth media and had relatively low yields of growth on this substrate. They attributed this to the possession of a limited amylolytic system. Among the 59 species studied further, the highest yields (biomass formed per unit mass of starch supplied) were obtained with the strains of the genus *Lipomyces,* and, in particular with strains of the species *starkeyi* and *kononenkoa*e.

In later comparative surveys of amylolytic yeast strains and their extracellular debranching enzymes, carried out in 1984a, 1984b and in 1987, De Mot and Verachtert confirmed the results of Spencer Martins and Van Uden. They showed that *L. starkeyi*, a member of the family Lipomycoideae, possessed a highly active amylolytic system, and was thus an attractive candidate for future industrial exploitation.

One of the first groups to explore the amylolytic system of *L. starkeyi* (CBS 1807) further was Moulin and Galzy (1979). They detected the presence of an α-amylase, which was mainly associated with the cell wall, (as very little was released into the starch culture medium). Washing the cells in 20mM phosphate buffer, pH 7.6 at 4°C, under constant stirring for approximately 3 hours, released the majority (40-65%) of

the cell-bound activity into the extracellular supernatant. The addition of 85-90% (w/v) ammonium sulphate precipitated all the enzyme activity and led to a 2-3-fold increase in specific activity. Closer inspection showed the amylase had a pH optimum of 3.5, a temperature optimum of 50°C and produced glucose as the end-product of hydrolysis. The release of glucose, a rare end-product for an α-amylase acting in isolation, pointed to the presence of another enzyme activity, either a glucoamylase or an α-glucosidase. However, they were later to dismiss the presence of other activities when they found that separation was impossible and the ratio between the two enzyme activities remained constant, despite varying the ammonium sulphate concentration, assay conditions and extraction techniques.

Contrary to the published findings of Moulin and Galzy (1979), which had suggested a single cell-bound amylolytic system, Kelly *et al.,* (1985), resolved the amylolytic activity of *Lipomyces starkeyi* (CBS 1809) into two amylolytic components: α-glucosidase and α-amylase. Using a specially designed culture medium containing a mixture of maize starch and soya bean meal, they were able to induce the highest level of both enzyme activities.

Maximal production of α-glucosidase was obtained when using the substrates cellobiose, pullulan and to a lesser extent, starch, maltose and dextrin as sole carbon sources. The highest production of α-amylase production was obtained with starch, maltodextrin and maltose as carbon sources. Sucrose and pullulan failed to induce any activity. These results support the earlier findings by Moulin and Galzy (1979), which showed that the α-amylase was equally induced by starch and maltose.

33% of the total α-glucosidase activity was detected in the cell-free supernatant after twenty hours, with the majority (52%) of activity bound to the cell wall and 15% located intracellularly. After forty-four hours, 70% of the total glucosidase activity was located extracellularly, with 20% bound to the cell surface and only 10% present intracellularly. Extracellular α-amylase was first detected after 20 hours, with 96% of the total amylase activity recovered at 44 hours, no α-amylase activity bound to the cell surface, and intracellular amylase not detected. These results contradicted the findings of Moulin and Galzy, who had only detected an α-amylase associated with the cell wall.

Kelly *et al.* harvested the cells by centrifugation and were then able to isolate the two carbohydrases from the growth medium by solvent precipitation using isopropanol (1:1), followed by a 50% (w/v) ammonium sulphate precipitation. They found that salt precipitation alone - without the initial solvent precipitation step, did not resolve the two enzyme activities, and furthermore, recovery was poor.

Solvent precipitation resulted in enzyme recoveries from the medium of 70% for α-glucosidase and 90% for α-amylase, these fractions showing 14% and 39% increases in purity respectively. The solvent pellet was then resuspended and brought to a concentration of 50% (w/v) ammonium sulphate, yielding an overall 202-fold purification for the α-glucosidase and 95-fold purification of the α-amylase.

The Kelly group's initial attempts to separate and purify the two enzymes used adsorption chromatography and gel filtration chromatography using a Sephadex G100 column. Although these were unsuccessful, separation was later achieved using ion-exchange chromatography on a DEAE-Biogel-A column. A linear gradient of 0.05-0.1M phosphate buffer, pH 7.0, completely separated the two activities: inert protein eluted at a concentration of 0.01M phosphate buffer, α-glucosidase eluted at 0.06M and the α-amylase eluted at 0.092M.

Kinetic studies were performed on the purified enzyme products. Analysis of the substrate specificities of the α-glucosidase showed highest activity on maltose (100%) and isomaltose (100%) with % relative rates of activity on maltotriose, isomaltotriose and p-nitrophenol glucopyranoside (PNPG) of 59, 48 and 22 respectively. The α-glucosidase did not hydrolyse sucrose. Using starch (0.2% w/v) and amylopectin (0.2% w/v), the enzyme gave % activities of 31 and 42 respectively. While this ability to degrade amylopectin or starch had been detected in a number of mould and bacterial glucosidases (Yamasaki *et al.*, 1977b; McWethy and Hartman, 1979), this was the first time this property had been reported for an extracellular yeast glucosidase.

Analysis of the substrate specificity of the purified α-amylase revealed percent relative activities for amylose, starch and amylopectin of 100, 77 and 75 respectively. Maltose, maltotriose and higher dextrins were the main products of the α-amylase activity, and glucose was not detected. These results suggested a typical endo-acting α-amylase mechanism of action, and contrasted with the results of Moulin and Galzy (1979), who had only identified glucose monomer as the end-product.

Kelly *et al.* also reported that the purified α-glucosidase and α-amylase from *Lipomyces starkeyi* (CBS 1809) had, respectively, pH optima of 4.5 and 4.0, unusually high temperature optima of 60°C and 70°C, and molecular weights of 35,000 and 76,000 Daltons. As Table 1.5 indicates, 35,000 Daltons is an unusually low molecular weight for an α-glucosidase.

The most recent study of the amylolytic system of *L. starkeyi* was carried out by Pungpeng *et al.*, (1992) on a strain (HN-606), isolated in Thailand from loogpang lauw, a traditional fermented food. They found that this strain gave an equivalent digestion of raw starch to that of the commercially used *Aspergillus* but with an unusually lower culture temperature. For optimal growth, *Lipomyces starkeyi* cells were grown at 15°C, a significantly cooler temperature in comparison to a more traditional growth temperature of 26°C for *Aspergillus*.

Seed cultures were grown at 20°C for two days in a growth medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) potato starch. For the main culture, large capacity fermenters containing 120L of YPS were sterilised and inoculated with 5% (v/v) of the two-day seed culture. *Lipomyces starkeyi* (HN-606), was grown on a specially modified medium of 2% (w/v) potato starch, 1% (w/v) yeast extract, 1% (w/v) peptone at 15°C; both α-amylase and glucoamylase activities were detected extracellularly after two days and reaching a maximum after six days.

The two carbohydrase activities were isolated from the growth medium by a 60% (w/v) ammonium sulphate fractionation. The precipitate was then re-dissolved, dialysed against deionized water and lyophilised. The lyophilised pellet was resuspended in 0.1ml acetate buffer pH 5.5 and applied to a DEAE-Sephadex A-50 ion-exchange column previously equilibrated with the same buffer. Using a linear gradient of 0.0 to 0.5M NaCl in 0.05M acetate buffer, pH 5.5, at a flow of 70ml/hr, the α-amylase separated from the glucoamylase activity. Active fractions were pooled and applied to a Sephacryl S-300 gel filtration column, previously equilibrated with 0.5M NaCl in 0.05M acetate buffer, pH 5.5, and eluted at a flow rate of 24ml/h. Again, active fractions were pooled. The isolated α-amylase had a molecular weight of 56,000, and could be adsorbed onto raw corn starch and could digest it to release maltose, maltotriose and glucose. The presence of glucose as an end-product supported the findings of Moulin and Galzy (1979), but was in contrast to the results of Kelly *et*

al., (1985). Kelly *et al.* had identified an α-amylase with a molecular weight 76,000, which did not hydrolyse starch to glucose, (although maltose and maltotriose were identifiable end-products). Pungpeng *et al.*, (1992) did not perform any further characterization studies on the isolated glucoamylase.

1.5.3.1. *Lipomyces starkeyi:* Dextranase Activity

An unusual feature of *L. starkeyi* is its ability to use α-1,6 linked glucans – dextrans – as a sole carbon source. Webb and Spencer-Martins (1983) investigated an extracellular dextranase produced from a strain of *Lipomyces starkeyi* (IGC 4047). Using a minimal medium, which was supplemented with 0.5% (w/v) dextran and vitamins, the dextranase produced was identified by its ability to hydrolyse blue dextran and Sephadex G-100. After six days the cells were harvested and the protein was recovered by solvent precipitation, using 2 volumes of isopropanol to 1 volume of cell free supernatant. This resulted in a five-fold increase in purification and represented a 69% recovery of activity. The pellet was resuspended in 50mM citrate buffer, pH 5.5 and applied to an anion exchange column, followed by a gel filtration column and finally concentrated by ultrafiltration. They determined that the dextranase was electrophoretically homogeneous after the first single purification step and estimated the size of the enzyme as 23,000, much smaller than those dextranases described from other bacterial and fungal species. The enzyme had a pH optimum of 5.0 and a temperature optimum of 50°C. Investigations of the end products of hydrolysis revealed the presence of glucose, isomaltose, and isomaltotriose with other isomaltooligosaccharides up to isomaltohexaose.

In 1988, Koenig and Day examined optimal growth conditions for the production of dextranase from a different strain of *L. starkeyi*, - ATCC 12659. Their results differed from those of Webb and Spencer-Martins (1983), as they discovered that the optimum growth rate of this strain to produce the highest dextranase yield was at pH 3 and 30°C. Exploring oxygenation levels, they found that below 1.0vvm (volume of air/volume of batch/min), and above 1.5vvm, dextranase activity was reduced (possibly due to oxygen toxicity). This confirmed the fact that *L. starkeyi* is an obligate aerobe.

Subsequently, Koenig and Day (1989) attempted to purify the dextranase, initially following the method previously described by Webb and Spencer-Martins (1983); again, purifying the enzyme by solvent fractionation only led to a disappointing 5-fold increase in specific activity. This preparation contained contaminating material and did not electrophorese uniformly on a native gel, but gave rise to a smear of activity throughout the gel, a phenomenon previously noted by Hattori *et al.* (1981) for a dextranase purified from *Chaetomium gracile*. Koenig and Day therefore adopted an alternative purification strategy.

Culture supernatant was concentrated from 20L to 500ml by ultrafiltration and then applied to a Carboxymethyl-Sepharose cationic ion-exchange column, and enzyme eluted using 0.5M NaCl buffer. Active fractions were pooled, concentrated again by ultrafiltration, and then applied to a gel filtration column equilibrated with 0.05M citrate/phosphate buffer (pH 5.5) containing 0.15M NaCl. The active protein eluted as a single peak, and at this point had been purified 43-fold. This preparation was then used for physical and kinetic studies. The temperature and pH optima were reported as 55°C and 5.0 respectively. The molecular weight of the native protein was estimated at 68,000 by gel filtration, but four bands - of molecular weights 74,000, 71,000, 68,000 and 65,000 Daltons - were detected by SDS/PAGE. Native gel electrophoresis was unsatisfactory, probably because of the interaction between dextranase and polyacrylamide, and therefore bands were not assayed for dextranase activity.

In an attempt to confirm that the four bands identified in SDS/PAGE were dextranases, they performed isoelectric focusing gels. The gel-filtration purified fraction yielded five bands by IEF, with pIs between 5.6 and 6.1; all five bands were found to have dextranase activities and were catalytically similar, exhibiting the same *K*m's when using dextran as substrate. End products of hydrolysis reactions were found to be typical for an endodextranase – glucose and isomalto-oligosaccharides to isomaltotetraose; four residues was the minimum glucan chain length that could be hydrolysed by the enzyme.

Temperature and pH profiles were similar to the dextranase purified by Webb and Spencer-Martins (1983), although the molecular weight estimation was significantly different – 68,000 versus 23,000 for the Webb and Spencer-Martins dextranase. However, both dextranases produced very similar end-products, indicative of endodextranase activity.

1.8 Objectives of Project

The main aim of the work reported in this thesis was to purify and characterise the α-glucosidase secreted by *Lipomyces starkeyi* NCYC 1436. Once kinetic studies had been performed on the purified enzyme, and the substrate specificity determined, the α-glucosidase could then be used to study the synthesis of novel oligosaccharides.

Chapter 2: Materials and Methods

2.1. Chemicals and Enzymes

All chemicals, enzymes and materials used during this work were supplied by Sigma Chemical Company, Poole, Dorset, unless otherwise stated. Growth and culture media constituents were supplied by Difco. Silver staining kits were purchased from BioRad Laboratories Limited and the premixed acrylamide solution from National Diagnostics. All percentage concentrations were prepared (w/v), unless otherwise stated.

2.1.1 Strain

Lipomyces starkeyi NCYC 1436 was obtained from the National Collection of Yeast Collections (Food Research Institute, Colney Lane, Norwich).

2.2. Growth of Yeast Strains

Yeast strains were routinely grown in YEPD, which contained 2% glucose, 1% yeast extract and 1% Bactopeptone. Solid media was prepared by the addition of 1.5% agar prior to autoclaving. All media was sterilised in an autoclave at 121°C for 15 minutes prior to use. *L. starkeyi* cultures were grown on a starch medium (YEPS), containing 2% soluble starch, 1% yeast extract and 1% Bactopeptone, for either 6-7 days or 15 days, and incubated at 30° C, in Gallenkamp orbital shakers at 120 rpm.

Pre-cultures were prepared by inoculating 50 ml of YEPD in a 250ml conical flask with a single colony of *Lipomyces starkeyi* from a slope culture maintained on YEPD medium containing 1.5% agar. The cultures were shaken at 100rpm in a Gallenkamp orbital shaker at 25°C for three days.

Over-vigorous rotation was reduced to avoid bubble formation, which can lead to the denaturation of protein molecules caught up in foam, due to surface tension effects.

After three days the pre-culture had entered late logarithmic phase, and an inoculum of 1ml of this log-phase pre-culture, containing ca. 1.2×10^5 cells/ml, was aseptically

transferred to a 2 litre conical flask containing 500ml of YEPS media. 1-5 litres of culture media was inoculated at any one time, with the standardised inoculum, allowing each flask to contain the same optical density.

Cells were harvested by centrifugation at 4°C and 10,000 rpm (6,300 x *g*), using the Sorvall RC-5B refrigerated superspeed centrifuge.

2.2.1 Fermenter Cultures

8 litres of YEPS were prepared (see section 2.2) and sterilised *in situ* in an Anglicon Alfa Laval fermenter. The air inlet valve was switched to the 'off 'position for sterilization and the pressure adjusted to 1bar to prevent medium entering the pH probe. An outer insulating jacket was placed around the glass housing of the fermenter and the water pump was disconnected. The fermenter was connected to a central controller (Chemap), the stirrer was switched on and autoclaving conditions defined, the temperature being set to 121°C. Upon reaching this temperature, the timer was activated, the temperature was maintained for 15 minutes, and then the medium was allowed to cool down.

Once the temperature dropped below 60°C, the insulating jacket was removed and, to affect a more rapid temperature drop, the water pump was re-attached to the cold water circulation pipe and turned on. The air exhaust valve was also opened to let out steam. The air filter (previously autoclaved individually) was attached and, the fermenting conditions were established: the rate of the stirrer was adjusted to avoid a froth building up, the temperature was set at 30°C, and the air inlet valve was opened.

To the cool sterilised medium, the yeast inoculum was added in the following way: a single port on the top of the fermenter was opened, its aperture (concealed by a blue rubber seal) was flamed, and the inoculum was injected using a sterile 50ml syringe.

The inoculum had been prepared as in section 2.2 and in a volume amounting to 400ml (i.e. 5% volume of the volume of medium).

The inoculation port was then closed and the fermentation process was underway. Samples of medium were withdrawn daily, using stringent aseptic techniques, and assayed for α-glucosidase, α-amylase and total protein.

2.2.2. Haemocytometer

The number of cells per ml in a suspension was determined using a Neubauer haemocytometer, which has two 1mm square grids, each divided into 26 large squares, further subdivided into 16 smaller squares, making a total of 400 small squares per grid. A drop of suspension was placed onto each of the two grids and a cover slip pressed firmly on top (the area around the grids having been slightly wetted). The gap between the cover slip and slide was exactly 0.1mm deep, as judged by the presence of coloured diffraction rings. The slide was then placed under a microscope (x400) and the number of cells within the millimetre square of each grid counted using a hand counter.

The number of cells counted in one 1mm square is the number of cells in a volume of 1mm x 1mm x 0.1mm = 1/10 cubic millimetre. As there are 1000 cubic mm in 1cubic centimetre, multiplying the number of cells within the grid by 10,000 (10^4) gave the cell number/ml.

2.3. Polyacrylamide Gel Electrophoresis (PAGE)

All PAGE gels were run on the Pharmacia LKB 2050 Midget Electrophoresis Unit. Typically, the acrylamide solution consisted of 30% (w/v) acrylamide and 0.6% (w/v) bis-acrylamide, unless otherwise stated. Normal gel running conditions utilised a 7.5% or 9% separating gel with a 4% stacking gel. Both the separating and stacking gels were prepared according to the formula detailed in section 2.3.11.

Gels were run at 150 mV for an hour and a half and then stained using 1% (w/v) Coomassie Blue R-250 solution (section 2.3.9).

2.3.1 Acrylamide/bis acrylamide Stock Solutions

30g acrylamide was mixed with 0.8g bisacrylamide and diluted to 100ml with deionized water. The solution was stored in the dark, up to 3 months at room temperature. Alternatively, Protogel (National Diagnostics), a premixed, pre-filtered acrylamide/bisacrylamide (37.5:1), stock solution was used. When kept in the dark at room temperature, the solution was stable up to one year.

2.3.2 Lower Gel Buffer (1.5M Tris-HCl, pH 8.8)

45.41g Tris (MW 121.1) was made up to 250ml with deionized water and the pH adjusted to 8.8 with concentrated HCl.

2.3.3 Upper Gel Buffer (0.5M Tris-HCl, pH 6.8)

6.05g Tris was dissolved in 100ml of deionized water and the pH adjusted to 6.8 with concentrated HCl.

2.3.4 20% (w/v) Sodium Dodecyl Sulphate (SDS)

20g SDS was diluted to 100ml with distilled water and the stock solution stored at room temperature.

2.3.5 Running (reservoir) buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3)

7.2g glycine was mixed with 1.5g Tris base, 0.5g SDS (or 2.5ml of 20% SDS stock solution) and made up to 500ml with distilled water.
For native, non-denaturing gels, SDS was not added to the running buffer.

2.3.6 Sample buffer (0.125M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2M β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8)

1.25ml upper gel buffer was mixed with 4.0ml of 20% SDS (2.3.4), 6ml glycerol, 4mg bromophenol blue and 1ml β-mercaptoethanol. This solution was then diluted to 20ml with distilled water and stored at room temperature. For native, non-denaturing gels, non-reducing sample buffer contained, upper gel buffer, glycerol and bromophenol blue only.

2.3.7 TEMED, (N,N,N',N'-tetramethylethylenediamine) (catalyst).

Typically, 10µl of TEMED was added per gel.

2.3.8 Ammonium Persulphate (initiator)

0.1g ammonium persulphate was made up to 1ml with distilled water. 75µl of this 1% (w/v) stock solution was added per gel. Alternatively, 5µg ammonium persulphate was added per gel.

2.3.9 Protein Staining Solution

250ml of methanol and 50ml acetic acid was made up to 500ml with distilled water. This stock solution could be stored indefinitely at room temperature; prior to gel staining, 0.25g of Coomassie Blue was dissolved in 50ml of the stock, dissolved immediately and the staining solution was then applied to the PAGE gel.

2.3.10 Destaining Solution

100ml of methanol was mixed with 200ml acetic acid and diluted with 1700ml distilled water. This solution was stored at room temperature indefinitely.

2.3.11 Formulations of Different Percentage Gels

	Resolving (lower) gel						Stacking (upper) gel		
	7.5%	8.0%	9.0%	10%	12%	15%	3%	4%	5%
Acrylamide (ml)	2.5	2.67	3.0	3.33	4.0	5.0	0.6	0.8	1.0
Tris pH 8.8 (ml)	2.5	2.5	2.5	2.5	2.5	2.5			
Tris pH 6.8 (ml)							2.4	2.4	2.4
Water (ml)	4.9	4.72	4.4	4.07	3.4	2.4	3.0	2.8	2.6
SDS (20%) (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.05	0.05	0.05
TEMED	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl
Ammonium Persulphate	5µg	5µg	5µg	5µg	5µg	5µg	5µg	5µg	5µg

2.3.12 SDS Molecular Weight Markers

SDS High Molecular Markers, Mark VI (Sigma), containing six proteins with molecular weights of 29,000 (carbonic anhydrase), 45,000 (chick egg albumin), 66,000 (bovine serum albumin), 97,400 (phosphorylase B), 116,000 (β-galactosidase) and 205,000 (myosin), were prepared by reconstituting the lyophilised contents with 1.5ml of buffer (62mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.001% bromophenol blue). A 15µl aliquot was pipetted into a sterile microfuge tube and incubated at 100°C for five minutes prior to electrophoresis. 2 x 5µl of this marker solution was applied per gel.

Low Molecular Weight SDS markers (Sigma Dalton Mark VII-L kit), contained a 3.5mg lyophilised mixture of the following seven proteins: bovine serum albumin (66,000), chick egg albumin (45,000), glyceraldehyde-5 phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and α-lactalbumin (14,200). The markers were prepared by the addition of 1.5ml of sample buffer (section 2.3.6), followed by thorough mixing; the solution was then dispensed into working aliquots of 10µl and frozen at -20°C, (to avoid repeated freezing and thawing cycles of marker solutions). Prior to electrophoresis, an aliquot

was incubated at 100°C for five minutes. 2 x 5µl of this marker solution was applied per gel, alongside lanes containing denatured samples.

2.3.13 Native Molecular Weight Markers

For native PAGE gels, protein samples were mixed 1:1 with non-denaturing sample buffer (section 2.3.6.), but not heated. Non-denaturing Molecular Weight Markers (Mark V1 Kit, Sigma) contained five proteins: urease hexamer (545,000) and trimer (272,000), bovine serum albumin dimer (132,000) and monomer (66,000), chicken egg albumin (45,000), carbonic anhydrase (29,000), and α-lactalbumin (14,200). Each protein-containing vial was reconstituted with 1ml of 50mM NaCl, 1mM sodium phosphate, pH 7.0, (except the vial containing urease, which was dissolved in 5ml of distilled water). Again, stock solutions were dispensed into working aliquots of 10µl and frozen at -20°C for future use. 2 x 5µl of this marker solution was applied per gel.

2.3.14 Preparation and Running of PAGE Gels

Polyacrylamide gels were prepared using the LKB Midget Multicast Gel Casting Unit. A maximum of 10 gels of 0.75mm thickness could be made at any one time. Conversely, when fewer gels were required, the unused space in the chamber was filled with glass or perspex plates.

An individual gel sandwich was assembled according to the manufacturer's instructions, using a single, clean, aluminium oxide plate, single glass plate and two plastic 0.75mm spacers. Care was taken to keep the plates and spacers flush against a flat surface. Additional gel sandwiches could then be added using grease-proof paper to separate each gel.

Polyacrylamide solution was de-aerated under vacuum using a water aspirator and the initiator and catalyst were added, mixed in without creating bubbles (excessive aeration interferes with polymerisation). The solution was poured directly into the stack of gel sandwiches until the desired height was obtained (approximately 3/4 of the height of the glass plate).

After pouring the separating gel, iso-butanol was added to the top of each gel to prevent evaporation and to reduce curvature of the gel from surface tension.

Once the lower, resolving gel had polymerised, the iso-butanol was decanted and the gel rinsed gently with distilled water, in preparation for the stacking gel. A gel comb providing ten sample wells was inserted, and the upper gel solution was added using a Pasteur pipette.

After the upper gel had polymerised, the gel sandwich was removed from the casting chamber, and an individual gel was detached from the stack of gels by sliding a razor or scalpel blade between the sandwiches.

The outside of the gel was rinsed with distilled water to remove bits of excess gel that may have adhered to the plates, and blotted dry. The comb was removed and the sandwich carefully clamped to the inner core unit. Reservoir buffer was added to the sample wells and the two upper buffer chambers were filled.

Experimental samples were prepared for SDS-PAGE by adding an equal volume of reducing buffer (section 2.3.6) in a 1:1 ratio and then boiling at 100°C for five minutes; samples of high and low molecular weight markers - prepared as previously described in section 2.3.12 were treated in the same way.

Using a 50µl Hamilton micro-syringe, 5-10µl of sample was gently loaded beneath the buffer in each well, maintaining a sharp interface between the sample and reservoir buffer. Care was taken to thoroughly rinse the micro-syringe with methanol and then distilled water, in between samples. Empty wells were filled with sample buffer to avoid spreading and diffusion of adjoining samples. The lid was replaced on the gel unit and connected to a power supply.

Gels were run at 150 mV at 4°C for an hour and half or until the bromophenol blue solvent front reached the bottom of the gel. The gel sandwich was disassembled and the gel stained with 1% (w/v) Coomassie Blue solution (section 2.3.9). Fresh stain was prepared and the gel was immersed for a minimum of one hour (or overnight). The gel was then destained in several changes of destain solution (section 2.3.10), until the blue-stained protein bands appeared against a clear background.

2.3.15 Silver Staining of PAGE Gels

Silver staining of PAGE gels allows the detection of proteins down to a 0.1ng/mm^2 limit (Bio-Rad Silver Stain instruction manual). Silver staining reagents (oxidizer and silver reagent from Bio-Rad) were prepared on the same day that staining was to be performed, by dilution of the supplied concentrate (1: 10) with deionized water. The oxidizer reagent consisted of potassium dichromate and nitric acid, and the silver reagent contained silver nitrate. Silver staining developer was prepared by dissolving 32g of developer (sodium carbonate plus parformaldehyde from Bio-Rad), in 1 litre of deionized water and warming the solution to 30-50°C prior to use. Care was taken to use deionized water at all stages of the silver staining procedure, as contaminants such as Cl^- ions precipitate silver ions, causing reduced sensitivity and an increase in background colour.

Immediately after the electrophoretic run was completed, gels were placed in fixative solution, (40% methanol/10% acetic acid (v/v)), for a minimum of 30 minutes.

The gel was then immersed in oxidizer for five minutes and then washed for a maximum of fifteen minutes in deionized water, undergoing 6-7 changes of water (thorough washing of the gel was needed to flush away excess oxidizer reagent from the gel). The gel was then placed in silver reagent and allowed to equilibrate for twenty minutes. After a quick deionized water wash, developer solution was added. The gel was closely examined until a smoky precipitate appeared, at which point the initial developer solution was replaced with fresh developer. The developer was replenished with fresh developer until the desired band intensity was obtained. The developing process was halted by addition of stop solution (5% acetic acid (v/v)). Gels were preserved as described in section 2.3.16.

2.3.16. Gel Preservation

Polyacrylamide gels were preserved using the Jouan GF10 gel dryer. This uses heat and reduced pressure to dehydrate PAGE gels and bond them permanently to filter paper or transparent porous cellophane.

The gel dryer was attached to an evaporation system consisting of a concentrator, a trap (to remove liquid) and a vacuum pump. The gel was placed on a cellophane or filter support, which was then placed upon a perforated stainless steel support screen. For encapsulation of the gel, a second sheet of cellophane was laid over the gel. A porous polyethylene or Mylar sheet was then placed on top of the gel, followed by a clear, silicone rubber flap, which covered the whole assembly. When the vacuum was applied, the rubber flap formed an air-tight seal and air and moisture were drawn from the gel through the perforated screen.

The temperature-setting dial was pre-set to 80°C and drying time set to approximately 45 minutes, sufficient for drying a 10% gel. The vacuum was set to switch off 30 minutes after the heater timer, to eliminate curling of the gels.

2.4. Protein Concentration Methods

2.4.1 Ammonium Sulphate Precipitation

Precipitation of proteins was achieved at 85% (w/v) saturation of ammonium sulphate, equivalent to 55.7g per 100ml (Dawson *et al.*, 1986). The solution was kept at 4°C, with the salt being added slowly and stirred in gently until the salt was dissolved.

The mixture was then centrifuged at 11,000 rpm (16,500 x *g*) for 30 minutes and the harvested precipitate was either kept as a pellet and stored at 4°C or resuspended in 10mM sodium phosphate buffer, pH 6.8.

2.4.2 Ultrafiltration

Ultrafiltration uses semi-permeable membranes to separate species by molecular size and shape. Concentration of proteins by ultrafiltration was achieved by forcing the solvent in a protein solution through a membrane, which retained the protein of interest. Elevated pressure has now been largely superseded by centrifugal force as a means of forcing liquid through the membrane but both techniques were used, depending on the volume size - as either concentration method is less likely to cause protein denaturation than, for example, precipitation.

Small volumes (up to 2ml) were reduced to 50µl using the Amicon Centricon system. The Centricon Microconcentrator was assembled using a 10KD molecular weight cut-off membrane, also supplied by Amicon. 2.0ml of sample was added to the sample reservoir, and the whole assembly was centrifuged at 5000 x *g* for 30 minutes using a microcentrifuge with a fixed angle rotor. The filtrate cap was removed and the retentate cup attached to the sample reservoir. The Centricon unit was inverted and then centrifuged at 1000 x *g* for 2 minutes. The concentrated sample was recovered in the retentate cup. A small amount of 10mM sodium acetate buffer was used to wash the membrane to try and ensure complete recovery of protein.

Sample volumes greater than 100ml were concentrated using Amicon Ultrafiltration Stirred Cells. This approach uses a pressurised and stirred cell system capable of rapid concentration and de-salting within the same unit. Amicon stirred cells (models 8050 and 80200, with capacity volumes of 50ml and 200ml respectively) were fitted with Diaflo membranes (10,000 KD cut-off); solute recovery was maximised using the Diaflo YM 10 advanced hydrophilic membranes, which have exceptionally low protein binding properties. Dilute protein solution was added to the cell, which was then placed on a magnetic stirrer and stirred gently. Finally, the whole system was attached to a nitrogen cylinder, pressure was applied and solute was slowly concentrated as water was expelled from the cell.

For sample volumes greater than 500ml and smaller than 2 litres, the concentration of dilute protein solutions (typically cell-free culture supernatant) was achieved using a Hollow Fibre Concentrator. The system used was the Amicon DC10, which consists of

cylindrical tubes of polysulfone, bundled together and sealed in transparent cartridges. The PM 10 membrane used here was suitable for retaining proteins above 10,000 KD.

Cell-free supernatant was placed inside the internal glass core cylinder and pressure applied at a maximum of 40 psi (using compressed air or nitrogen); this caused solvent (water) and microsolutes to be transported through the membrane fibre, while retained solutes were progressively concentrated within the fibre lumen.
At room temperature, volumes up to 3 litres were reduced to 400ml within 1-2 hours, depending on the viscosity of the solution.

2.4.3 Reverse Dialysis

Sample volumes greater than 50ml were often concentrated by reverse dialysis. Dialysis tubing from Medicell International Ltd (14,000 KD cut-off) was prepared by boiling for 1 hour in 10mM sodium bicarbonate (double distilled water), 1mM EDTA. Using gloves and clean forceps the tubing was then thoroughly rinsed in double distilled water and one end sealed using a dialysis clip. The sample was delivered into the dialysis tubing using a pipette, and the open end sealed. The tubing was then placed in a flat tray and covered in PEG (polyethylene glycol, MWT 15-20 000 KD) and kept at room temperature for 3-4 hours. By adding more PEG, the desired reduction in volume was speeded up. The sample was removed once the tubing had been rinsed outside to remove any contaminating PEG. The inside of the tubing was rinsed carefully with buffer for maximum enzyme recovery.

2.4.3.1 Dialysis: to exchange buffers or remove contaminants

Dialysis tubing was prepared as described in section 2.4.3 and the protein sample added carefully, avoiding air bubbles formation, using a pipette. The open end was then firmly sealed with a knot, care being taken to leave space for liquid volume expansion (as a result of high solute concentration and osmotic forces, buffer enters, swelling occurs and the subsequent high pressure could result in leakage or the bursting of the dialysis bag). The secure dialysis tubing was placed in a large volume of the chosen buffer, which was stirred continually at 4°C. After two or three changes of buffer during a minimum sixteen hour period, dialysis was assumed to be complete.

2.4.4 Freeze-Drying (lyophilization)

All samples were prepared in low ionic strength buffers and, depending on sample volume size, placed in either flat-bottomed glass vessels or in 1.5ml microfuge tubes. Glass vessel openings were covered in parafilm, perforated with small air-escape holes, and lids of microfuge tubes were pierced. Samples were then frozen in an appropriate freezer at minimum of -25°C or a -80°C freezer.

The refrigeration unit of the freeze-drier (Birchover Instruments Ltd. Freeze Dryer Model 3.5.), was switched on 40 minutes prior to use and the temperature set to -40°C. Once the latter temperature was reached, frozen samples were placed inside the internal chamber and the vacuum pump switched on. Samples were freeze-dried overnight under vacuum and subsequently stored at 4°C.

2.4.5 Organic Solvent Precipitation

Cold isopropanol (-20°C) was slowly added to stirred ice-cold, culture medium, to a final concentration of 50% (v/v). The mixture was left stirring in an ice-bath overnight and then centrifuged at 10000 rpm, (16,300 x *g*) for 30 minutes. The supernatant was decanted and the pellet resuspended in a minimum volume of 10mM sodium phosphate buffer, pH 6.87.

2.5 Detection of Enzyme Activity in Polyacrylamide Gels

2.5.1 Assay of Enzyme Activity in Gel Slices

A 7.5% native polyacrylamide gel - used for an electrophoretic protein separation as described in section 2.3.14 - was cut into two halves. One half was stained with Coomassie Blue (section 2.3.9), while the other half of the gel was immersed for one hour in 10mM sodium citrate buffer (pH 5.0), to remove the electrophoresis running buffer. The unstained gel portion was cut into strips corresponding to the sample wells, and these strips were then sectioned at right angles into equal segments. Each segment was placed into separate 1.5ml microfuge tubes, crushed using a sterile spatula, and 1ml of 10mM sodium acetate buffer (pH 5.0) was added. Tubes were incubated at 37°C for a known period of time and then centrifuged at 12,000 rpm, (7,200 x *g*) for 5 minutes. The supernatant was removed and subsequently assayed for α-glucosidase activity and α-amylase activity as described in sections 2.10.1 and 2.10.2.

2.5.2 Activity Staining for α-Glucosidase

A 10% native polyacrylamide gel loaded with two identical sets of samples (so that when cut, the two gel halves were exact duplicates) was run at 100mV for 2 hours. The gel was then cut into two halves, one half stained in the normal way using Coomassie Blue (section 2.3.9); and the other half being left to equilibrate in 10mM sodium acetate buffer, pH 5, for two hours. The latter gel-half was then laid onto a thin (2mm), 1% (w/v) agarose gel containing 1mM PNPG, covered with a sponge pre-soaked in sodium acetate buffer, pH 5.0, followed by a glass plate bearing a 50-100g weight. This gel "sandwich" was left for 2 hours at room temperature after which the polyacrylamide gel was removed and the agarose was stained by flooding with 0.2M borax (disodium tetraborate). The activity stain developed within a few minutes and active bands stained yellow. The gel was examined and/or photographed within one hour of development, as the colour dissipated and completely diffused away after two hours.

2.5.3 Activity Staining for α-Amylase

A 10% non-denaturing gel was run at 100mV for two hours, with identical samples loaded across the two halves of the gel. After electrophoresis the gel was bisected and one half stained with Coomassie Blue (Section 2.3.10), while the second half was left to equilibrate in 10mM sodium acetate buffer, (pH 5.0) for two hours at room temperature. This portion of the gel was then laid onto a thin 1% (w/v) agarose gel containing 1% (w/v) soluble starch and covered with a sponge pre-soaked in buffer, a glass plate with a 50-100g weight then being placed onto the sponge. This gel "sandwich" was left for 2 hours at room temperature, after which the polyacrylamide gel was removed and the agarose underlay was stained by flushing with 5% (w/v) iodine solution.

Active α-amylase bands were immediately visible as clear bands against a dark blue background. The agarose gel was stable and could be kept for several days, allowing a ready comparison between the agarose underlay bands and Coomassie Blue-stained bands of the original native gel.

2.6 Kinetic Studies of α-Glucosidase

*K*m and *V*max were determined for α-glucosidase using a coupled enzyme assay system section 2.7. A suitable fixed amount of α-glucosidase, providing an easily measurable activity in each sample, was incubated with 1ml of pre-equilibrated glucose/hexokinase assay kit, (see section 2.7) and progress curves were determined.

The enzyme activities (μmol s^{-1} min^{-1}) at several substrate concentrations were calculated from the progress curves, and used to construct Michaelis-Menten, and Hanes plots, from which *K*m and *V*max were determined.

Hanes plots were graphs of $[S]/v_o$ against [S] (substrate concentration). *K*m was determined by the intercept on the X-axis and *V*max was determined by the reciprocal of the slope of line.

2.7 Hexokinase Assay for the Determination of Glucose

The hexokinase-based (HK) enzymatic assay has been extensively used in clinical laboratories and is recognized as highly specific for glucose determinations. Glucose is first phosphorylated by adenosine triphosphate (ATP), in the reaction catalysed by hexokinase (HK). The glucose-6-phosphate (G-6-P) formed is then oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD^+). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). During this oxidation, an equimolar amount of NAD^+ is reduced to NADH. The consequent increase in absorbance at 340nm is directly proportional to glucose concentration.

The HK Kit from Sigma comprised of six vials, each containing a mixture of active ingredients (the concentrations after re-constitution are given in parentheses): NAD^+ (1.5mmol/l), ATP (1.0 mmol/l), HK (1000 units/l), G-6-PDH (1000 units/l), magnesium ions (2.1 mmol/l), buffer pH 7.5, non-reactive stabilizers and fillers, and sodium azide (0.05%). Each vial was reconstituted by adding 10ml of deionized water, stoppered and then mixed by inversion. Reconstituted reagents were kept on ice during use and subsequently stored at 4°C. A UV/VIS spectrophotometer capable of maintaining two restricted (1ml) quartz cuvettes at a constant temperature of 37°C, (Perkin Elmer Lambda 2), was set to 340nm. Assay blanks were set up containing 0.5ml of HK reagent and 0.5ml sodium acetate buffer (pH 5.0). Cuvettes were mixed and the absorbance was set to zero.

In a standard assay, 0.5ml of hexokinase solution and 0.4ml of buffer were placed in the reaction cuvette, 0.1ml of substrate (dissolved in 10mM sodium acetate buffer, pH 5.0) and 0.02ml of sample were added rapidly, mixed by inversion and then the cuvette was immediately placed in the spectrophotometer. The change of absorbance with time was electronically logged by the Perkin Elmer Lambda 2. The reaction was usually monitored for approximately 5 minutes.

Enzyme activity was determined from the linear increase in absorbance at 340nm. One glucosidase enzyme unit was defined as the amount of activity that releases 1μmole of

glucose from the substrate per minute at 37°C. This was calculated using the following formula:

$$\text{Enzyme units} = \frac{A_{340} \text{ change min}^{-1}}{6.22^*}$$

* (millimolar extinction coefficient of NADH at 340nm)

A calibration curve was constructed using a set of known glucose standards to check the linearity of the assay.

2.8 Protein Assays

2.8.1 Absorbance at 280nm

A_{280} was used primarily as a tool for approximately estimating protein concentrations in column fractions. A dual-beam spectrophotometer (Perkin Elmer Lambda 2 UV/VIS) was set to zero using buffer in matched quartz cuvettes and samples were subsequently read against the reference cell containing buffer. Protein concentrations were calculated assuming that A_{280} of 1.0 corresponds to 1mg protein per ml.

2.8.2 Bradford Assay for Total Protein

Total protein concentration was estimated using the dye-binding method developed by Bradford (1976). The assay involves the binding of Coomassie Brilliant Blue G-250 in acid conditions to protein molecules, thus causing a shift in the absorption maximum of the dye from 465nm to 595nm. It is this increase in absorbance at 595nm, which was measured.

The working Bradford reagent was made up by dissolving 100mg of Coomassie Brilliant Blue G-250 in 50ml 95% (w/v) ethanol and 100ml 85% (w/v) phosphoric acid. This solution was diluted to a final volume of 1 litre, filtered through Whatman

3MM paper and stored at room temperature in a dark reagent bottle. For assays of protein solutions with concentrations in the range of 0.1-1mg/ml, a ratio of 1ml of Bradford reagent to 20µl of sample was used. For samples containing approximately 10-100µg protein/ml, the ratio used was 1ml of reagent to 100µl of sample. In both cases the total assay mixture was mixed carefully and after 15 minutes incubation time, the absorbance was read at 595nm. All samples were assayed in duplicate.

2.8.3 BCA Protein Assay

The 'BCA' protein assay kit from Pierce (Chester, Cheshire) is a highly sensitive method for determining protein concentration. This assay is essentially a modified version of the well-known biuret reaction where, in alkaline conditions, Cu^{2+} is reduced to the cuprous ion (Cu^{1+}) by proteins. The resultant Cu^{1+} then interacts with two molecules of BCA (bicinchoninic acid) to produce a purple coloration, which exhibits a strong absorbance at 592nm.

An advantage of the assay is the flexibility of the protocol, allowing the sensitivity to be increased significantly by increasing the incubation time and/or temperature.

Protein concentrations were determined following manufacturers' instructions. Assays were carried out in a microtitre plate. A calibration curve using BSA standards (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2mg/ml concentrations prepared from a 2mg/ml stock solution), was prepared in parallel with unknown experimental samples. A new standard curve was generated on each assay occasion.

Standards and experimental samples were assayed in duplicate. 10µl of sample was mixed with 200µl of the reagent mix, composed of 50ml reagent A (containing sodium carbonate, BCA detection reagent (bicinchoninic acid) and sodium tartrate in 0.2M NaOH), mixed with 1ml of reagent B (4% (w/v) copper sulphate). For protein concentrations in the range of 20-1200ug/ml, samples were then incubated at 37°C for thirty minutes. For samples of low protein concentrations, between 5 and 250µg/ml, assay solutions were incubated at 60°C for 30 minutes. The absorbance of the samples was then measured at 570nm, using a microtitre plate reader, and the concentration of the samples calculated from a graph of absorbance against protein concentration for the standards.

2.9 Isoelectric Focusing

Ampholine "PAGplate" precast polyacrylamide gels (Pharmacia), able to generate a pH gradient of 3.5 to 9.5, were used. Each 1mm thick (110x 245mm) gel, precast onto a plastic support film, allowed a maximum of 32 samples to be separated using appropriate electrophoretic conditions: these were provided by a Pharmacia Multiphor Electrophoresis Unit which was connected to a thermostatic circulating water bath. The water bath was set to 4°C and switched on 20 minutes before starting an electrophoretic run. The gel was carefully placed on top of a template located on top of the flat bed cooling plate of the Multiphor unit. The gel was held in place using some light paraffin oil. The electrode wicks were evenly soaked in the appropriate solutions, the cathode electrolyte solution being 1M sodium hydroxide and the anode electrolyte solution being 1M phosphoric acid. Both wicks were then applied to the long edges of the gel, ensuring correct polarity.

Prior to sample application, the gel was pre-focused to allow ampholines to establish a pH gradient. When using the whole gel, the prefocus conditions were 750V, 25mA, and 14W for fifteen minutes; when using only half a gel, current and power setting were halved.

2.9.1 Preparation of pI Markers

An isoelectric focusing marker mixture purchased from Sigma (I 3018) and supplied as a lyophilised powder, contained enzymes with pIs ranging from 3.6 to 9.3. The contents of a vial were reconstituted with 250µl of deionized water to yield a solution containing 3.6mg of total protein/ml in 0.1M glycine, pH 6.0.

Each vial contained 0.9mg of the mixture of marker proteins below and approximately 1.5mg of glycine. The marker protein mixture contained amyloglucosidase from *Aspergillus niger* (pI: 3.6), trypsin inhibitor from soyabean (pI: 4.6), β-lactalbumin A from bovine milk (pI: 5.1), carbonic anhydrase II from bovine erythrocytes (pI: 5.9), carbonic anhydrase I from human erythrocytes (pI: 6.6), myoglobin from horse heart (pI: 6.8, 7.7), lentil lectin from *Lens culinaris* (pI: 8.2, 8.6, 8.8), and trypsinogen from bovine pancreas (pI: 9.3).

2.9.2 Sample Preparation and Application

Samples were prepared in PBS (Dulbecco's phosphate buffered saline, 0.1M, pH 6.0) and diluted to a protein concentration of 1mg/ml. Care was taken to ensure that the salt concentration (e.g. sodium phosphate) of the samples was below 50mM, to avoid band disturbance.

Dry application wicks were positioned along the gel, 2cm from the cathode. Typically, 10µl of a 1mg/ml protein solution was applied to each applicator wick. pI markers were prepared according to manufacturer's instructions (see section 2.9.1) and 7.5µl of this marker solution was applied to an applicator strip and run in parallel with the unknown samples.

After sample application, the electro-focusing lid was positioned across the width of the gel and the terminals of the Multiphor connected. The Multiphor safety cover was placed over the unit and the pressure bars fixed into place. The electrode leads were attached to the power supply and the running conditions set to 1500V, 50mA and 30W for an hour and a half, (when only half a gel was used, the current and power setting were reduced by a factor of two).

After approximately half the electrofocusing time had expired, sample application pieces were removed with care, using fine forceps.

Immediately after isoelectric focusing, the gel was placed into fixative solution (12% (w/v) trichloroacetic acid and 3.5% (w/v) 5- sulphosalicylic acid in deionized water), for a minimum of half an hour, to precipitate the proteins and allow the ampholines to diffuse away. The gel was then rinsed for five minutes with destaining solution (25% (v/v) ethanol, 8% (v/v) acetic acid). The destaining solution was then discarded and replaced with staining solution (0.46g Coomassie Brilliant Blue R-250 in 400ml destaining solution), which had previously been heated to 60°C. The gel was immersed in staining solution for ten minutes and the staining dish covered with aluminium foil to retain warmth. The gel was destained by removing the staining solution and washing the gel in several changes of destaining solution until a clear background emerged.

After focusing and staining, the isoelectric point of the unknown was estimated using the marker proteins. Migration distances from the cathodic edge of the gel to the different marker bands were plotted on the Y-axis and corresponding pIs of the marker proteins plotted on the X-axis. A calibration curve was then drawn and by measuring

the migration distance of the unknown protein, it was possible to interpolate the isoelectric point of the protein from the curve.

2.10 Enzyme Assays

2.10.1 α-Glucosidase Assay

α-Glucosidase activity was determined using p-nitrophenol α-D-glucopyranoside (PNPG) as the colorimetric assay substrate. α-Glucosidase hydrolyses PNPG releasing p-nitrophenol, which is yellow in alkaline solution (Halvorson and Ellias, 1958).

0.2 ml of sample was added to 1ml of 5mM PNPG, in 10mM phosphate buffer, pH 6.8 in a sterile microfuge tube; this was sealed, vortexed briefly, and then the mixture was incubated at 37°C for two hours. The reaction was terminated by the addition of 2ml of 0.2M disodium tetraborate (borax). The absorbance was measured at 400nm.
Individual blanks appropriate for different types of sample were set up, as the reaction product is yellow and has an absorbance peak at 400nm: growth medium absorbs at this wavelength, and various other types of sample were also found to be coloured. To avoid errors in over estimating enzyme activity within coloured samples, and to compensate for any non-enzymatic hydrolysis of substrate during the assay, duplicate tubes were set up. 0.2ml of sample was added, to one tube only, and then both tubes were incubated at 37°C. After two hours, 0.2ml of sample was added to the second ("blank") tube, and the reaction was immediately stopped in both tubes by the addition of 2ml of 0.2M borax.
A_{400} was measured for all tubes and values for "zero time" blanks subtracted from the absorbance values of the experimental tubes.

One unit of enzyme activity was defined as the amount of enzyme, which hydrolysed 1nmol of substrate per minute per ml.
(Absorbance unit/nanomolar extinction coefficient of PNPG x dilution factor) ÷ time =U/ml

2.10.2 α-Amylase Assay

α-Amylase activity was determined using Phadebas, a cross-linked covalently dyed starch substrate from Pharmacia. This product, initially intended for clinical α-amylase determinations, is a highly sensitive and specific, colorimetric assay substrate, resistant to exo-acting hydrolysis by glucoamylases and β-amylases (Pharmacia Diagnostics, technical product insert: 52-1393-00/4).

Individual Phadebas tablets were carefully crushed using a pestle and mortar and the fine powder dispensed as 20mg aliquots into sterile 1.5ml microfuge tubes. To a tube, 0.2ml of sample was added together with 0.8ml of 10mM sodium phosphate buffer, pH 7.0. The tube was sealed and vortexed briefly. This mixture was then incubated at 37° C for 1 hour, centrifuged at 12,000 rpm (7,200 x *g*) for 5 minutes to remove any undigested blue-dyed starch and 0.6ml of the clear supernatant was removed, diluted 1 in 5 with distilled water, and the absorbance read at 620nm. Blank tubes, containing 0.2ml of distilled water instead of 0.2ml of the sample were treated in the same way. One unit of enzyme activity was defined as one absorbance unit/min at 37°C:

(Absorbance unit x original dilution factor x dilution factor (5)) ÷ time =U/ml

Preliminary experiments (section 3.3.1) have shown that a cheaper alternative to the Phadebas substrate may be prepared by dyeing soluble starch with the dye Cibacron Blue, and crosslinking this with 1,4-butanediol diglycidyl ether (Ceska *et al.*, 1969).

2.10.3 Measurement of Neutral Carbohydrate Content

The amount of neutral carbohydrate covalently attached to the glucosidase enzyme was estimated by the phenol-sulphuric acid method described by Dubois *et al.*, (1956): phenol in the presence of sulphuric acid is used in the quantitative measurement of sugars, their methyl derivatives, oligosaccharides and polysaccharides.

Using identical 10 ml Pyrex test tubes, with a uniform internal diameter not less than 16mm, 2ml of calibration curve standards (containing between 10-80 μg of glucose) or

2ml of unknown sample was added to 1ml of 5% aqueous phenol. 5ml of 98% sulphuric acid was then rapidly added, with the stream of acid directed against the inside of the test tube. The contents were thoroughly mixed and then allowed to stand in a fume cupboard at room temperature for 30 minutes. The absorbance of the characteristic yellow-orange colour was measured at 490nm. A blank was prepared by substituting the glucose sample with 2ml of distilled water. All samples were prepared in triplicate.

2.11 Chromatography

2.11.1 Hydrophobic Interactive Chromatography (HIC)

The hydrophobic chromatographic medium Phenyl Sepharose CL-4B (Pharmacia) was used. In this matrix, the phenyl group is covalently coupled to a cross-linked 4% agarose matrix by ether linkages, thus generating straight chain alkyl ligands and producing a gel lacking in ionic properties.

Phenyl Sepharose CL-4B is supplied pre-swollen in 20% (v/v) ethanol. Ethanol markedly reduces hydrophobic interactions therefore all traces of it were removed by washing the gel with ten volumes of distilled water. A slurry was then prepared using binding buffer (i.e. 1M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8) and the gel slurry was de-gassed.

Mini-columns were prepared in 1ml syringes, stoppered with Micropore fine filters (Whatman), and packed by pouring the slurry down the inside walls of the column in one continuous motion, avoiding the introduction of any air bubbles and allowing packing under gravity. Those salts which cause salting-out, e.g. ammonium sulphate, also promote binding to hydrophobic ligands, therefore, each column was equilibrated by passing through it 10 aliquots of 1ml, 1M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8, after which the concentrated protein sample (usually a redissolved ammonium sulphate pellet), was loaded onto the column.

HIC was performed at room temperature, as hydrophobic interactions usually decrease with decreasing temperature.

Loaded columns were washed with two column volumes of the initial loading buffer, and then the proteins were eluted with a decreasing salt gradient.

For mini-columns, 1ml fractions were collected manually into clean Pyrex test tubes (and monitored for total protein, α-glucosidase and α-amylase activity (sections 2.10.1 and 2.10.2).

For larger columns, the matrix was prepared as just described, with the columns being connected to a peristaltic pump (Pharmacia- Model P3) and fraction collector (BioRad-Model 2110). The flow rate was approximately 60ml/hour for the first 2 hours and then increased to 100ml/h for the rest of the gradient.

2.11.2 Ion Exchange Chromatography (IEC)

Two anion exchangers, DE52 from Whatman, and Q Sepharose from Pharmacia, were used to perform ion exchange chromatography. Q-Sepharose has a quarternary amine ligand and uses small uniform beads of agarose, permitting a fast elution rate. Q Sepharose is supplied pre-swollen and minicolumns were prepared as described in section 2.11.1.

DE 52 is a pre-swollen microgranular matrix with a diethylaminoethyl (DEAE) ligand, which was fully equilibrated prior to use. Firstly, DE52 was washed in a buffer of substantially higher ionic strength (1M phosphate buffer, pH 6.8), than that of the required initial loading buffer (10mM phosphate buffer, pH 6.8). The slurry was gently stirred, left for half an hour to settle and the supernatant containing any fines, decanted off. Mini-columns were then set up as described in section 2.11.1.

Protein samples made up in a small volume (1ml) of the initial (low salt) buffer were then applied, and proteins were eluted from the columns using an increasing, stepped salt concentration gradient (e.g. 0.01-0.5M phosphate buffer, pH 6.8).

Using DE52, the method was scaled up to a 30ml column volume (column with an internal diameter of 2.5cm). Firstly, air was eliminated from column dead spaces by flushing the end pieces of the column with buffer and closing the column outlet while a few centimetres of buffer remained in the column. Pre-equilibrated DE52 slurry was then poured into the column, allowing the matrix bed to pack under gravity. Once the column was packed, without air-bubbles, buffer was added to fill the rest of the column to form a meniscus at the top, and an adapter was inserted at an angle, avoiding the introduction of air bubbles. The adapter plunger was pushed gently down

the packed matrix, displacing excess buffer, and then locked in position on the gel surface.

The column and tubing were attached via a peristaltic pump to a UV flow-cell plus chart recorder and the column was left to equilibrate in new buffer for a minimum of two hours at a flow rate of 60cm/h, until a stable baseline at $A_{280}$was obtained.

Samples were prepared in a total volume of 5ml of 0.01M sodium phosphate buffer, pH 6.8, and applied to the column via a three-way valve. The column was then washed in two bed-volumes of starting buffer and the flow rate was reduced to ~25ml/h, before starting the elution gradient.

All linear gradients were produced by placing an equal volume of buffers representing the upper and lower concentration limits of the gradient, in two separate but identical beakers. The beaker containing the lower limit buffer was placed on a magnetic stirrer, a magnetic flea was added, and tubing was put in place to connect the beaker via the peristaltic pump, to the ion-exchange column. The beaker containing the upper limit buffer was placed on a adjustable platform at exactly the same height as the beaker of lower limit buffer and a sterile plastic U-tube filled with lower limit buffer was used to connect to each beaker and form a siphon. As the column outlet valve was released and liquid passed through the column, buffer was siphoned from the upper limit buffer and thoroughly mixed with the lower limit buffer, so initiating the formation of a concentration gradient, which was then applied to the column. Care was taken to ensure that no air bubbles were present in the tubing, so a constant flow rate was maintained and an accurate linear gradient was formed.

The eluate was passed through a UV-transparent flow cell (UV-1Pharmacia) and A_{280} recorded on a chart recorder. Fractions were collected using the BioRad Fraction Collector (Model 2110). All column operations were conducted at 4°C.

2.11.3 Gel Filtration Chromatography

Gel filtration chromatography was performed using Sephacryl 300 Superfine (Pharmacia), a gel supplied pre-swollen, ready to use and with high resolving power (Sephacryl is manufactured by covalently cross-linking allyl dextran with N,N-methylene bisacrylamide).

A long and narrow column, with a 10ml bed volume, was set up in a 10ml graduated pipette. The sample was applied in a volume of between 1-5% of the bed volume, using 10mM sodium phosphate buffer, pH 6.8. Concentrated protein samples were preferred, with dilute samples being avoided, due to subsequent further dilution on elution.

It was possible to accurately measure the void volume and separation time by pre-calibrating the column with a solution of blue dextran. Elution of the protein was carried out at 4°C, using 10mM sodium phosphate buffer, pH 6.8. A peristaltic pump attached to the column maintained a constant flow rate of approximately 60ml/hour, and 1ml fractions were collected and assayed for total protein, α-glucosidase and α-amylase activity.

2.11.4 Affinity Column Chromatography

A column of Cibacron Blue 3GA (Sigma), consisting of a matrix of 4% cross-linked beaded agarose bearing a covalently liganded A-ring o-sulphonic acid structure, was used in an initial attempt to purify α-glucosidase and α-amylase.

Typically, a mini-column was set up using a 1ml syringe, which was packed with Cibacron Blue 3GA. The matrix is supplied from the manufacturers in 0.5M NaCl, and this was displaced with an initial wash of 2 column volumes of methanol, followed by a 50% (v/v) solution of methanol and water. A third wash of distilled water was followed by a 1M NaCl wash, and finally the column was equilibrated in 10mM sodium phosphate buffer, pH 7.0. A sample of crude protein preparation was applied to the column, and the column was washed with 2ml batches of starting buffer, fractions being monitored for protein, until most of the (inert) unbound protein was removed from the column. Protein was then eluted from the column using a linear gradient of increasing salt concentration i.e. from 0.01 to 1M sodium phosphate buffer, pH 6.8.

Chapter 3: Assay Development and Growth and Production of α-Glucosidase and α-Amylase Activities

3.0 Enzyme Assay Development

This section describes a series of experiments performed to select specific and sensitive assays, appropriate for the determination of α-glucosidase and α-amylase activities from *Lipomyces starkeyi* cultures. Purified enzyme stocks purchased from Sigma were used as controls: α-glucosidase (derived from baker's yeast), α-amylase (Type X-A, fungal crude from *Aspergillus oryzae),* and glucoamylase (derived from *Aspergillus niger,* containing less than 0.02% glucose).

3.1 α-Glucosidase Assays

α-Glucosidase enzyme activity can be measured in a variety of ways (Halvorson, 1966; Khan *et al.,* 1967; Bergmeyer, 1974), but two approaches are most commonly used. The first is based on coupled enzyme reactions, typically using maltose as a substrate, enzyme activity being measured by the release of glucose. Glucose can be measured using highly specific, enzymatic, diagnostic clinical kits (purchased from Sigma) - based on the assay methodology of Somogyi-Nelson, (Bergmeyer, 1974), the hexokinase method (section 2.7), or the DNS (3,5-dinitrosalicylic acid) reducing sugars assay.

Alternatively, α-glucosidase activity can be measured using the chromogenic substrate *p*-nitrophenol glucopyranoside (PNPG), which is a quick and relatively sensitive assay (Oda *et al.*, 1993; Sheu *et. al.*, 1994).

Enzyme activity is quantified by measuring the release of the leaving group, *p*-nitrophenol, which in its ionised form is a good chromophore, with a molar extinction coefficient of 18,300 at 400nm; the p-nitrophenyl group is widely used in aryl esters of amino acids and other related compounds, and exploited in colorimetric assays. It is subject to nucleophilic attack by OH^-, so the pH of the final assay mixture should not be too basic, and amine buffers such as Tris-HCl should not be used (Reese, *et al.*, 1968).

3.2 PNPG Assays - Experimental Work

The linearity of the PNPG assay, with respect to the rate of product release at a fixed substrate concentration of 5mM, was examined using a purified α-glucosidase preparation derived from bakers yeast (Sigma no: G5003). Duplicate tubes containing a mixture of substrate (PNPG) in 10mM phosphate buffer, pH 6.8, and 200µl of the enzyme at a concentration of 0.001U/ml, were incubated for fixed time intervals at 37°C, (one unit liberates 1.0µmole of D-glucose from PNPG per min). The assays were then stopped by the addition of 0.2M disodium tetraborate and the absorbance read at 400nm (section 2.10.1). Figure 3.1 shows the progress curve of α-glucosidase activity against time, for this experiment.

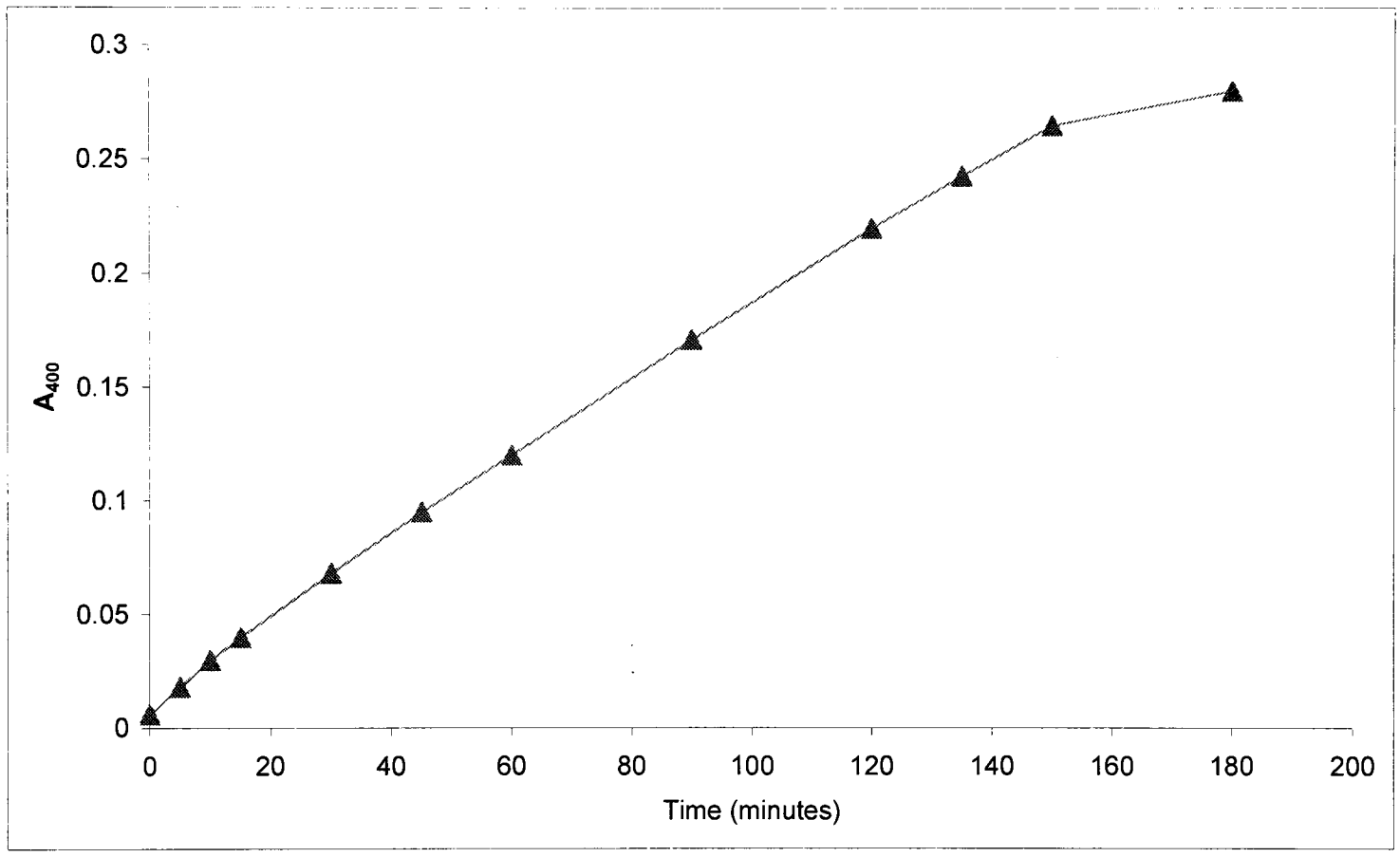
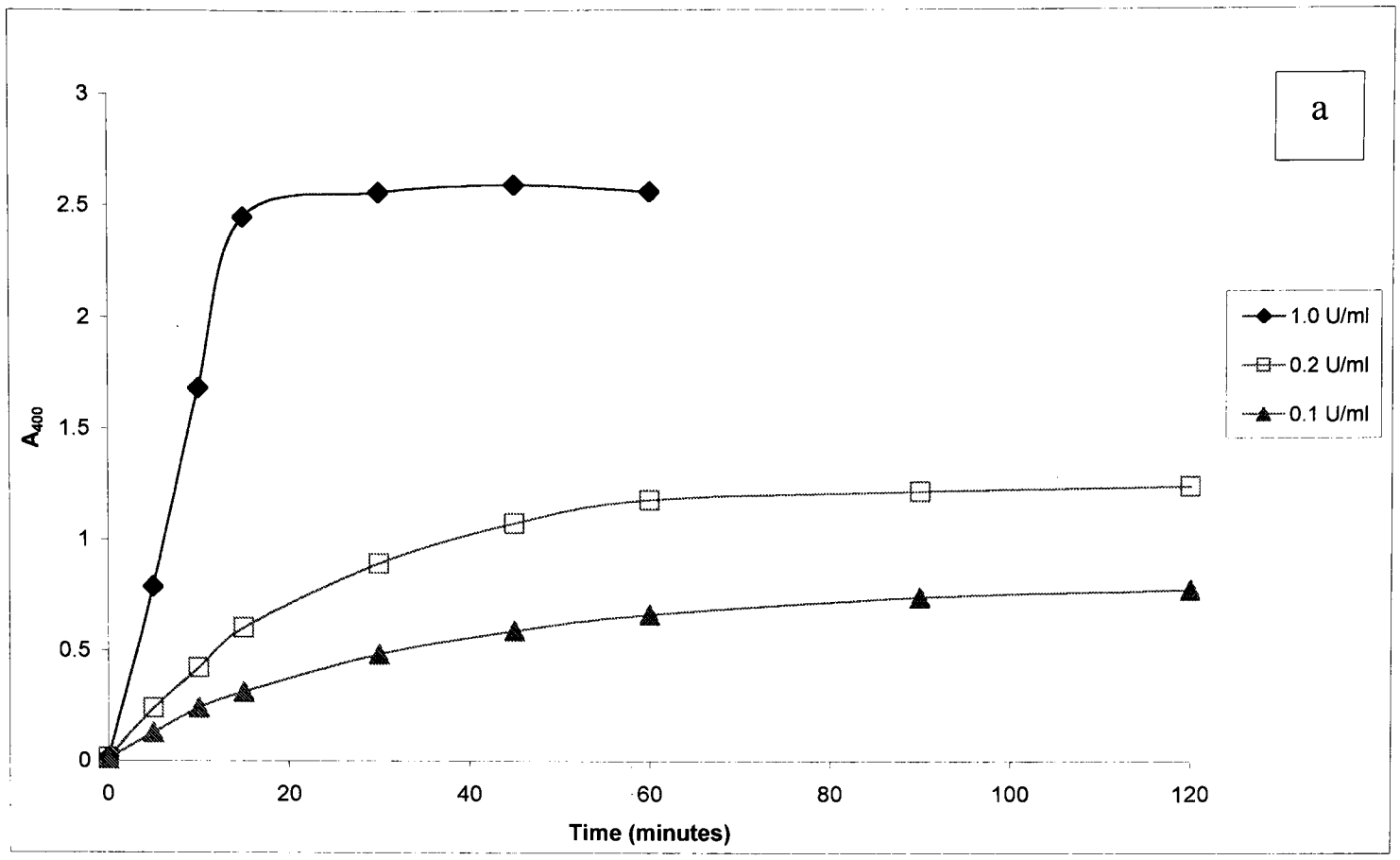


Figure 3.1: A typical progress curve using purified α-glucosidase Type 1: from baker's yeast (Sigma). *Each assay tube contained 1ml of 5mM PNPG, in 10mM phosphate buffer, pH 6.8 with the final enzyme concentration in each assay tube equivalent to 0.001U/ml. All assays were performed at 37°C. Datum points are the mean of two values.*

From the graph it can be seen that the assay was essentially linear over a two and half-hour incubation. This experiment was extended, using different enzyme concentrations at the same fixed substrate concentration of 5mM PNPG, to confirm linearity between the initial rate and enzyme concentration.



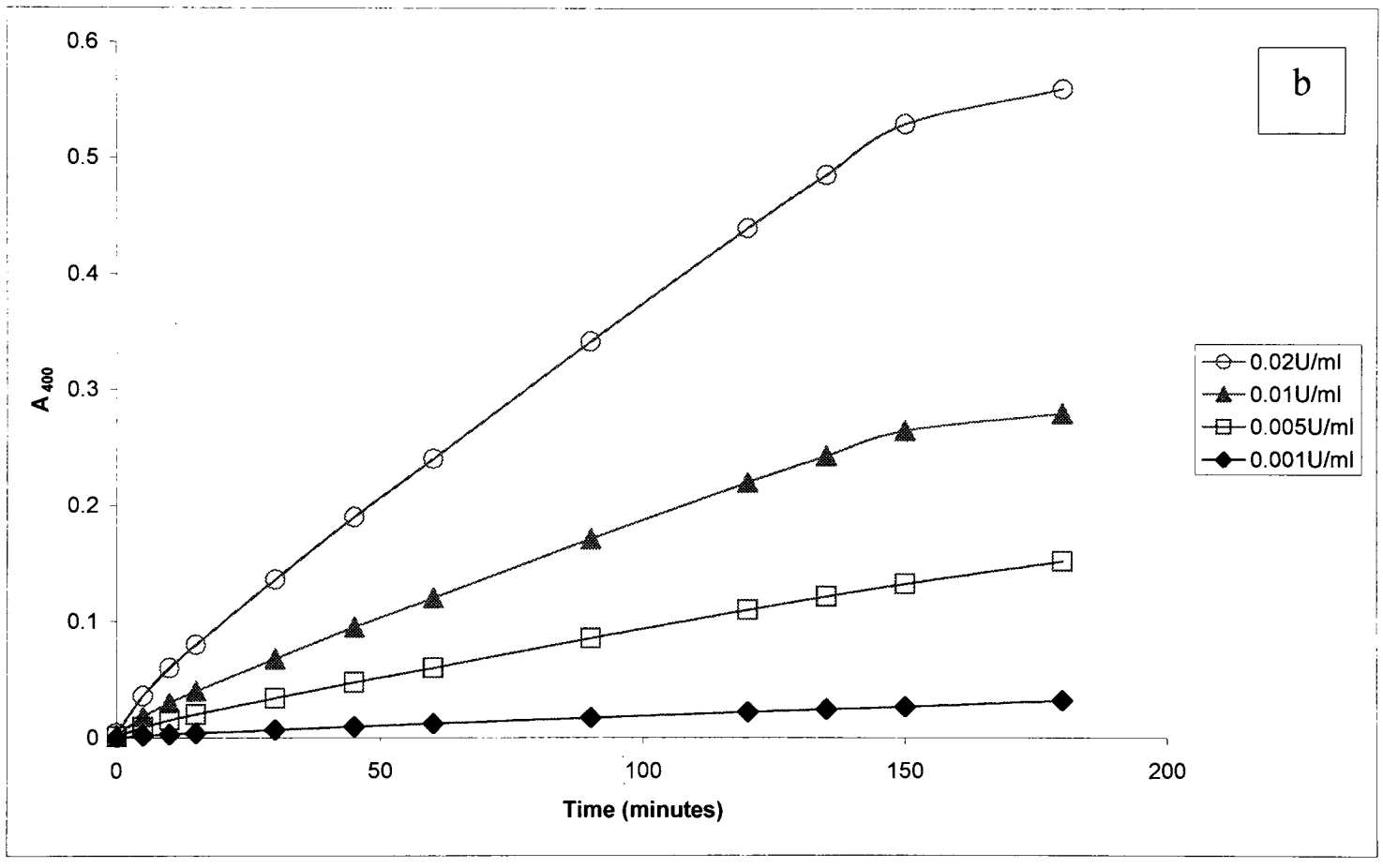


Figure 3.2(a) and (b): Activity progress curves using purified α-glucosidase (from Baker's yeast), at different enzyme concentrations from (a): 1-0.1U/ml and (b): 0.02-0.001U/ml. *One unit liberates 1.0μmole of D-glucose from PNPG per min.*

From the results shown in Figure 3.2(a), it can be seen that at the higher enzyme concentrations the assay was linear for ten to fifteen minutes, but then the substrate concentration became rate limiting. At lower enzyme concentrations – 0.02-0.001U/ml – Figure 3.2(b)), the progress curves were linear for 2 hours.

These findings suggested that for samples containing between 0.02-0.001U/ml, a two-hour incubation at 37 °C at a fixed substrate concentration of 5mM PNPG in 10mM sodium phosphate buffer, pH 6.8, are appropriate conditions for assaying α-glucosidase activity during the course of purification from *L. starkeyi*. Figure 3.3, shows that, for rates determined in the first fifteen minutes, under the given conditions, there is a linear relationship between enzyme activity and enzyme concentration up to 0.02U/ml.

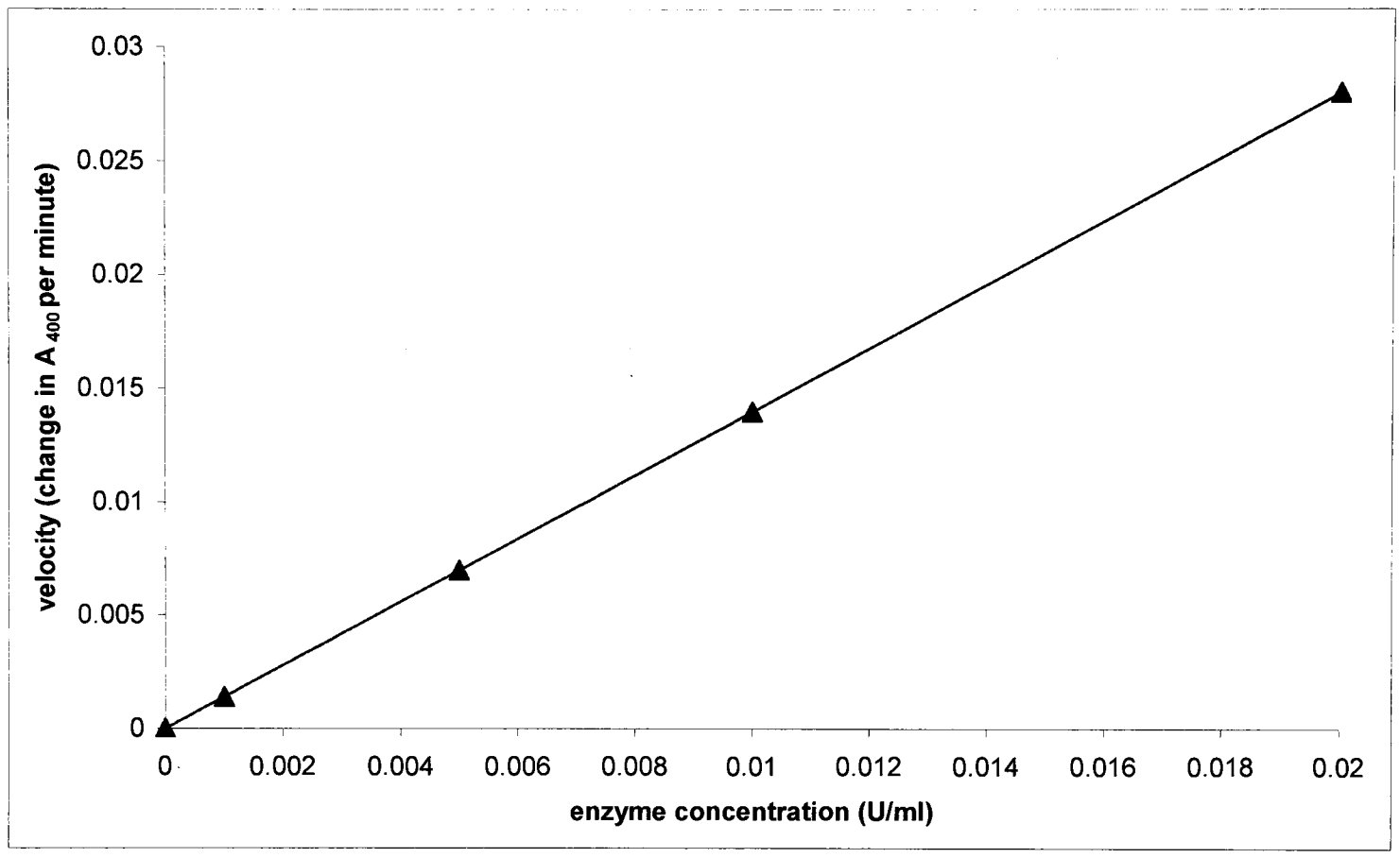


Figure 3.3: Plot showing initial velocity versus enzyme concentration. *One unit liberates 1.0μmole of D-glucose from PNPG per min.*

3.3 α-Amylase Assays

Traditional α-amylase assays are based on measuring a decrease in viscosity of starch solutions, a decrease in turbidity of a starch suspension, or a decrease in starch-iodine colour intensity (Ceska, 1969). However, a number of novel assays have been developed recently; in some of these, p-nitrophenyl glycosides are used as α-amylase substrates. For example, the Sigma Diagnostics Amylase Reagent (577-10) uses 4,6-ethylidene{G_7}-α,D-maltoheptaoside {ET-G_7PNP}as a substrate: α-amylase hydrolyses this substrate to release G_2-, G_3-, and G_4PNP fragments. α-Glucosidase incorporated into the kit, then hydrolyses G_2PNP and G_3PNP to yield p-nitrophenol and glucose. The release of p-nitrophenol is measured at 405nm and is directly proportional to the α-amylase activity in the sample.

Relevant to the work here, on an incompletely characterised system, is that non-α-amylase activities might be present (for example glucoamylase), which could act directly on the ET-G_7PNP. The second consideration is cost - coupled enzyme kits can be expensive when developing a new purification scheme.

Another novel α-amylase assay uses Phadebas: a cross-linked covalently dyed starch substrate from Pharmacia. This product is primarily intended for clinical α-amylase determinations; it is reported to be specific for α-amylase activity - being resistant to exo-acting hydrolysis by glucoamylases and β-amylases (Pharmacia Diagnostics, technical product insert: 52-1393-00/4). This well established assay was therefore the method of choice for quantitative detection of α-amylase activity during the analysis of the *Lipomyces* amylolytic system reported here. However, the cost of the Phadebas substrate was of some concern, so some experimental work on the feasibility of "in-house" preparation of a chromogenic α-amylase substrate was undertaken.

3.3.1 Synthesis of Chromogenic α-Amylase Substrate

Several attempts were made to produce a cheaper alternative to the Phadebas substrate, by dyeing soluble starch with Cibacron Blue, and cross-linking the product with 1,4-butanediol diglycidyl ether, following the work of Ceska *et al.*, (1969).

A 10% (w/v) suspension of soluble starch was prepared in deionized water and 0.1g of Cibacron Blue 3GA was added and then mixed under alkaline conditions - by adding 0.5ml NaOH of a 10M NaOH stock. This mixture was stirred magnetically overnight at room temperature, resulting in the formation of covalent bonds between the starch molecules and Cibacron dye molecules. The starch was then cross-linked by the addition of 1ml of 1,4-butanediol diglycidyl ether, and the mixture was then allowed to swell and was again stirred overnight at room temperature. The mixture was then extracted with industrial methylated spirits (IMS) to remove any unbound dye. If the blue-starch polymer had been successfully crosslinked, then a precipitate was formed (however, if the solution went cloudy on the addition of IMS, then the solution was judged not to have been successfully cross-linked and the material was discarded). The resulting insoluble blue starch polymer was then recovered by filtration, washed three times with distilled water, and then dried overnight in a temperature controlled cabinet at 37°C. After 24 hours, the dyed starch polymer was transferred to a sterile 15ml centrifuge tube and stored at room temperature.

Table 3.1 summarises the results of α-amylase assays using three different batches of dyed starch polymer. The assays were performed in triplicate: each tube contained 29mg dyed starch, 50U of α-amylase activity from *L. starkeyi* (collected after hydrophobic interactive chromatography and prepared in 0.2ml of sodium phosphate buffer), and 1.3ml of deionized water. A negative control tube was also set up with 29mg of dyed starch and 1.5ml of deionized water. Another tube was set up with 29mg of dyed-starch polymer and 50U of α-glucosidase activity. This tube was intended to confirm the specificity of the polymer and its use as an appropriate substrate for α-amylase assay determinations. The assays were then performed as described in section 2.10.2.

Table 3.1: Carbohydrase assays using three batches of Cibacron Blue dyed-starch polymer.

	Batch 1	Batch 2	Batch 3
Blank – no enzyme	off scale	0.002	0.004
α-Amylase activity (U)	off scale	0.010	0.120
α-Glucosidase activity (U)	off scale	0.002	0.004

Each batch was tested for α-amylase and α-glucosidase activity (sections 2.10.2 and 2.10.1 respectively).

Although background absorbance readings were detected in all tubes, including the blank and α-glucosidase-containing tubes, the assay was partially successful and appeared to be specific for α-amylase as compared to α-glucosidase. However, from the results in Table 3.1, it is clear that the process for producing a consistent quality of dyed starch polymer was unreliable.

When using Batch 1, within ten minutes of incubation at 37°C all four tubes including the negative control were blue. Excess dye not bound to the starch molecules apparently leached from the substrate into solution, or it was possible that this batch of substrate was not stable for long periods of time at 37°C.

Although it was possible to successfully cross-link and insolubilise the starch molecules, problems arose at the initial step of covalently binding the starch to Cibacron Blue. There was considerable batch-to-batch variability in the amount of dye that was covalently attached to the starch. This often resulted in very pale blue dyed-starch complexes, as in Batch 2.

This, and the problem of (slow) leaching of unbound dye from the starch causing high absorbance in enzyme-free controls, led to the abandonment of this approach. The Phadebas method was selected for all (routine) α-amylase estimations undertaken in this work.

3.4 Determination of Assay Specificity

Experiments were conducted to verify the specificity of the selected α-glucosidase and α-amylase assays; comparisons were also made with a maltose-based α-glucosidase assay and a glucoamylase assay. The results are shown in Table 3.2.

Table 3.2: Specificity of PNPG and Phadebas assays for α-glucosidase and α-amylase enzyme activities.

	Enzyme Activity (Absorbance Units/Time)		
Assay	**α -Glucosidase**	**α-Amylase**	**Glucoamylase**
α-Glucosidase assay using PNPG	2.64	0.005	0.015
α-Glucosidase assay using maltose	0.047	0.002	0.007
α-Amylase assay using Phadebas	0.030	1.125	0.050
Glucoamylase assay using maltotriose	0.000	0.000	2.090

α-Glucosidase enzyme activity using PNPG was measured as described in section 2.10.1. Section 2.7 describes assay conditions when measuring α-glucosidase using maltose. α-Amylase enzyme activity was measured as described in section 2.10.2. Glucoamylase enzyme activity was measured using the substrate maltotriose with a coupled hexokinase assay, as described in section 2.7. The four assays were compared using purified preparations of α-glucosidase, α-amylase and glucoamylase, diluted with 10mM sodium phosphate buffer, pH 6.8 to 0.2U/ml.

Evidently, the PNPG assay is a sensitive assay for α-glucosidase - more so than the maltose-based assay, and it does not respond significantly to purified α-amylase and glucoamylase preparations. The α-glucosidase, purchased from Sigma and derived from Baker's yeast, possessed a higher activity towards PNPG than maltose (2.64 absorbance units/time for PNPG compared to 0.047 absorbance units/time when using maltose as the substrate). Glucoamylase did not significantly hydrolyse this substrate and α-amylase activity appeared to be repressed in the presence of maltose (possibly end-product regulated).

Fungal glucoamylase can cleave PNPG (0.015 absorbance units/time), but at a neutral pH the rate of hydrolysis is slow, this is possibly due to possessing a different catalytic

reaction mechanism from α-glucosidase (Chiba, 1997) or simply be due to poor binding.

The Phadebas α-amylase substrate was readily cleaved by the purified α-amylase (1.125 absorbance units/time), but not by the α-glucosidase or glucoamylase. Furthermore, the glucoamylase assay appears to be highly specific to glucoamylase (2.09 absorbance units/time).

Thus, the two assays chosen for use in the bulk of the work undertaken here appear to be reasonably sensitive and selective for α-glucosidase and α-amylase activities.

3.5 Growth and Production of α-Glucosidase and α-Amylase Activities

3.6 Introduction

Several groups have reported on the growth conditions and production of carbohydrases from *Lipomyces starkeyi.* Moulin and Galzy (1979), were the first to explore a strain of *L. starkeyi* (CBS 1807), and demonstrated the presence of α-amylase activity when using soluble starch in the growth medium.

Using a different strain of *Lipomyces starkeyi*, CBS 1809, Kelly *et al.,* (1985) detected the existence of distinct α-glucosidase and α-amylase activities. Initially, they examined the effect of different carbon sources on enzyme production. Replacing 2% (w/v) starch with 2% (w/v) of each of a range of carbohydrates, they found that the highest yields of α-glucosidase were obtained with cellobiose, pullulan, and to a lesser extent, with starch, maltodextrin 01913, and maltose. No activity was detected with glucose, sucrose, fructose or lactose. Highest production of α-amylase was obtained with starch, maltodextrin 01913, and maltose as carbon sources. No activity was induced using sucrose and pullulan, and any minor activity induced by other sugars disappeared after 48 hours.

Kelly *et al.* also examined the effect of different nitrogen sources on enzyme production by *L. starkeyi*. They discovered that soya bean meal gave the highest yield for α-amylase, and the highest α-glucosidase activity was obtained with yeastex and corn steep liquor. Eventually they developed a special complex medium, combining maize starch and soya bean meal, for optimum production of both enzymes.

Punpeng *et al.,* (1992) reported the existence of an α-amylase and a glucoamylase from a strain of *L. starkeyi* isolated in Thailand from loogpang lauw, a traditional fermented food. The strain was grown for enzyme production on a specially modified medium of 2% potato starch, 1% yeast extract, 1% peptone, at 15°C, with both enzyme activities detected extracellularly after two days and reaching a maximum after six days of growth. The culture was harvested after seven days. The two enzyme activities were isolated from the growth medium by a 60% (w/v) ammonium sulphate fractionation.

Oda *et al.* (1992), purified an α-glucosidase from the yeast *Torulaspora pretoriensis* YK-1. The yeast was grown to late logarithmic phase in 400ml of a medium containing 2% bactopeptone, 1% bacto-yeast and 2% maltose in a 2L Erlenmeyer flask at 30°C.

3.7 Cell Growth and Secretion of Enzyme Activity in Rich and Minimal Media

Growth, α-glucosidase and α-amylase secretion by the yeast, *L. starkeyi* (NCYC 1436) was investigated using standard yeast culture media. Between 1 and 5 litres of culture media were inoculated with the standardised inoculum (section 2.2). 3ml aliquots were withdrawn daily from each flask and checked for bacterial contamination. A number of protein and enzyme assays and tests were then performed on the sample (sections 2.8 and 2.10). The pH of the culture medium was measured; the number of cells per ml counted using a haemocytometer (section 2.2.2), and the absorbance of the sample at 620nm was also determined, as a parallel indication of cell density, calibrated by the cell counts.

α-Glucosidase activity present in the culture supernatant was assayed by the PNPG assay, (section 2.10.1) and α-amylase activity was measured using the Phadebas assay (section 2.10.2). Protein assays were attempted using the $A_{280,}$ Bradford and BCA methods (section 2.8), but the high background from the rich medium was a problem and the data are not presented here.

The growth and carbohydrase secretion patterns of *Lipomyces starkeyi* were measured on five separate occasions. Figure 3.4 illustrates a typical growth curve pattern observed where it was found that the culture entered log phase after one to two days of growth on YEPS and stationary phase after six to seven days. Figure 3.5 illustrates the typical secretion patterns of both enzymes measured in more than five batch culture experiments.

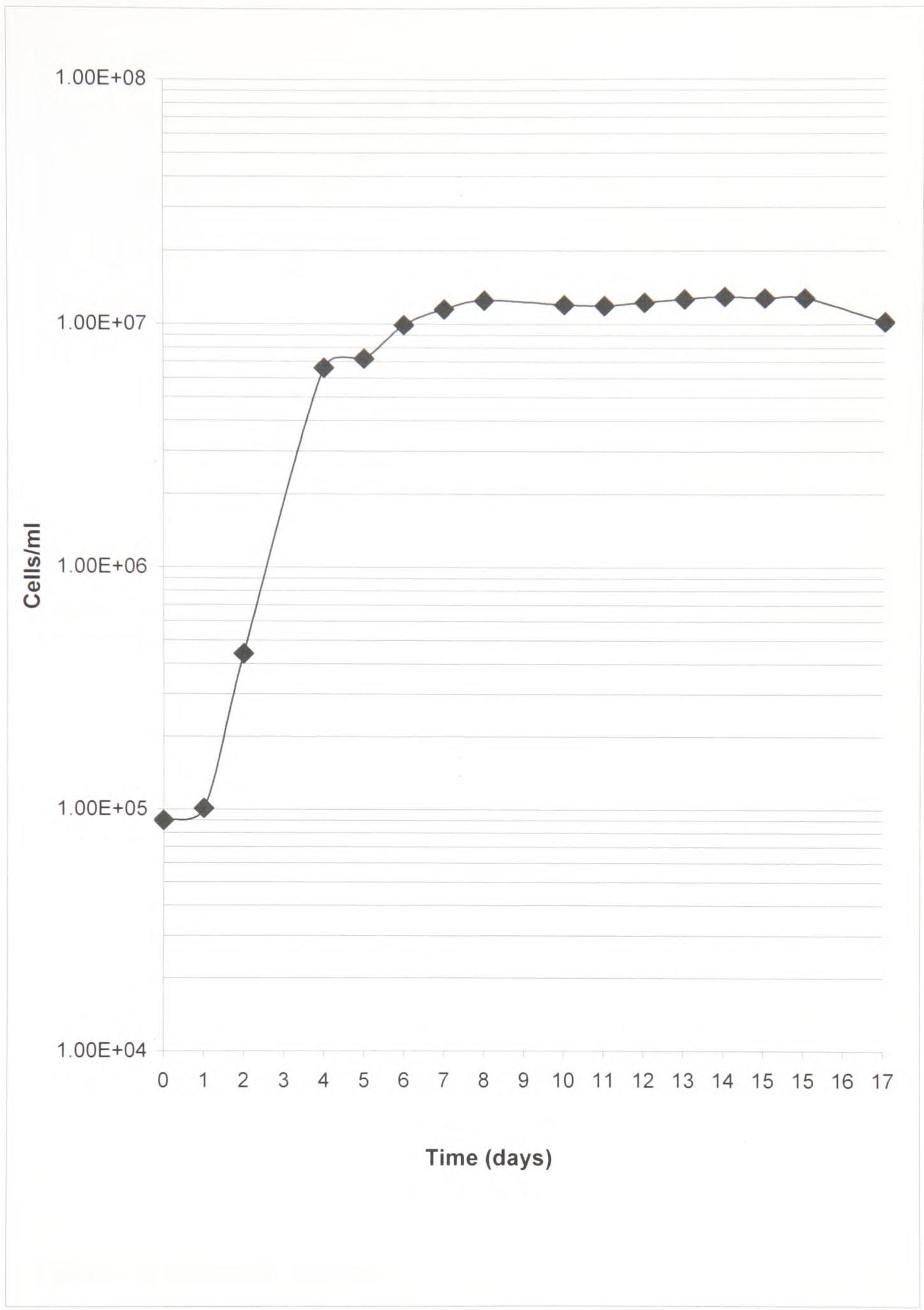


Figure 3.4: Growth curve of *Lipomyces starkeyi* NCYC 1436

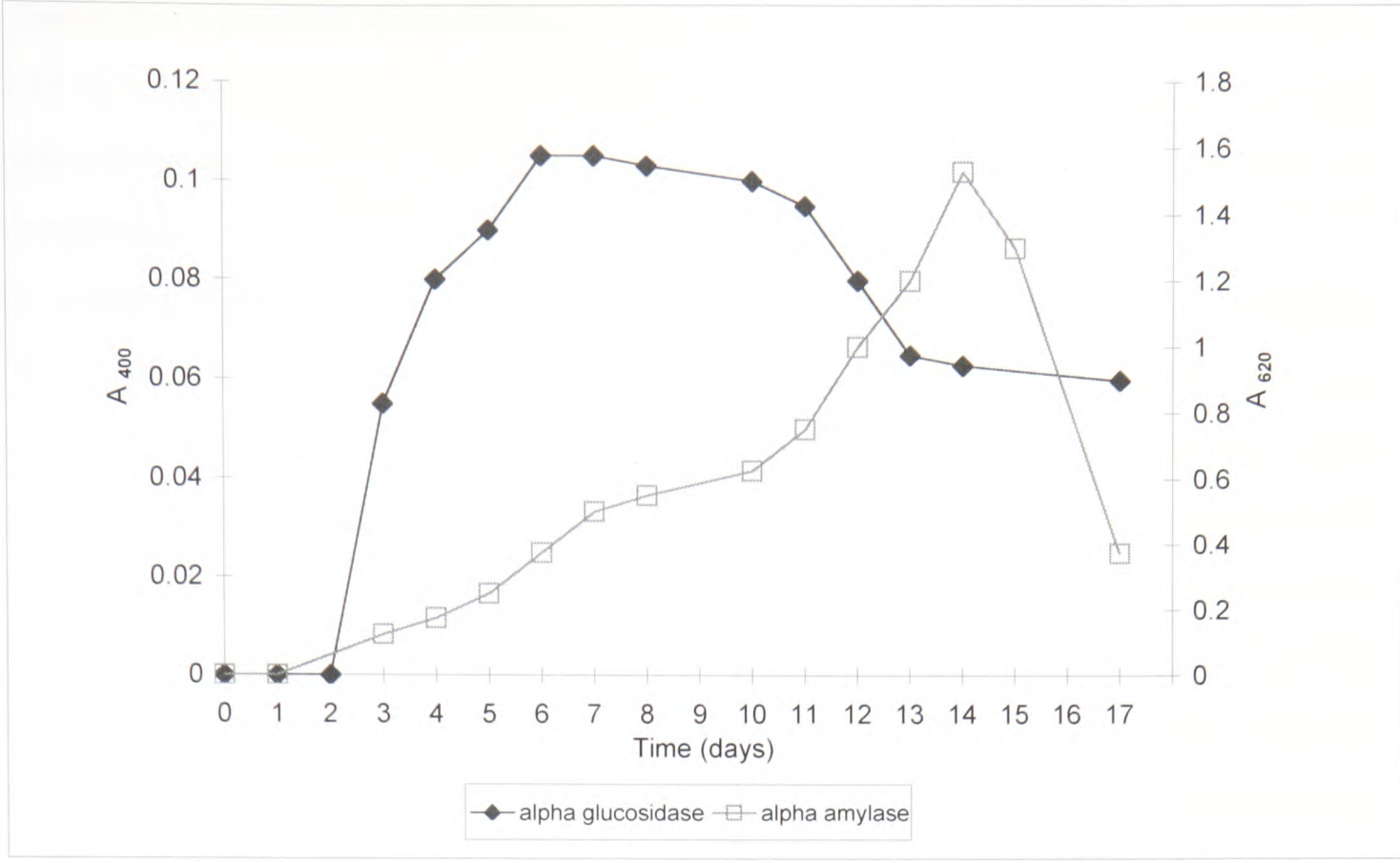


Figure 3.5: α-Glucosidase and α-amylase activity in a *Lipomyces starkeyi* (NCYC 1436) starch medium batch culture over a 17-day period.

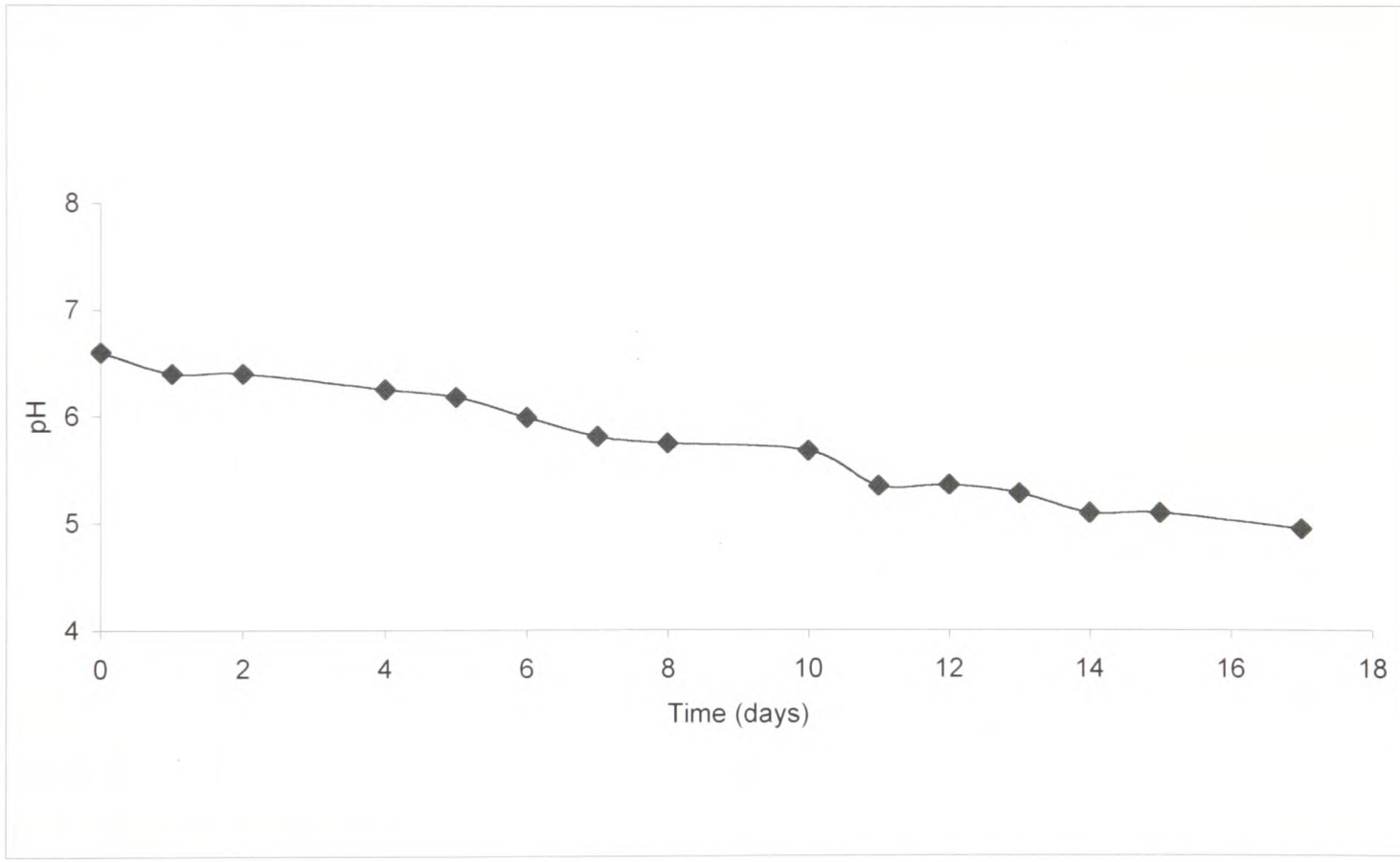


Figure 3.6: pH profile of a culture of *Lipomyces starkeyi* NCYC 1436 in YEPS.

The batch culture reached stationary phase after six days (Figure 3.4) and the pH dropped to below 6.0 (Figure 3.6). α-Glucosidase activity was detected after two days of growth and reached a peak around six to seven days. α-Amylase activity was

observable from three days with maximum activity seen around fourteen days (Figure 3.5). It is clear that peak levels of α-glucosidase in the culture medium appear several days before the maximum levels of α-amylase, though the reasons for this pattern are not obvious.

In contrast, when Kelly *et al.* used their complex medium of maize starch and soya bean meal, they reported that α-glucosidase and α-amylase were both detected extracellularly after 20 hours; 33% of α-glucosidase and 100% of α-amylase being extracellularly located, whereas 52% of α-glucosidase was associated with the cell wall and 15% found intracellularly. After 44 hours, 70% of the total α-glucosidase activity was detected extracellularly, 10% was present intracellularly and only 20% was cell bound. At this time, all the α-amylase activity was still extracellular. After 44 hours, 96% of the total α-amylase was located extracellularly, with 4% located bound to the cell surface. No α-amylase activity was detected intracellularly.

The Kelly group found that the level of α-glucosidase activity increased sharply up to 168 hours, with the level of reducing sugars (maltose, maltotriose and higher dextrins) increasing up to 72 hours, then rapidly decreasing as fermentation reached its end-point. α-Amylase activity increased linearly from 24 to 120 hours and the pH fell during the growth period from an initial starting pH of 6.8 to a final pH of 5.8 after 168 hours.

These results, as well as those reported here, differed with those found by Moulin and Galzy (1979), where the culture was harvested after 5-7 hours, and α-amylase activity was not detected extracellularly.

For enzyme purification purposes, growth of *Lipomyces starkeyi* cells on a minimal medium – protein/peptide free - could be preferable to rich medium for the isolation of extracellular enzymes, as there should be fewer contaminants. Simple trials were set up to directly compare growth and enzyme accumulation patterns on MMS (minimal medium consisting of 0.67% yeast nitrogen base without amino acids plus 2% (w/v) starch) and YEPS growth media, (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) soluble starch).

The growth curves are shown in Figure 3.7, and the culture levels of α-glucosidase and α-amylase in Table 3.3.

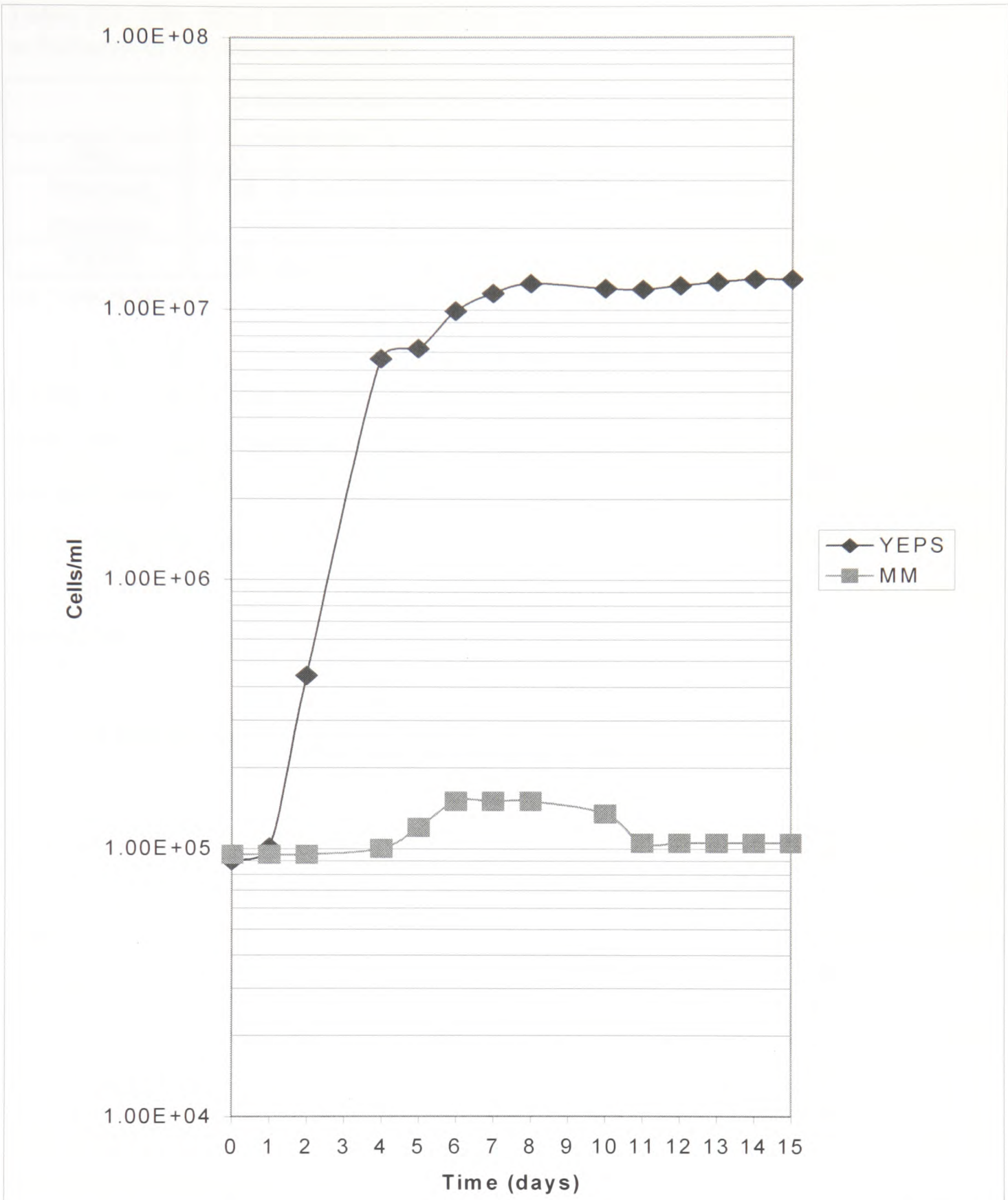


Figure 3.7: Growth curves of *Lipomyces starkeyi* NCYC 1436 on YEPS and Minimal Medium (MM).

Table 3.3: The effect of culture medium on levels of α-glucosidase and α-amylase in cultures of *Lipomyces starkeyi.*

	α-Glucosidase Activity (U)				α-Amylase Activity (U)			
Day	1	5	10	15	1	5	10	15
Minimal medium	nd	nd	nd	nd	nd	nd	nd	nd
YEPS	nd	0.74	0.67	0.44	nd	0.25	0.63	1.53

nd = not detected.

Figure 3.7 shows that growth in minimal medium was very poor so the lack of measurable α-glucosidase and α-amylase activity in the minimal medium cultures is not surprising. The results for the YEPS culture were consistent with the previous results (Figures 3.4 and 3.5) and confirmed that YEPS was an appropriate growth medium for *L. starkeyi* cultures intended for the isolation and purification of amylolytic enzymes.

3.8 Determination of the Presence of Cell-Bound α-Glucosidase Activity

Several groups have purified intracellular yeast α-glucosidases (e.g. Chiba *et al.,* 1973, Oda *et al.,* 1993) but until Kelly *et al.* published their findings on *L. starkeyi* (CBS 1807) in 1985, extracellular production of the enzyme by a yeast, had not been reported.

Kelly and colleagues found that substantial quantities of α-glucosidase were released into the starch culture medium by *L. starkeyi,* and that 20% of the total extracellular α-glucosidase activity was associated with the cell wall, being released by washing the cell pellet in phosphate buffer.

A previous study on the same strain of *Lipomyces starkeyi* used in the present work had shown the presence of cell-bound α-amylase activity (Bignell, PhD thesis). It was therefore thought worth examining the partitioning of α-glucosidase between the cell-bound and extracellular fractions in case large amounts of activity, useful for the enzyme purification work, were found to be cell-bound.

A culture of *L. starkeyi* was harvested after six days of growth on YEPS at 30°C. The cell-free supernatant was separated from the cell pellet by centrifugation at 6,300 x *g*. The cell pellet was then briefly washed in a small volume of double distilled water, weighed and then resuspended at a ratio of 10ml of 10mM sodium phosphate buffer, pH 6.8, per gram wet weight of cells. The wet weight yield of cells was about 26g per litre. Following this, the suspension was kept at 4°C and stirred overnight to extract any α-glucosidase activity loosely bound to the cell wall, (Kleinman *et al.*, 1988). The cell suspension was then re-centrifuged, and protein present in the supernatant was precipitated by ammonium sulphate (85% w/v), (salt precipitation was necessary as the enzyme activity in the supernatant and cell wash was too dilute to assay directly). Finally, the pellet and supernatant were dialysed against sodium phosphate buffer, to remove residual ammonium sulphate, resuspended in phosphate buffer pH 6.8 and assayed for α-glucosidase activity (as described in Section 2.10.1). As Table 3.4 shows, 69% of α-glucosidase activity was released into the medium. Using the same procedure it was found (Table 3.4), that 82% of the α-amylase activity was secreted into the cell-free supernatant - in the case of a batch culture harvested after fifteen days.

Table 3.4: Distribution of α-glucosidase and α-amylase activity in *Lipomyces starkeyi* (NCYC 1436)

	α-Glucosidase Activity U	α-Amylase Activity U
Extracellular activity recovered from the CFS after an initial precipitation with 85% (w/v) ammonium sulphate	128.2	16.0
Lipomyces starkeyi cells were then washed with distilled water, weighed and resuspended in phosphate buffer to extract activity associated with the cell wall, and protein was precipitated from the extract by a second 85% (w/v) ammonium sulphate precipitation		
Supernatant	nd	nd
Pellet	16.8	0.6
% Total enzyme activity released into the medium	69%	82%

nd = not detected; CFS = cell free supernatant

Brief washing of the cell pellet in double distilled water to remove any excess salt prior to the re-suspension in sodium phosphate buffer may have resulted in some loss of total recoverable extracellular enzyme activity, nevertheless, the data presented in Table 3.4, indicated that the bulk of both carbohydrases was released to the medium during growth.

It therefore seemed reasonable to use the – easily isolated - cell free supernatant as a starting point for enzyme purification, and ignore any cell-associated activity.

Chapter 4: α-Glucosidase and α-Amylase Isolation and Purification

4. 1 Ammonium Sulphate Precipitation

4.1.1 Introduction

Ammonium sulphate precipitation has been a common first step for the recovery of many extracellular enzymes from a cell-free supernatant. Salting out is an analytical technique, which utilises the unique hydrophobic characteristic of each individual protein. A typical protein has hydrophobic patches, side chains of isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan and valine. Forcing these amino acids into contact with water causes an ordering of water molecules, effectively freezing them directly around the side chains. This ordering of water molecules is thermodynamically unstable since it represents a large decrease in entropy compared with the unsolvated protein and free water molecules. As salt is added and salt ions become solvated, freely available water becomes scarce and there is a tendency to pull off the ordered "frozen" water molecules from the hydrophobic side chains. This causes an interaction and subsequent aggregation of protein molecules. Proteins with a large number or bigger clusters of non-polar residues will aggregate sooner than proteins with a few nonpolar surfaces (Scopes, 1987).

Punpeng *et al.,* (1992) precipitated the enzymes from the supernatant of a culture of *L. starkeyi (HN-606),* using a 60% (w/v) saturation prior to using a DEAE-Sephadex A-50 column. Prieto *et al.,* (1995), working on *Lipomyces kononenkoae* (CBS 5608), also used a 0-60% cut on the cell-free supernatant before applying the preparation to an affinity column packed with a cross-linked starch matrix.

In 1995, Steyn and Pretorius, working on a mutant strain of *Lipomyces kononenkoae* (IGC4052B), initially purified their α-amylase by a 0-75% (w/v) ammonium sulphate precipitation before adsorption chromatography using a Spectra/Gel Fast flow DEAE column.

However, Kelly *et al.* (1985) found that 0-80% (w/v) ammonium sulphate precipitation did not recover all the available enzyme activity from the cell free supernatant of *Lipomyces starkeyi* (CBS 1809). Their results differed from those of Moulin and Galzy

(1979), who found that ammonium sulphate precipitated the great bulk of enzyme activity. Only after using solvent precipitation with isopropanol (1 volume isopropanol to 1 volume CFS), prior to using a second precipitation with ammonium sulphate 0-50% (w/v) on the re-dissolved precipitate, were Kelly *et al.,* successful in recovering 90% of the available α-amylase and 70% of the α-glucosidase activity. These steps showed an overall 95-fold purification for the α-amylase and a 202-fold increase for the α-glucosidase.

Work carried out on other yeast species for the purification of intracellular α-glucosidases e.g. from *Saccharomyces cerevisiae,* has also used salt precipitation to recover the available protein from the supernatant of the crushed cell extract (Khan and Eaton, 1967; Matsusaka, 1977).

4.1.2 Results

After six days of growth on YEPS, the *Lipomyces starkeyi* NCYC 1436 cells were harvested, and the α-glucosidase and α-amylase activities were precipitated from the cell free supernatant as described in section 2.4.1. A variety of different percentage saturation ranges were initially attempted to precipitate the proteins of interest, the results of which are summarised in Table 4.1.

Table 4.1: α-Glucosidase and α-amylase activity after ammonium sulphate precipitation

Ammonium Sulphate Percentage Saturation Range	α-Glucosidase activity units (nmol/ml/min)	α-Amylase activity units (absorbance units/ml/min)
0-30	0	0
30-60	0	0
60-90	170	22
0-85	161	18

The results in Table 4.1 show that the 0-30% and 30-60% cuts were not successful in precipitating either carbohydrase. The third cut (60-90%) precipitated substantial amounts of both activities. A single precipitation step (85% (v/w)) was virtually as effective, and there was a clear advantage to precipitating the enzyme in one step rather than in two. Although salt precipitation did not separate the two components, a single salt precipitation step - 85% (w/v) - seemed a simple way to recover carbohydrase activities from the culture supernatant, also acting as a useful concentrating step prior to chromatography.

4.2 Solvent Precipitation

Solvent precipitation is a recognised method for extracting proteins from a solution. It works on the principle that adding organic solvent to a solution decreases the dielectric constant of the solution and hence increases electrostatic forces. Aggregation is caused by electrostatic and Van der Waals forces as hydrophobic attractions are less involved. Large proteins precipitate at lower solvent concentrations than smaller proteins. Temperature is a very important factor, as proteins are more readily deactivated at increased temperatures in the presence of organic solvents. Certain organic solvents are more effective at denaturing proteins than others; it has long been recognised that

longer-chain alcohols are more potent in this regard than short-chain ones. At low temperatures there is lack of conformational flexibility, so organic molecules cannot penetrate the internal protein structure and cause destabilisation. As the temperature rises to above 10°C, small organic molecules can enter though "cracks" in the surface that occur spontaneously due to natural flexing of the structure. Solvent molecules attach themselves through hydrophobic forces to internal residues such leucine, isoleucine, Tyrosine, phenylalanine and valine. Above 10°C, internal hydrophobic forces that help maintain protein structure need to be much stronger in maintaining the proteins' integrity, otherwise the loss of these hydrophobic interactions would lead to denaturation (Scopes, 1987).

Experiments were conducted to precipitate the protein from the cell-free supernatant by organic solvents, following the method reported by Kelly *et al.* (1985), and described in section 2.4.5.

Table 4.2: α-Glucosidase and α-Amylase activity after 50% (v/v) organic solvent precipitation using isopropanol.

	α-Glucosidase activity U *	**α-Amylase activity U ***
Pellet	0	8
Supernatant	149	10

** The precipitate was re-suspended in 10mM sodium phosphate buffer, pH 6.8 and then dialysed against this buffer prior to measuring enzyme activity.*

The results in Table 4.2 show that a 0-50% (v/v) saturation with isopropanol was ineffective in precipitating the α-glucosidase activity, and only approximately 44% of the α-amylase activity precipitated out. The white precipitate formed was difficult to resuspend, possibly due to the presence of denatured protein. The addition of more buffer to resuspend the precipitate diluted the sample and difficulties arose in re-concentrating it. Evaporating the solvent using a rotary evaporator was problematical as it was difficult to control the temperature, and as the temperature increased, frothing of the sample was seen. The sample was found to have lost activity – presumably because of denaturation. As these initial solvent precipitation results were unsatisfactory, this approach was discarded in favour of salt precipitation.

4.3 Preliminary Chromatographic Experiments

4.3.1 Introduction

Early purification experiments used ion exchange chromatography (IEC) with the weak anionic exchanger, DE52, from Whatman. The crude ammonium sulphate pellet was dialysed to remove the salt, and then concentrated as described in Section 2.4, prior to being applied to the ion exchange column. Enzyme recoveries in these experiments were poor, so anionic IEC was dismissed as a first column step - various other chromatographic approaches were then appraised. Reverse phase chromatography was not considered here, as harsh organic buffer systems – using, for example, acetonitrile - often denature the proteins of interest (Scopes, 1994), but affinity dye columns and gel filtration, were considered in some initial experiments. Affinity dyes were attempted as they offer a 'specific' affinity medium that is reusable, resistant to enzyme attack and low in cost. It is believed that affinity dyes mimic biological substrates (substrate co-factors and effectors) and thereby produce the affinity effect (Lowe *et al.*, 1981); because protein binding by affinity-dyes can often be highly specific, this approach has the appeal that a protein of interest may be highly purified in just one step.

In gel filtration chromatography, proteins can typically be separated according to their size and the molecular weight can also be determined (once the column has been calibrated with known standards). This seemed an appropriate approach to separate two enzyme activities. Hydrophobic interactive chromatography proved to be a useful technique in this work and is a relatively new approach in protein purification, and so is introduced in more detail in section 4.4.

4.3.2 Affinity Chromatography Results

Figure 4.1 shows the results when using a small-scale affinity dye column, Cibacron Blue 3GA, with a matrix of cross-linked 4% beaded agarose (section 2.11.4). The column was equilibrated with 10mM sodium phosphate buffer, pH 6.8 prior to adsorption of the enzyme preparation. The proteins were eluted by a series of steps of increasing ionic strength buffer.

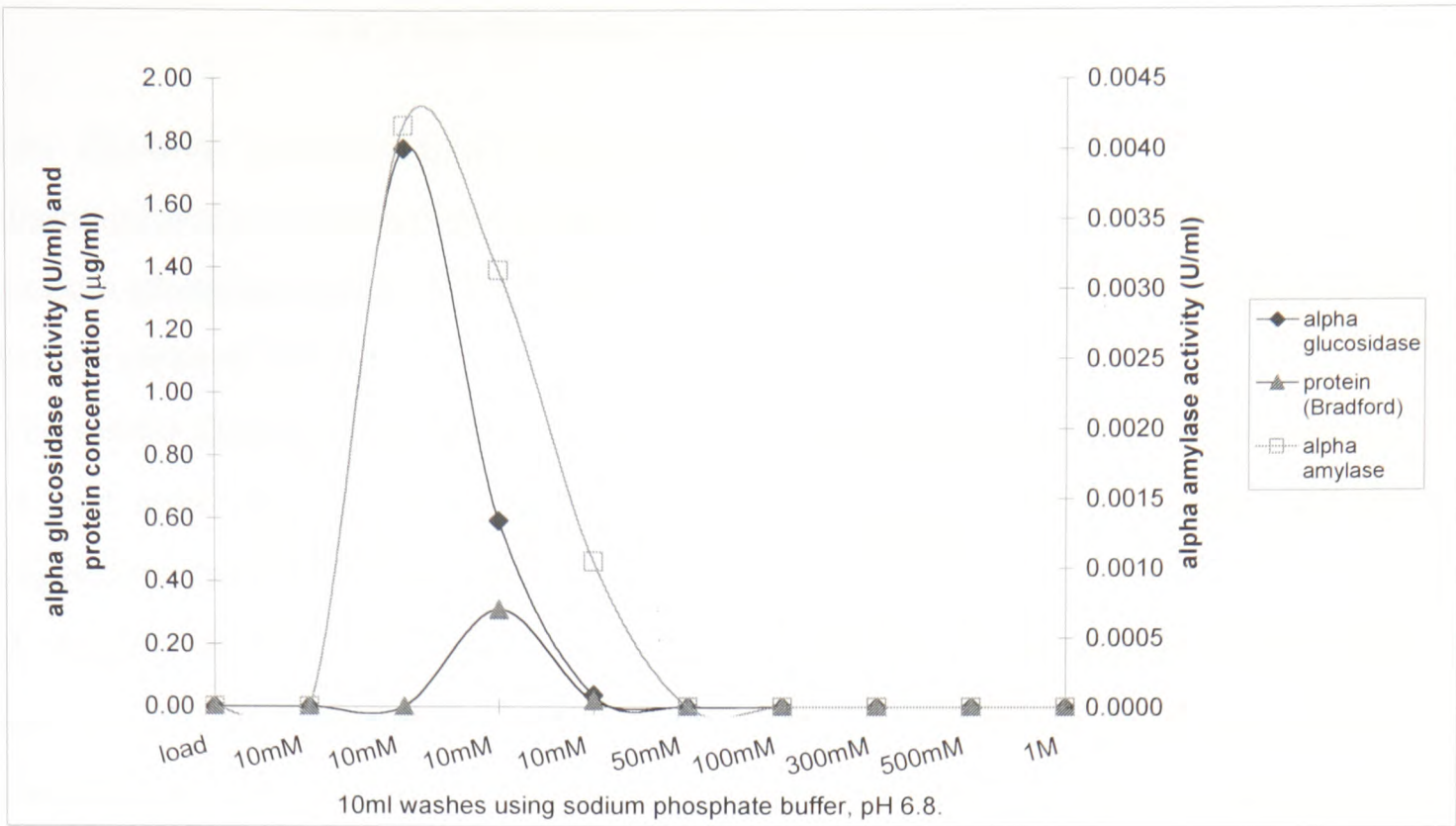


Figure 4.1: Affinity dye binding chromatography using Cibacron Blue 3GA

Both enzyme activities co-eluted with the column wash, though some purification was achieved, as the bulk of the inactive protein eluted separately with the third 10mM sodium phosphate buffer wash. Despite eluting with high ionic strength buffer washes, only 56% of the α-glucosidase, 33% α-amylase, and 26% of the total protein was recovered. The experiment was repeated and similar results were obtained.

The poor resolution was possibly attributable to other components in the crude sample, which appeared to be interacting with the column. The crude sample contained traces of yeast extract and peptone - capable of competitively binding irreversibly to affinity sites on the ligand. As the affinity column is charged at low ionic strength, the column could also act as anion exchanger and this would allow for the binding of a number of unwanted proteins. But using higher ionic strength buffer can also be problematical allowing undesirable hydrophobic interactions to occur. Therefore, an alternative approach would have been to use a pH gradient for elution.

Regardless of which elution method was followed, nonspecific adsorption is always a factor in affinity chromatography, unless a highly specific adsorbent (i.e. one with a unique ligand) is available.

Preliminary experiments with other affinity matrices were also disappointing - with problems of recovery and repeatability - so the affinity chromatography approach was not pursued.

4.3.3 Gel Filtration Chromatography Results

Gel filtration chromatography using Sephacryl 3000 was attempted with the re-dissolved dialysed ammonium sulphate pellet. The column was equilibrated in 10mM sodium phosphate buffer, pH 6.8 and the enzymes were eluted using an isocratic buffer system (section 2.11.3).

The results displayed in Figure 4.2, show that α-glucosidase and α-amylase co-eluted as two main activity peaks, though both activity profiles were complex, possibly suggesting multiple enzyme species.

A recovery of 68% total protein was achieved, and 95% of the applied α-glucosidase and 92% of the applied α-amylase were recovered. While the good recoveries of carbohydrase activities from the column were encouraging, the lack of resolution of the main α-amylase and α-glucosidase peaks indicated that gel filtration was not useful at this early stage of purification.

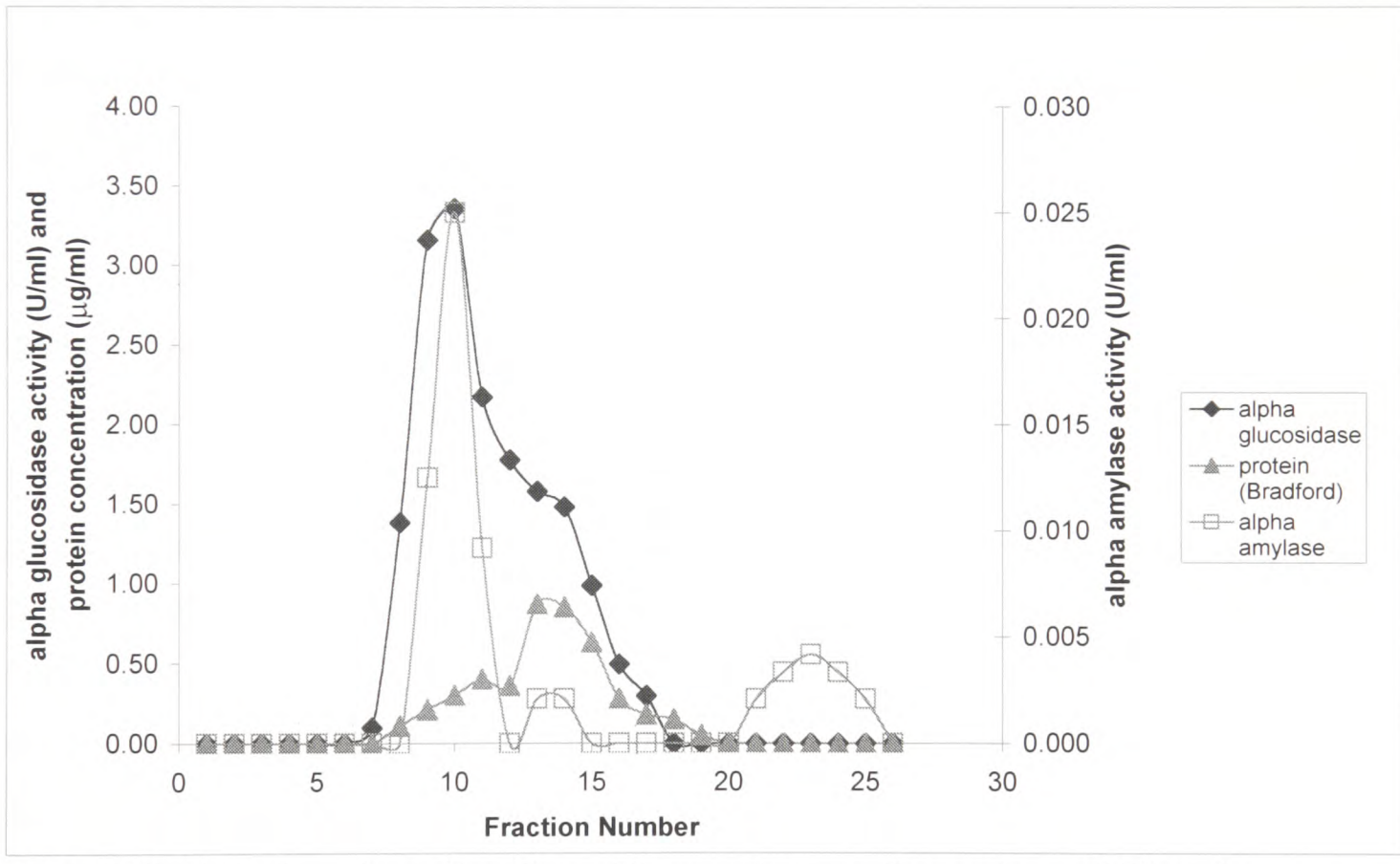


Figure 4.2: Fractionation of α-glucosidase and α-amylase using gel filtration chromatography. *The re-suspended ammonium sulphate pellet (~1mg protein, 16U α -glucosidase and 1.8U α-amylase) was loaded in a total volume of 3ml. Elution was carried out at a flow rate of 60ml/hour, and 1ml fractions were collected.*

4.4 Hydrophobic Interaction Chromatography (HIC)

4.4.1 Introduction

It has been estimated that as much as 40-50% of the accessible surface area of proteins is non-polar (Klotz, 1970; Lee and Richards, 1971). Hydrophobic Interactive Chromatography (HIC) exploits these differences in the surface hydrophobicity of proteins and peptides to separate biomolecules by virtue of the fact that in aqueous solvents, hydrophobic patches on proteins seek out other hydrophobic surfaces preferentially.

HIC requires minimal sample pre-treatment and can be used with traditional protein precipitation techniques and in combination with ion exchange chromatography. It fits in naturally after ammonium sulphate precipitation, where the sample has a high ionic strength, and if the sample is subsequently eluted in a low ionic strength buffer, it can often be directly applied to an ion exchange column without additional de-salting or dialysis steps. This makes HIC a versatile liquid chromatography technique and a logical part of a rational purification strategy.

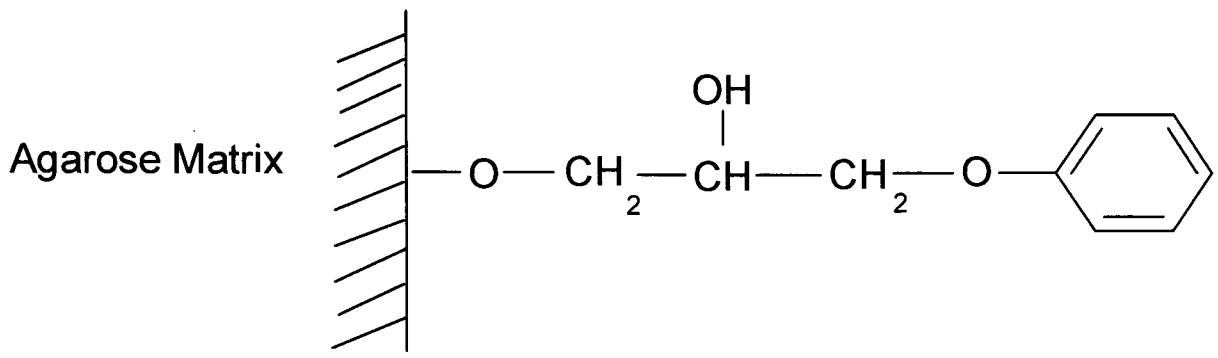
The type of ligand and the degree of substitution of the different HIC adsorbents are important factors in optimising hydrophobic interaction chromatography.

The type of immobilized ligand determines the primary adsorption selectivity (Hofstee, 1973). If the ligand is a straight chain alkyl (hydrocarbon), then the interaction is purely hydrophobic in nature. By contrast, aryl ligands show mixed mode behaviour, and it is possible for aromatic and hydrophobic interactions to occur (Hofstee and Otillio, 1978).

Most proteins should bind at a reasonable low salt concentration – around 1M - but if the protein doesn't bind then a medium of higher hydrophobicity is required. The protein binding-capacities of the HIC adsorbents increase with the increased degree of substitution of immobilized ligand and with the length of the alkyl chain. At a sufficiently high degree of substitution of phenyl groups per ml of gel, a plateau is reached where binding capacity of the adsorbent remains constant but the interaction continues to be very strong. Solutes bound under these circumstances are difficult to elute, due to multipoint attachment, and their migration is slow (Jennissen, 1978).

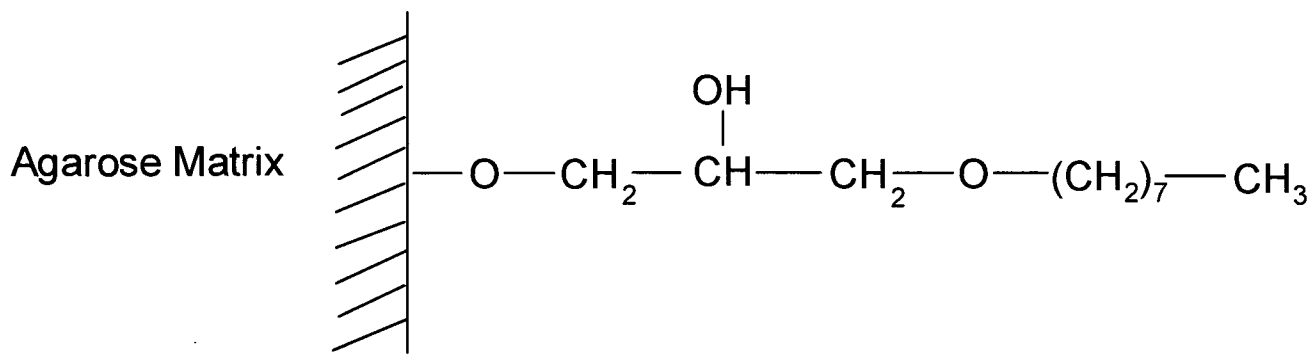
HIC media are manufactured using a glycidyl-ether coupling procedure, producing a hydrophobic gel that has no ionic properties and should allow for only hydrophobic interactions with proteins (Pharmacia Biotechnology, 1993). Figure 4.3 illustrates the straight chain alkyl ligand, Phenyl Sepharose CL-4B. All of the phenyl-based media (Phenyl Sepharose 6 Fast Flow (high substitution), Phenyl Sepharose Fast Flow (low substitution) and Phenyl Sepharose High Performance) have the same ligand backbone as Phenyl Sepharose CL 4B.

Figures 4.4 and 4.5 illustrate the alternate ligands of Octyl Sepharose CL-4B and Butyl Sepharose 4 Fast Flow.



Phenyl Sepharose CL-4B

Figure 4.3: Phenyl Sepharose CL-4B. *The ligand is covalently coupled to a cross-linked 4% agarose matrix by ether linkage. Adsorption to the matrix is via phenyl groups and is suitable for samples with unknown hydrophobicity.*



Octyl Sepharose CL-4B

Figure 4.4: Octyl Sepharose CL-4B. *Adsorption to the ligand depends solely on the number of hydrophobic amino acids in the proteins. This is typically used for the separation of proteins of low hydrophobicity and those labile at high ionic strength.*

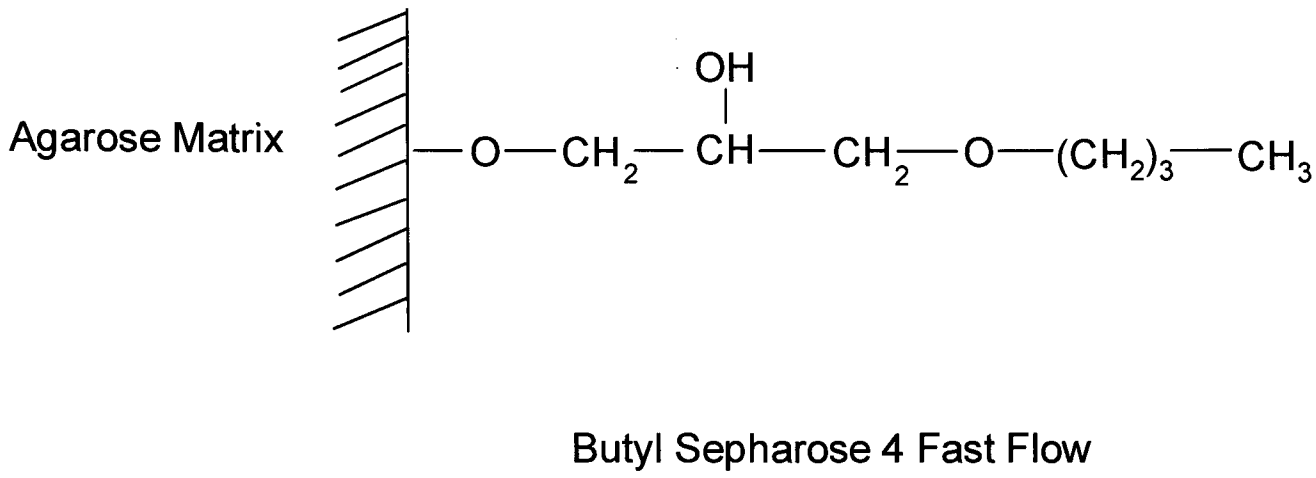


Figure 4.5: Butyl Sepharose 4 Fast Flow. *The ligand is covalently coupled to a cross-linked 4% agarose matrix by ether linkage. The adsorption and desorption mechanism is different from phenyl-based ligands; this ligand is used in the standard aliphatic matrices.*

The adsorption selectivity of the phenyl-based media is different from the other types of media, with adsorption occurring via aromatic amino acids - though π-π interactions. The retention of proteins is also affected by the presence or absence of surface charges on the sample molecules and also to the number of hydrophobic amino acids in the proteins, whereas, protein purification using Octyl Sepharose CL-4B (Figure 4.4) depends solely on the number of hydrophobic amino acids in the proteins.

The adsorption and desorption mechanism of proteins using Butyl Sepharose 4 Fast Flow (Figure 4.5), is different from octyl and phenyl based ligands: the butyl ligand is more hydrophobic than phenyl-based ligands and is a suitable medium for separating weakly hydrophobic proteins.

In all cases the hydrophobic matrices are physically stable, due to the highly cross-linked Sepharose backbone; this results in very good flow rates and reduced backpressures, hence, the term "Fast Flow".

4.4.2 Results from HIC Experiments

4.4.2.1 Hydrophobic Interaction Chromatographic Media – Screening Results

Selection of appropriate hydrophobic media (and method development work) was carried out on a small scale using six different media - Phenyl Sepharose CL-4B (which recommended for the separation of protein samples of unknown hydrophobic characteristics) and five alternatives in an HIC Media Test Kit (Pharmacia).

The media test kit consisted of five prepackaged 1ml HiTrap columns each containing a different adsorbent. Three of the five were phenyl-based ligands; Phenyl Sepharose High Performance, Phenyl Sepharose 6 Fast Flow (low sub), Phenyl Sepharose 6 Fast Flow (high sub). The final two adsorbents were Butyl Sepharose 4 Fast flow and Octyl Sepharose 4 Fast Flow. Each of the five kit media and the Phenyl Sepharose CL-4B adsorbents were tested as described in section 2.11.1.

The column performance of each adsorbent was not only assessed by the separation of α-glucosidase from α-amylase activity, but also by the removal of contaminating materials from the initial preparation, and by high enzyme recoveries.

Table 4.3 summarises the different media characteristics and outlines the results from the initial screening study.

Table 4.3: Phenyl Sepharose CL-4B and HIC Test Kit Media Characteristics.
Assessment of the (selectivity and capacity) of different HIC media in a purification scheme for α-glucosidase and α-amylase

Medium	Bead structure	Mean Particle size	Particle size range	Degree of substitution	Adsorption of α-glucosidase and α-amylase to ligand	Enzyme Recovery >55%
Phenyl Sepharose CL-4B	4%, cross-linked agarose, spherical beads	90μm	45-165μm	40μmol phenyl groups/ml gel	✓	✓
Phenyl Sepharose 6 Fast Flow (low sub)	6%, cross-linked agarose, spherical beads	90μm	45-165μm	20μmol phenyl groups/ml gel	✓	✓
Phenyl Sepharose 6 Fast Flow (high sub)	6%, cross-linked agarose, spherical beads	90μm	45-165μm	40μmol phenyl groups/ml gel	✓	✓
Phenyl Sepharose High Performance	6%, cross-linked agarose, spherical beads	34μm	22-44μm	25μmol phenyl/ml gel	✓	✓
Butyl Sepharose 4 Fast Flow	4%, cross-linked agarose, spherical beads	90μm	45-165μm	50μmol butyl groups/ml gel	×	×
Octyl Sepharose CL-4B	4%, cross-linked agarose, spherical beads	90μm	45-165μm	40μmol octyl groups/ml gel	✓	×

Of the three phenyl-based adsorbents supplied in the test kit, two - Phenyl Sepharose 6 fast flow (high sub) and Phenyl Sepharose (low sub) - displayed similar results in terms of higher selectivity and good recoveries compared to Phenyl Sepharose CL-4B. Flow rates were marginally slower compared to Phenyl Sepharose CL-4B, which was possibly due to differences in the bead structure, 6% cross-linked agarose gel compared to 4% cross-linked agarose for Phenyl Sepharose CL-4B. The 4% agarose is suitable for macromolecules up to $2.7x10^7$ whilst the 6% agarose is suitable for molecules up to $4x10^6$. Phenyl Sepharose High Performance medium - recommended by the manufacturer for use at an intermediate stage in the purification process - also

showed reduced flow rates and higher back-pressures when attempting to purify the ammonium sulphate pellet material.

With Butyl Sepharose 4 Fast Flow it was found impossible to elute the protein when using low salt buffer. The ligand binding is much more efficient at low salt concentrations, and as such the enzyme bound strongly to the ligand. Enzyme recoveries were poor when using Octyl Sepharose. Octyl Sepharose was suitable for weakly hydrophobic proteins and strongly hydrophobic proteins are not easily eluted from the column.

From the results obtained from the screening experiments, Phenyl Sepharose CL-4B was chosen as the preferred hydrophobic medium, for the initial purification of the ammonium sulphate pellet. Highly reproducible column performance, low cost and medium stability were all factors in the final selection.

4.4.3 Small to Medium Scale Hydrophobic Interaction Chromatography

After the initial screening of the different hydrophobic media, 1ml mini-columns were set up as described in Section 2.11.1, using the hydrophobic chromatographic medium, Phenyl Sepharose CL-4B. Working at room temperature, the columns were equilibrated using 1M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8, prior to adsorbing the resuspended ammonium sulphate pellet to the column. The column was then washed with two column volumes of the initial loading buffer (i.e. 1M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8), before the elution was carried out by sequentially washing the column with a decreasing salt step-gradient (Figure 4.6).

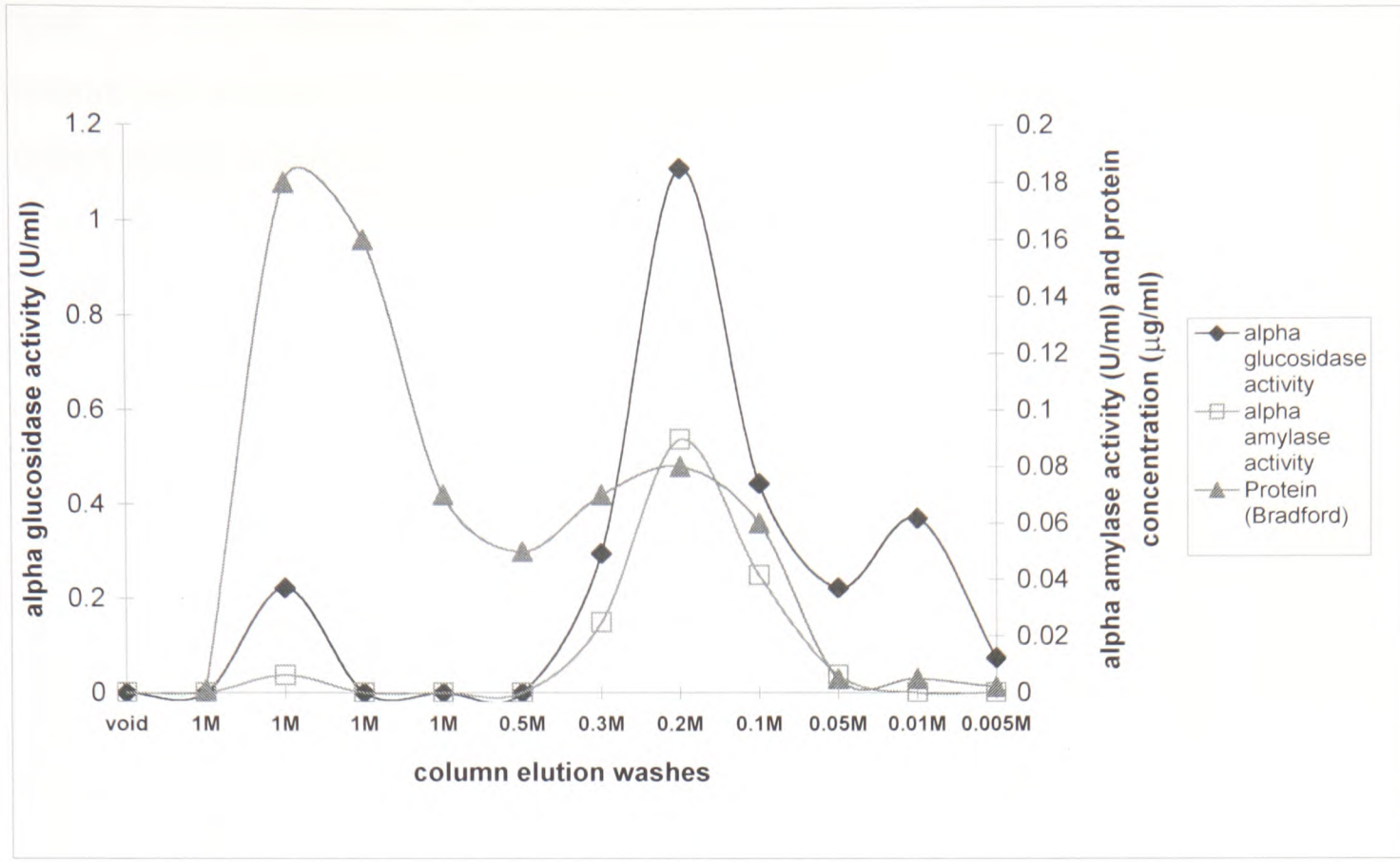


Figure 4.6: Batch elution of α-glucosidase and α-amylase from a Phenyl Sepharose CL-4B column, using a decreasing salt gradient of ammonium sulphate buffer. *Re-suspended ammonium sulphate sample (containing ~1.5mg of protein, 26U α-glucosidase and 3U α-amylase activity) was loaded and 1ml fractions were collected and assayed as in sections 2.10.1, 2.10.2 and 2.8.2).*

This experiment was repeated on four occasions with consistent results: the small-scale columns appeared to bind both the α-amylase and α-glucosidase, whilst consistently removing approximately 50% of the unwanted protein, either in the void volume or in the first few washes. However, the two enzyme activities also eluted at approximately the same salt concentration, in two broad coincident peaks with a second smaller α-glucosidase peak, significantly free of α-amylase activity, eluting separately at a lower salt concentration. This possibly indicates the presence of isoenzymes of both α-glucosidase and α-amylase.

Whilst the small scale Phenyl Sepharose CL-4B column did not result in a complete separation of the enzyme activities, it was effective in the removal of substantial amounts of unwanted proteins. Greatly increased resolution between the two enzymes was not expected at this stage, as the principle of the HIC method is similar to the salting-out technique, already carried out.

HIC using Phenyl Sepharose CL-4B was then scaled-up. The column procedure was scaled up in stages; firstly the column volume was increased 5-fold, (Figure 4.7): the cross-section of the column was increased without a significant increase in column

length. It was observed that as the sample loading was increased there was a proportional increase in elution time and the flow rate was adjusted. A typical column elution profile is shown in Figure 4.7.

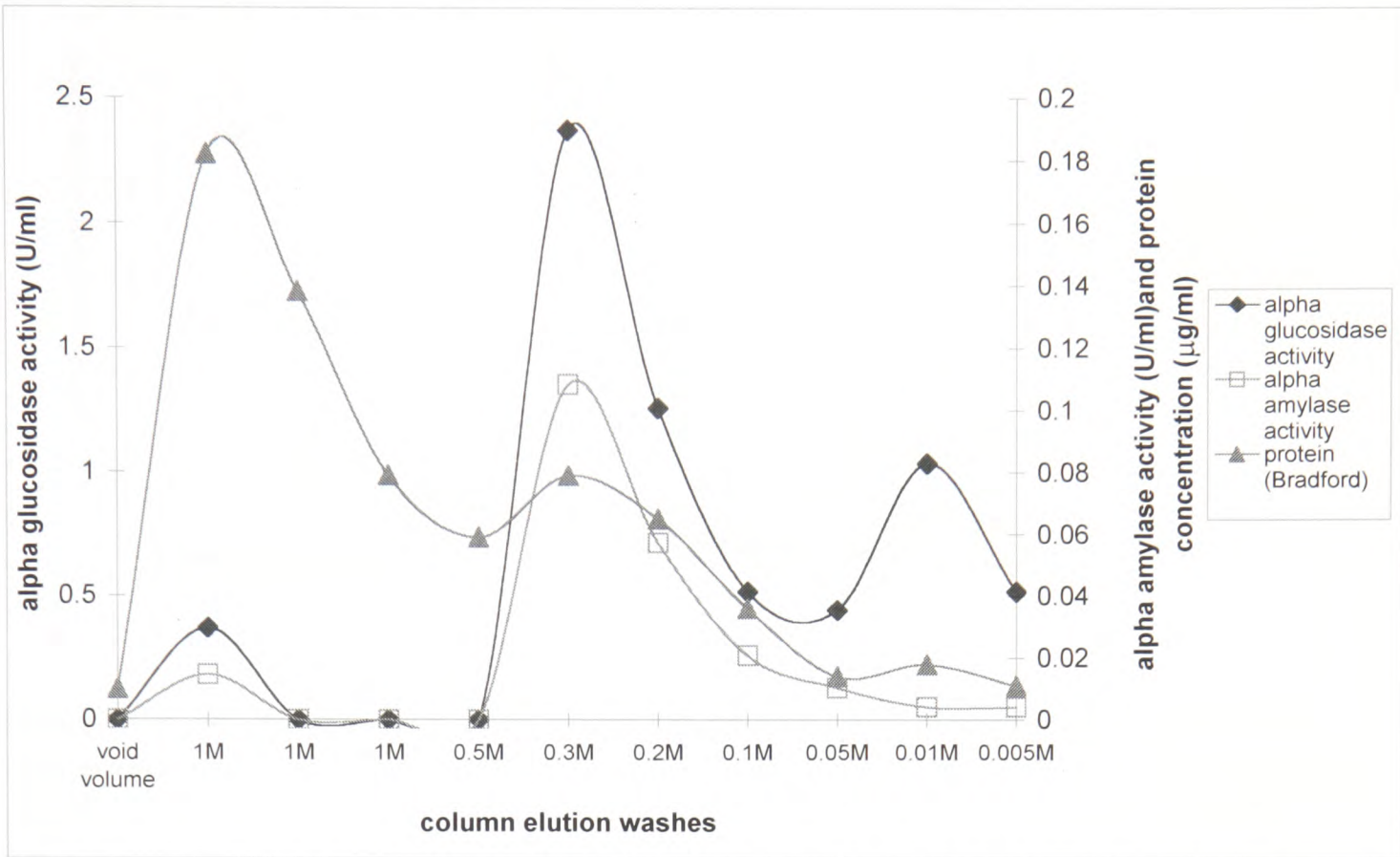


Figure 4.7: Intermediate (5-fold scaled-up) Phenyl Sepharose CL-4B column *(illustrating the batch elution profile of α-glucosidase and α-amylase enzyme activity).*

Scaling up the HIC step confirmed the initial findings that the α-glucosidase activity may in fact be composed of more than one species - or isoenzyme - of the same protein. The elution profile was identical to the small-scale column, with more than one peak associated with α-glucosidase activity. Some activity eluted from the column in the first 1M wash, possibly indicating that either the column was overloaded and that the maximum capacity had been reached or that the potential isozymes of both enzymes were not binding to the column. However, the column was effective in removing a substantial amount of protein (Table 4.4) and a further 4-fold column scale-up was attempted.

An intermediate column was considered appropriate for the application of the maximum intended load – derived from a 5L culture of *L. starkeyi* cells. However, batch elution for a column this size is inefficient, due to several, manual, time-consuming steps. Therefore, a linear gradient was attempted which would allow for the chromatography to take place in one step, and could improve the separation between the different enzyme activities. Representative elution profiles are shown in

Figure 4.8, 4.9 and 4.10.

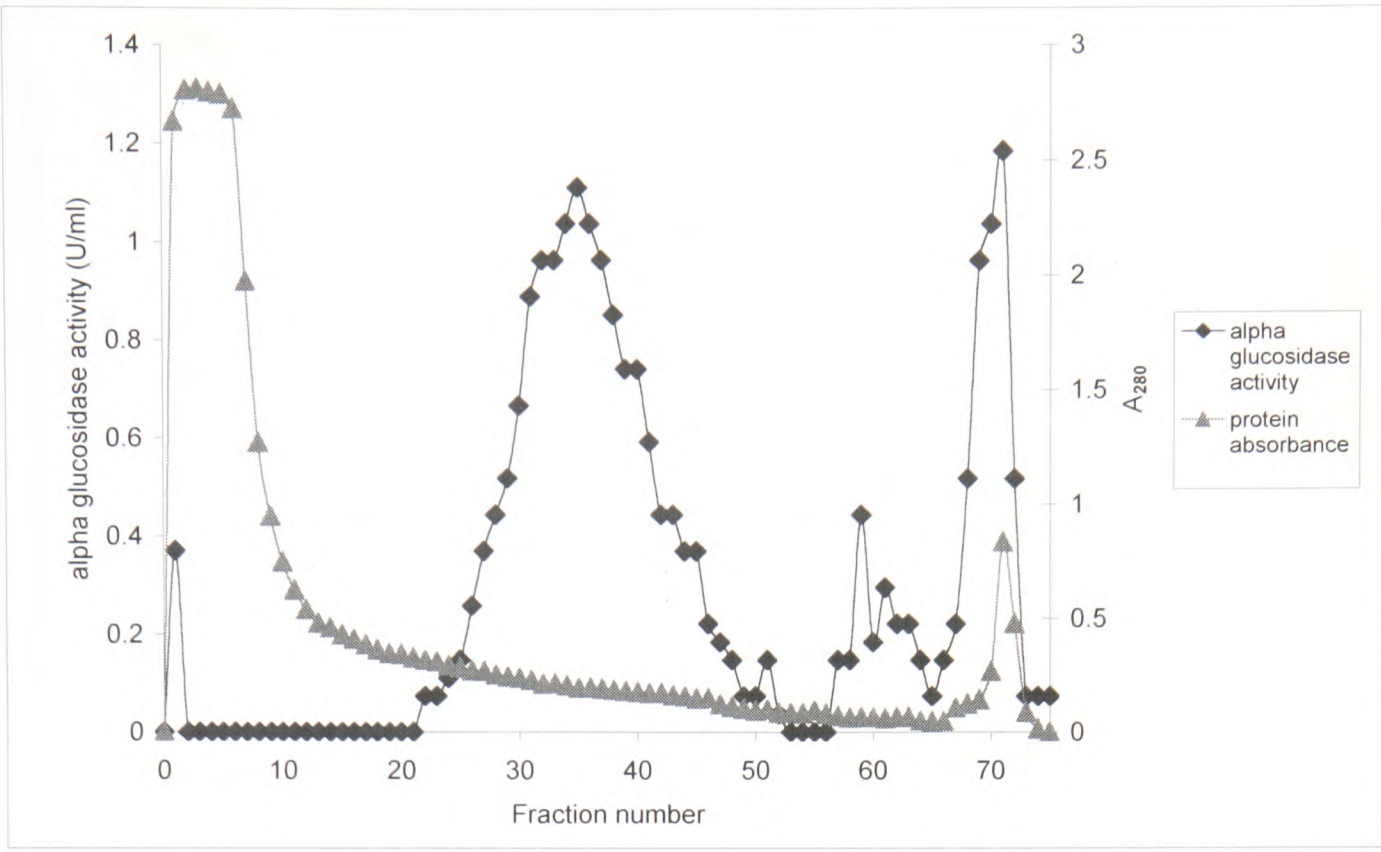


Figure 4.8: Medium scale: 20ml Phenyl Sepharose CL-4B column. *Elution of α-glucosidase and protein was achieved using a linear gradient of 1.0-0.01M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8. at a flow rate of approximately 60ml/hour 5ml fractions were collected and assayed for α-glucosidase activity and protein as described in sections 2.10.1 and 2.8.1 respectively.*

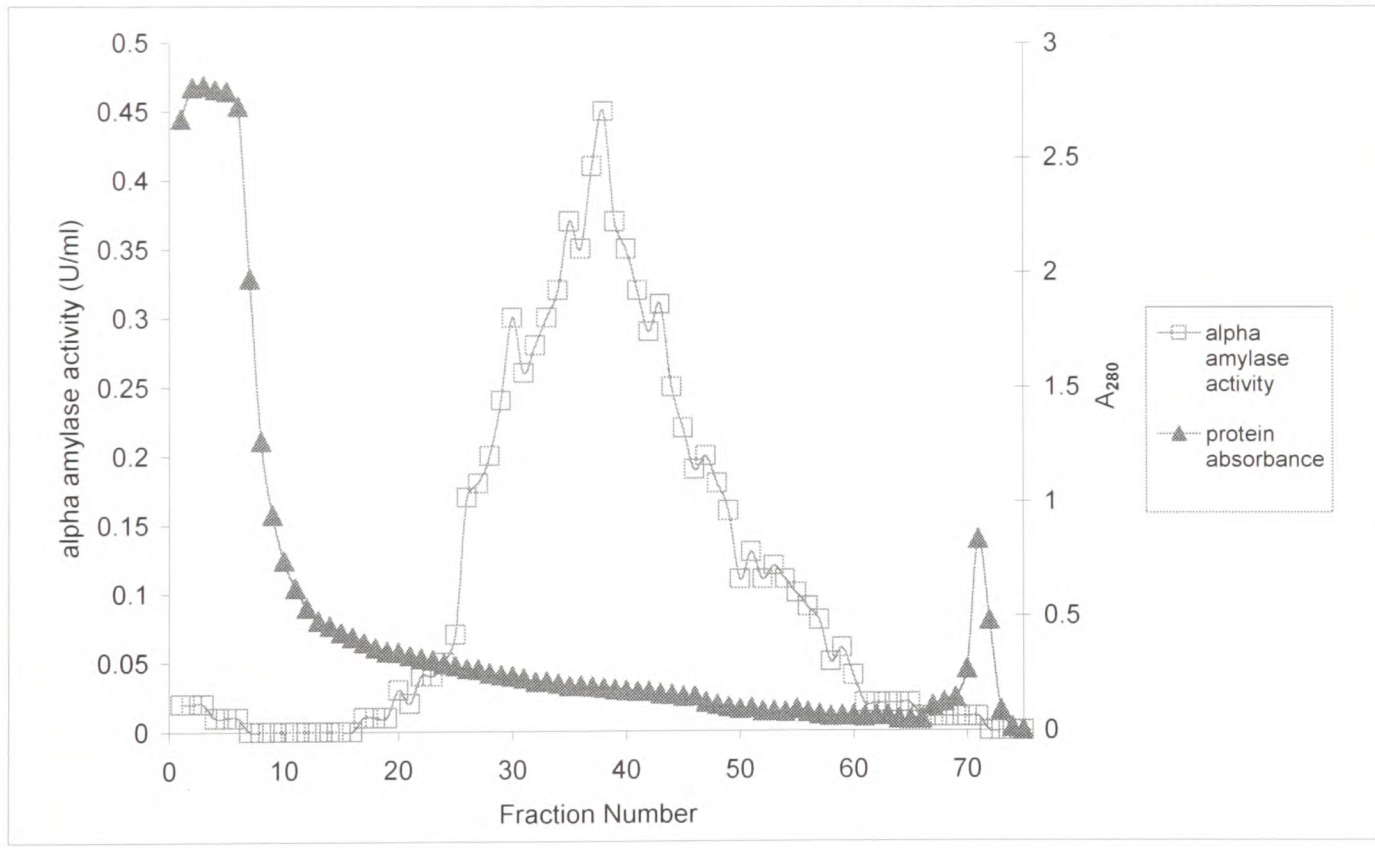


Figure 4.9: Medium scale: 20ml Phenyl Sepharose CL-4B. *Elution of α-amylase and protein was achieved by using a linear gradient of 1.0-0.01M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8. 5ml fractions were collected and assayed for α-amylase and protein as described in sections 2.10.2 and 2.8.1 respectively.*

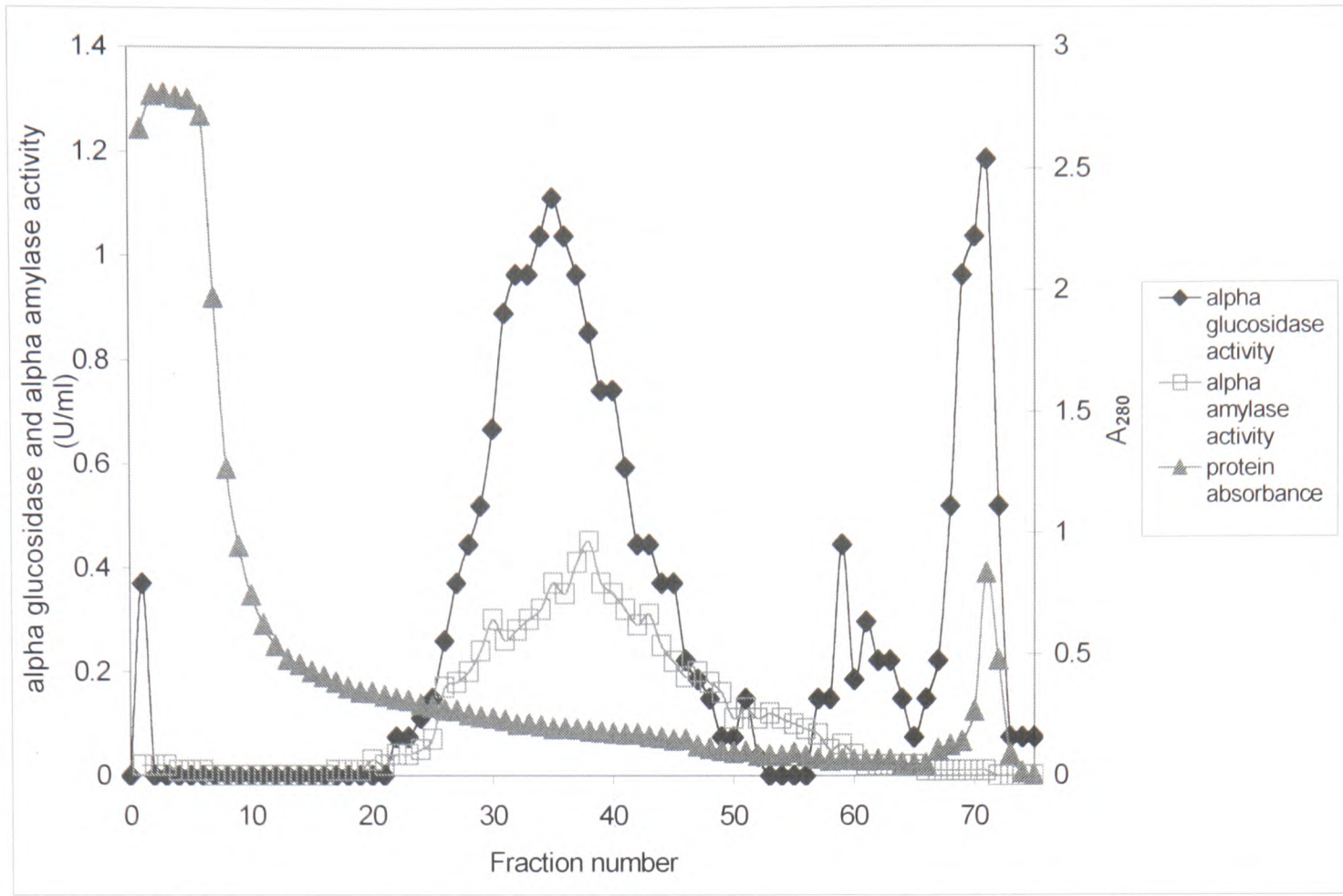


Figure 4.10: Combined activity plot of α-glucosidase and α-amylase. *150U of α-glucosidase, 16.8U of α-amylase and 5.4mg of protein were loaded, with maximum recoveries of 59% and 72% achieved for α-glucosidase and α-amylase activities, respectively.*

The elution profiles obtained from Figures 4.8 and 4.9 show that the linear gradient was effective in removing a significant amount of unwanted protein. The α-glucosidase and α-amylase eluted in the same fractions as broad overlapping peaks, centred around the middle of the gradient. This result is consistent with the results from small-scale columns (see Figures 4.6 and 4.7), though the modes of these activity peaks were now clearly separated by several fractions. Again, in other small-scale runs, a significant second peak of α-glucosidase activity eluted at low salt, but this time overlapping a minor α-amylase peak.

While the well-separated α-glucosidase peaks were suggestive of distinct α-glucosidase enzymes, the irregular, non-Gaussian major peak profiles obtained for both enzymes, could indicate the existence of isozymes for the main species of both α-glucosidase and α-amylase.

However, as this experiment shows, linear gradient elution did not significantly improve the separation of the main activity peaks of the two enzymes, compared to the

batch elution approach. Therefore, to avoid handling numerous dilute protein fractions and a subsequent concentration step, it was decided to return to the batch elution method, when using HIC. Batch elution was not only simple to implement but was also a precise and reproducible way of eluting the proteins of interest.

4.4.4 Purification data for α-glucosidase and α-amylase activities, from a typical medium-scale Phenyl Sepharose CL-4B column

Table 4.4: Enzyme activity and protein concentration data presented on selected fractions using batch elution techniques, representative elution profiles of which are given in Figure 4.7.

Fraction	Total volume (ml)	Activity applied (U)	Protein applied (mg)	Total activity recover-ed (U)	Total protein recovered (mg)	Specific activity U/mg	Recovery in selected fractions (%)	Purif-ication factor*
α-Glucosidase								
C.F.S.	1000			210.2	9.6	21.9	100	1
$(NH_4)_2SO_4$ Pellet	30	210.2	9.6	161.0	8.9	17.9	76.6	0.8
0.3M	50	=150.2	=8.9	14.8	2	7.4	=59	0.4
0.2M	30			33.3	1.1	31.8		1.8
0.1M	50			22.2	1.8	12.7		0.7
0.01M	50			18.5	0.5	37.1		2.1
α-Amylase	**Total volume (ml)**	**Activity applied (U)**	**Protein applied (mg)**	**Total activity recover-ed (U)**	**Total protein recovered (mg)**	**Specific activity U/mg**	**Recovery in selected fractions (%)**	**Purif-ication factor***
C.F.S.	1000			20.3	9.6	2.1	100	1
$(NH_4)_2SO_4$ Pellet	30	20.3	9.6	18	8.9	2.0	88.7	1.0
0.3M	50	=16.8	=8.9	2.5	2	1.3	=72	0.6
0.2M	30			5.4	1.1	5.1		2.6
0.1M	50			4.2	1.8	2.2		1.1

** The purification factor of each HIC fraction was calculated on the basis of the increase in specific activity after the ammonium sulphate fractionation step.*

The data collated in Table 4.4 represents the typical elution profiles of the two enzyme activities when performing batch elution on a Phenyl Sepharose CL-4B column. The total protein recovery from the HIC step (i.e. including data from enzymatically inactive fractions) was calculated to be greater than 90%. The column was effective in purifying the α-glucosidase from the bulk of the protein and identified more than one peak rich in α-glucosidase activity. Although HIC was not effective in separating α-glucosidase from α-amylase, some purification of the α-amylase was seen, with good recoveries of 72% achieved.

4.4.5 Investigation of High Salt Buffer Effects on α-Glucosidase Enzyme Activity

The ionic strength of 1M ammonium sulphate in 0.01M sodium phosphate buffer, pH 6.8 was calculated to ascertain whether there was a high salt buffer effect on the activity of the α-glucosidase enzyme, possibly causing artifactual elution profiles (Appendix 1). The ionic strength of 1M ammonium sulphate buffer in 0.01M sodium phosphate buffer is 3.54, compared to 0.10 for 0.01M-ammonium sulphate in 0.01M sodium phosphate buffer - a 35-fold difference. In order to check that the multiple peak pattern seen for α-glucosidase eluted from Phenyl Sepharose CL-4B columns (e.g. Figure 4.8) was not a salt-concentration artifact, an identical chromatographic experiment to that of Figure 4.8 was performed.

A linear gradient was run (Figure 4.11) and fractions were collected and assayed as normal, without any salt-adjustment (section 2.11.1). Stock solutions of ammonium sulphate buffer, prepared at concentrations ranging from 1 to 2M, were then added as equal volumes, to selected fractions - as shown in Table 4.5, so approximately equalizing the ammonium sulphate concentration – and hence the ionic strength - of all fractions at 1M; the fractions were then assayed again for α-glucosidase activity.

Table 4.5: Fraction number and corresponding salt additions

Fractions	**1 - 6**	**7 - 12**	**13 - 18**	**19 - 24**	**25 - 30**	**31 - 36**	**37 - 43**	**44 - 49**	**50 - 55**	**56 - 61**	**62 - 67**	**68 - 75**
Ionic strength of buffer before adjustment (M)	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.01	0
Ionic strength of adjusting buffer (M)	1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	1.99	2

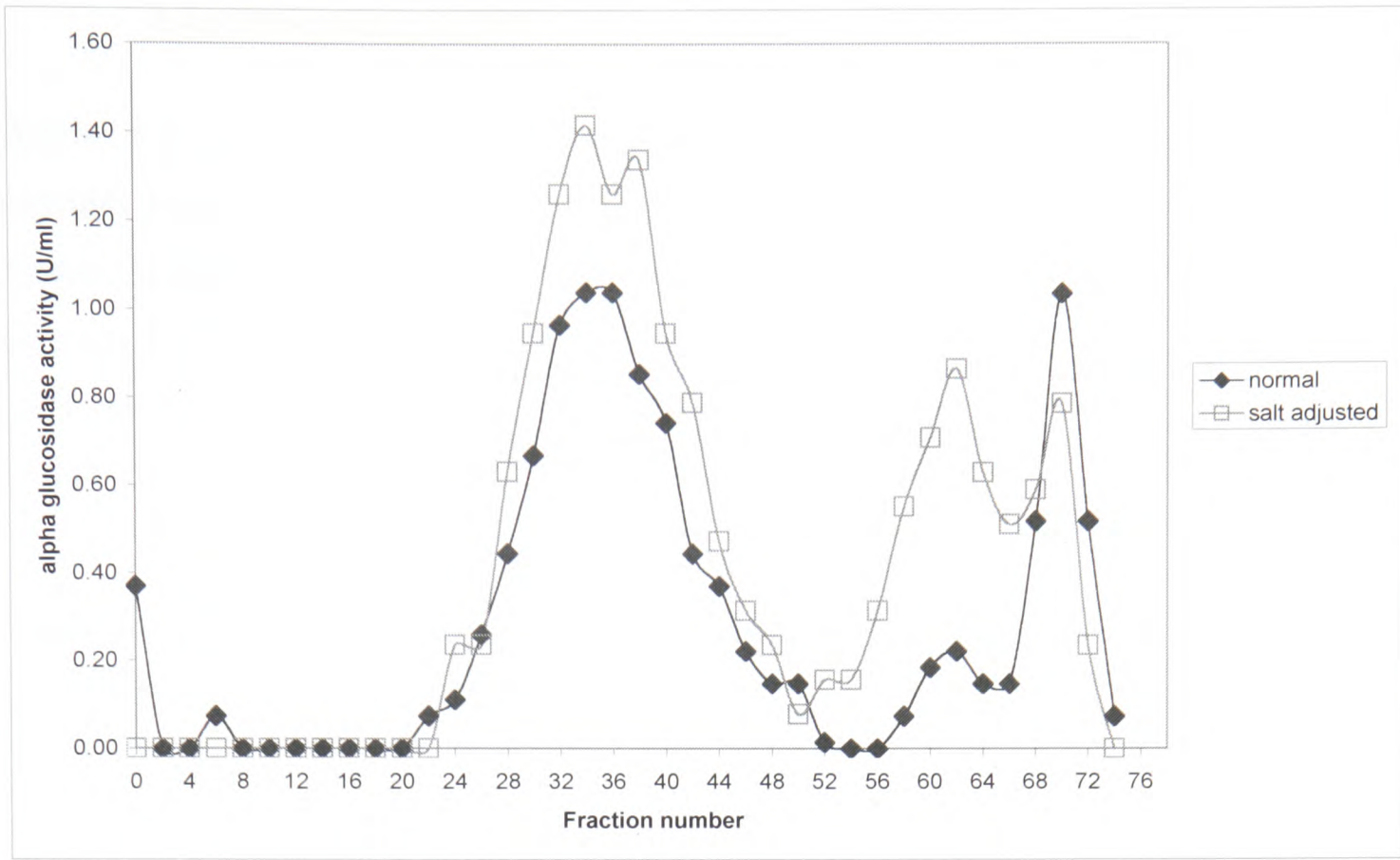


Figure 4.11: Activity profile of α-glucosidase, with and without salt adjustment to normal assay conditions. *Fractions assayed were previously collected from a Phenyl Sepharose CL-4B column using a linear gradient of 1.0-0.01M ammonium sulphate, 10mM sodium phosphate buffer, pH 6.8 buffer at a flow rate of approximately 60ml/hour. 5ml fractions were collected and assayed for α-glucosidase activity and protein as described in sections 2.10.1 and 2.8.1 respectively.*

As Figure 4.11 shows, the activity profiles for both sets of fractions were similar, though in most cases the α-glucosidase activities in the high salt fractions were elevated, sometimes very considerably. Clearly, the elution profile of the α-glucosidase is highly complex, and HIC appears to be separating 3 or 4 possible species. Interestingly, the α-glucosidase activities in the three obvious peaks all appeared to respond differently to the addition of salt: this could be indicative of different responses to high ionic strengths, or specific ions.

4.4.6 Processing of HIC Column Fractions and Enzyme Stability

Active fractions were collected from HIC columns, dialyzed against a low ionic strength buffer (2mM sodium phosphate, pH 6.8), and finally concentrated before further analysis or purification. Samples were concentrated by either freeze-drying (section 2.4.4) or by reverse dialysis (section 2.4.3). The former method was usually used, as the lyophilized protein preparations were shown to be stable over long periods, without any significant loss in enzyme activity (Table 4.6).

Table 4.6: Stability of the lyophilized ammonium sulphate precipitate

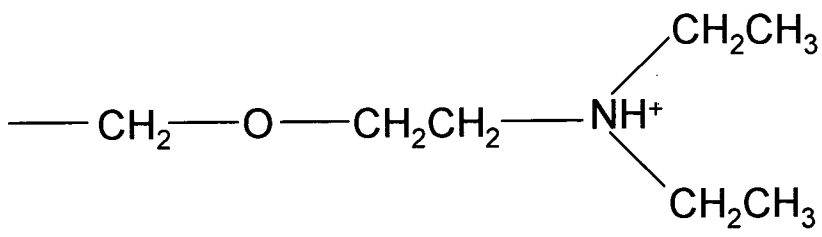
Time Point	α-Glucosidase Activity (U)	α-Amylase Activity (U)
Initial	161.0	18.0
1 Month	160.6	18.0
3 Months	158.2	17.7
6 Months	154.0	17.5
12 Months	153.1	17.1

4.5 Ion Exchange Chromatography (IEC)

4.5.1 Introduction

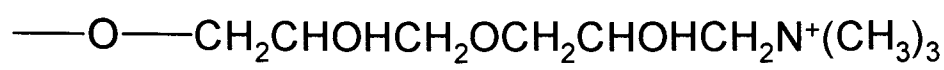
Ion exchange chromatography (IEC) separates molecules based on the differences in charge characteristics, and is therefore dependent on the pH and isoelectric point of the protein. IEC separates molecules based on their net charge. Negative or positively charged functional groups are covalently bound to a solid support matrix yielding a cation or anion exchanger. When a charged molecule is applied to an exchanger of opposite charge it is adsorbed by electrostatic forces while ions of the same charge are eluted in the void volume of the column. The binding of the charged molecules is reversible and adsorbed molecules are commonly eluted with a salt or pH gradient.

The ion exchange matrix and the choice of charged groups covalently bound to it have a profound effect on the overall performance of the ion exchanger. The type of group determines the type and strength of ion exchanger. Sulphonic and quaternary amino groups are used as strong ion exchangers whereas diethylaminoethyl (DEAE) groups are weak ion exchangers. The definition of a weak ion exchanger refers to the extent of the variation of ionization and not the strength of binding. Strong ion exchangers are ionized over a wide pH range and have almost constant ionic capacity within this range. Strong exchangers may also exhibit slightly better selectivity than weak ones. Weak ion exchangers are only ionized over a limited pH range and may start to lose their charge at pH values below 6 for cation exchangers, or above 9 for anion exchangers (Pharmacia, 1991).



Diethyl aminoethyl-, DEAE ligand

Figure 4.12: Partial structure of DEAE anionic exchange medium



Q Sepharose

Figure 4.13: Functional groups on Q-Sepharose anionic exchange medium

Initial experiments were conducted using DE52, an inexpensive and widely used weak anionic exchanger from Whatman (Figure 4.12). However, one clear advantage to using DEAE over a strong ion exchanger is that the DEAE adsorbent is partly uncharged at neutral pHs. Therefore, when using a buffer at pH 7.0, the DEAE adsorbent acts as a strong buffer, which can be beneficial when separating similarly charged species (Scopes, 1994). However, this necessarily means that the single positive charge on the nitrogen atom can dissociate if the pH fluctuates, and this in turn can lead to marked variability in the binding capacity of the adsorbent.

Parallel experiments, using the strong anionic exchanger Q-Sepharose, were therefore also conducted. Q Sepharose, an anionic column from Pharmacia, has a quaternary amine ligand (Figure 4.13). It is a "high performance" medium capable of a high sample throughput. The firm matrix manufactured from small uniform beads of agarose combines the two crucial properties for an efficient separation: fast flow rate and large binding capacity. As a strong ion-exchanger it is completely ionized over a wide pH range. This in essence means that sample loading is not affected by low or high pH values of the eluting buffer.

4.6 Ion Exchange Chromatography Results

4.6.1 IEC using DE52

Fractions collected from Phenyl Sepharose CL-4B columns, rich in α-glucosidase and α-amylase activity, were initially pooled together and prepared for ion exchange chromatography as described in section 2.4. The initial screening experiments were carried out on a small scale with 1ml columns equilibrated in 10mM phosphate buffer, pH 6.8, after which the protein sample was applied. Columns were washed by elution with batches of an increasing salt concentration buffer.

Figure 4.14 is a representative example of results from one of these columns.

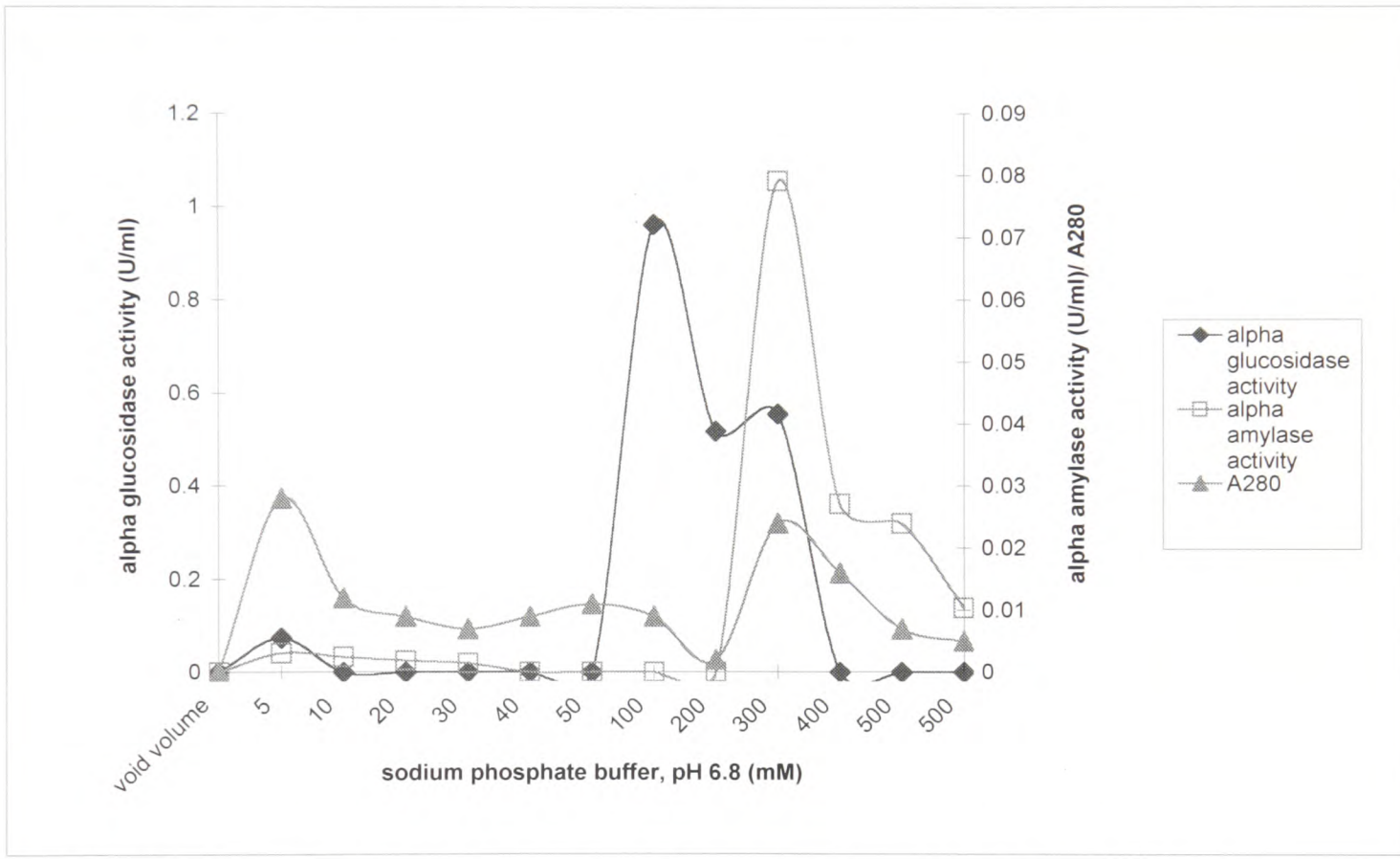


Figure 4.14: Batch elution of α-glucosidase and α-amylase using DE 52. *The sample applied was a highly concentrated aliquot of the pooled active fractions collected from a previous HIC column (Figure 4.7), and eluted using a linear gradient of 10-500mM sodium phosphate buffer pH 6.8.*

This small-scale experiment demonstrated that an anionic ion exchange column could effect some separation of the two enzyme activities, though there was also significant overlap. The multiple peak patterns could be indicative of isoenzymes or distinct enzyme species - as previously suggested by the HIC results (Figure 4.7).

In this experiment, any resolution offered by prior HIC was not effectively exploited as HIC fractions were pooled prior to IEC. Using individual HIC fractions might

therefore improve resolution at the IEC stage. Adjustment of the column and running conditions might also be expected to improve resolution. Increasing the column volume would increase the number of theoretical plates, so enhancing resolution (flow rates being adjusted accordingly). Also, a long, shallow linear gradient should be helpful with a longer column, minimizing band broadening. These approaches were taken in subsequent IEC work with DE52.

Therefore, specific HIC fractions that contained both α-glucosidase and α-amylase activity were applied separately to an anionic exchange column, and various elution conditions were evaluated to firmly establish the optimum separation conditions for the two enzymes. Firstly, the individual ("0.1M") fraction from an HIC column was applied to a medium scale (8ml) DE 52 column equilibrated in 0.005M sodium phosphate buffer, pH 6.8. The elution profiles of the HIC column used to provide the sample are shown in Figure 4.15.

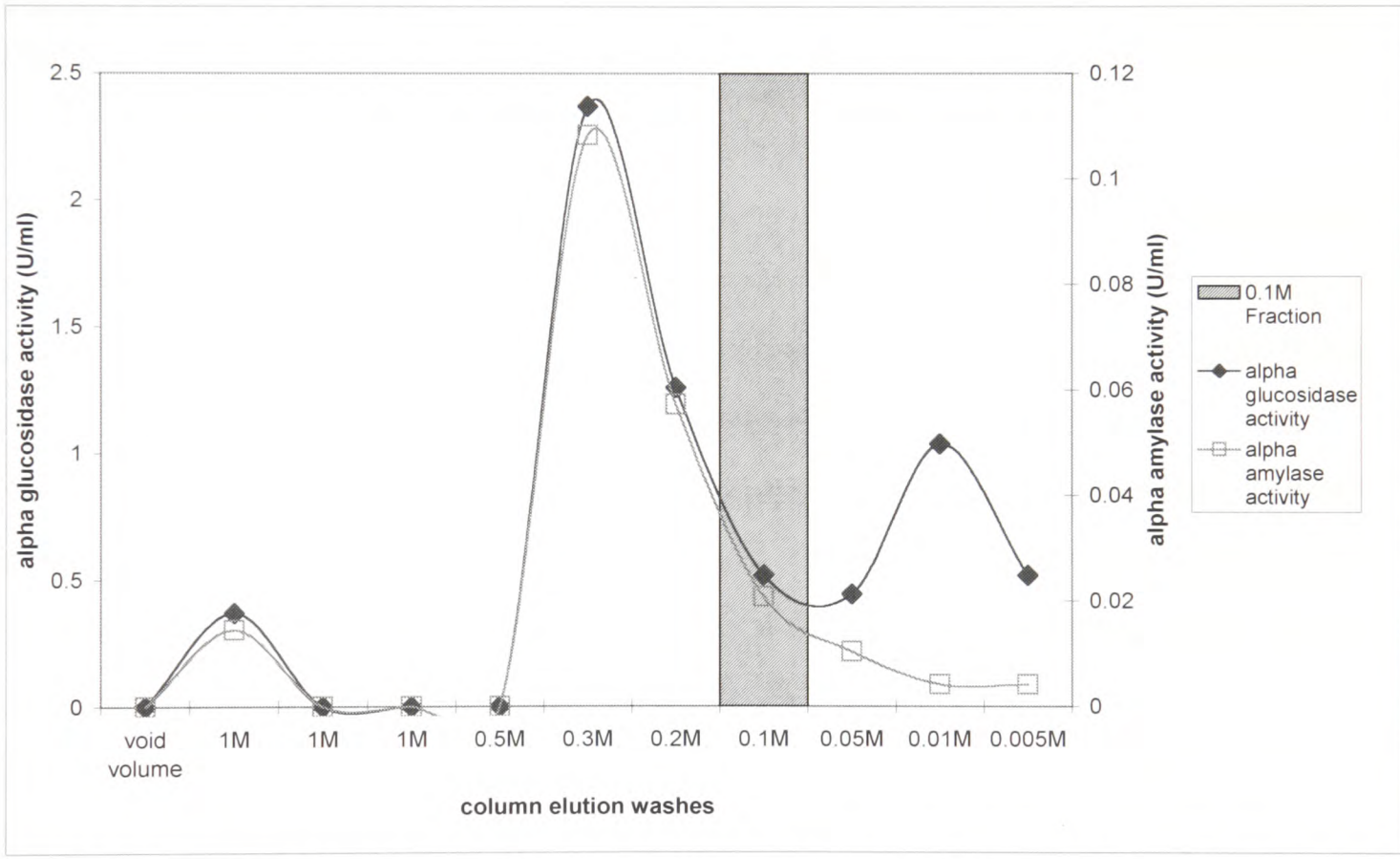


Figure 4.15: Chromatogram of α-glucosidase and α-amylase activities using Phenyl Sepharose CL-4B. *The shaded area denotes the 0.1M eluate subsequently applied to a DE52 column. 1.8mg of protein containing 22.2U of α-glucosidase and 5.4U of α-amylase activity were applied. Individual fractions were then selected for a second chromatographic step. α-Glucosidase and α-amylase were eluted from the DE52 column using a long, shallow gradient 10-500mM sodium phosphate buffer, pH 6.8. Estimated % recoveries of the α-glucosidase and α-amylase activities were 59% and 72% respectively.*

The elution profiles are shown in Figure 4.16.

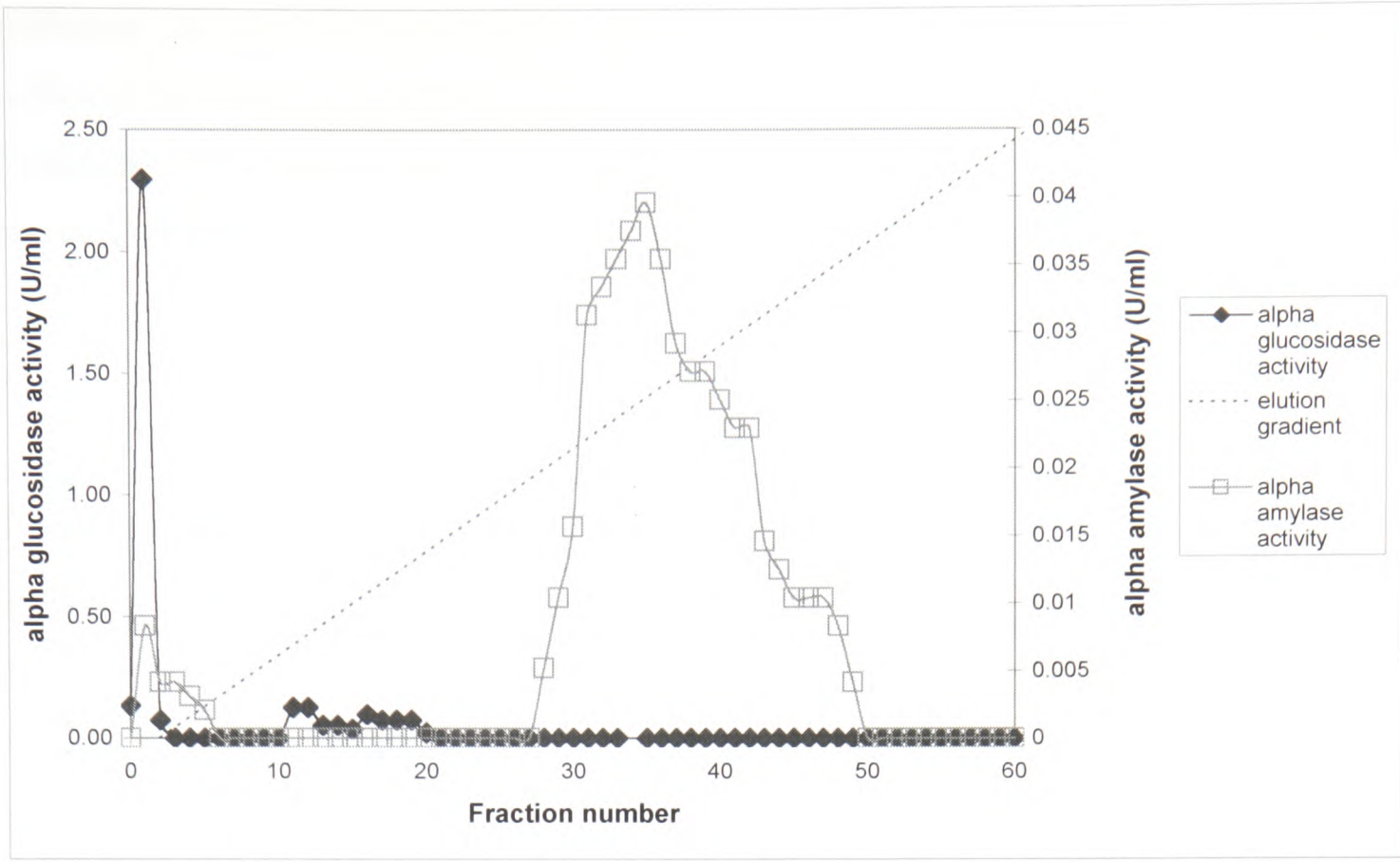


Figure 4.16: Chromatogram showing the elution of the 0.1M HIC fraction using DE52. *α-Glucosidase and α-Amylase activities are eluted using a linear gradient 10-500mM sodium phosphate buffer, pH 6.8 (as denoted by the dotted line).*

The majority of the α-glucosidase activity did not bind to the column, being found predominately in the void volume and first column washes. The small quantity of enzyme that did adsorb, appeared to have a mixed mode of binding, and eluted across several fractions.

The poor selectivity of the adsorbent for the α-glucosidase could be attributed to either a poor, unevenly packed column or to the structure of the adsorbent itself. Although α-amylase activity was completely segregated from the α-glucosidase peak, the α-amylase activity was spread across 20 fractions.

The experiment was repeated, in case technical error caused the poor performance of DE52. The column was carefully correctly packed - having no obvious flaws such as air bubbles, which would lead to channeling; also the column was checked for proper equilibration by ensuring that the conductivity of the buffer was constant and at a stable pH, prior to running the sample. Nevertheless, the results were similar to the data presented in Figure 4.16 with poor resolution and variable binding.

Zone broadening – as seen in the DE52 runs - is caused by longitudinal diffusion of the solute molecules. The diffusion effect is minimized if the distances available for

diffusion, in both the liquid phase and gel beads, are minimized, and this can be achieved by using an adsorbent with small beads of uniform size (Pharmacia, 1991). Sepharose-type matrices are such an adsorbent. It was therefore decided to assess the use of Q-Sepharose, a strong anionic exchanger.

4.6.2 Fractionation of Individual HIC Fractions using Q-Sepharose

A series of experiments were undertaken to investigate the separation of α-glucosidase and α-amylase activities originating in various HIC fractions, using Q-Sepharose with batch elution, with the intention of generating at least some fractions sufficiently pure for more detailed analysis and characterization - especially in the case of α-glucosidase. All the HIC fractions used as samples were derived from a single HIC column run (Figure 4.7), which was an intermediate Phenyl Sepharose CL-4B column, batch eluted with ammonium sulphate buffer.

4.6.2.1 IEC using 0.3M HIC Fraction

The 0.3M HIC fraction (Figure 4.16), containing 14.8U of α-glucosidase activity and 2.5U of α-amylase activity, was adsorbed to a medium scale Q-Sepharose column previously equilibrated with 0.01M phosphate buffer. The column was eluted using a linear gradient, and all fractions were assayed for α-glucosidase, α-amylase activity and protein. The activity profiles are shown in Figure 4.17

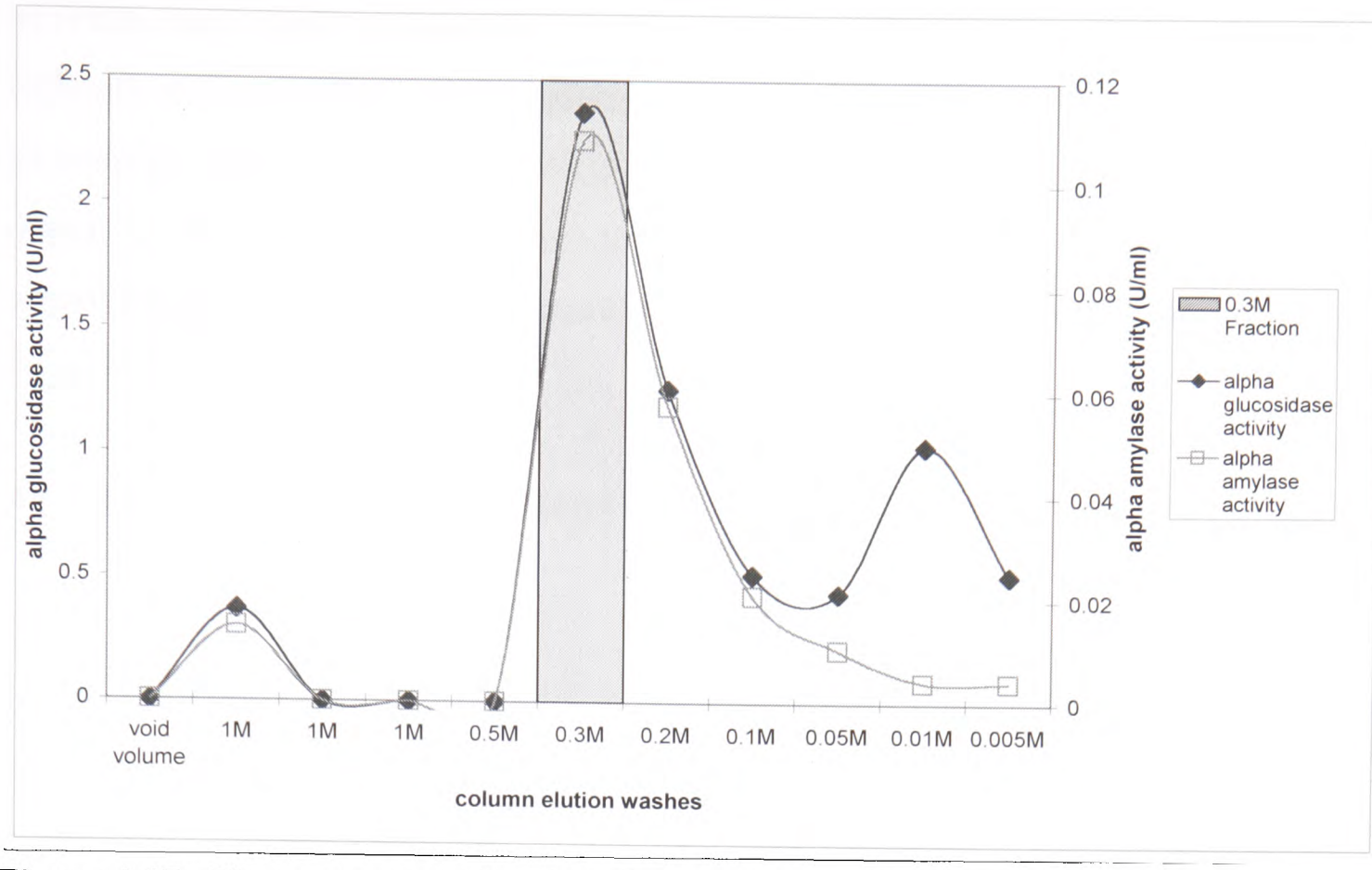


Figure 4.17: Chromatogram showing the separation of α-glucosidase and α-amylase enzyme activities using Phenyl Sepharose CL-4B. *The shaded area denotes the 0.3M HIC fraction, pooled and applied to Q-Sepharose anion exchange column.*

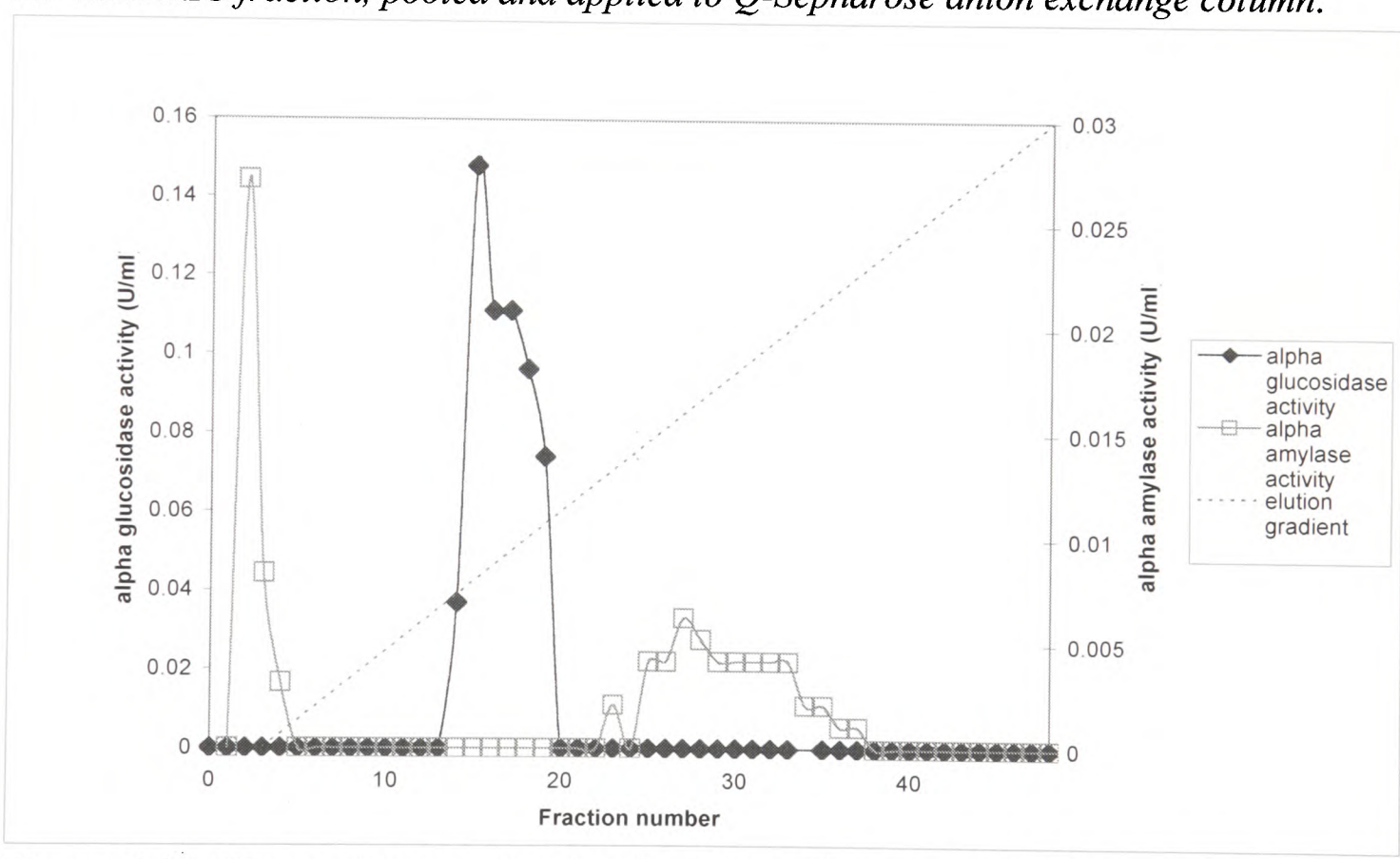


Figure 4.18: Chromatogram showing the separation of α-glucosidase and α-amylase enzyme activities from the 0.3M HIC fraction using Q-Sepharose. *Enzymes were eluted using a 0.01-0.5M phosphate buffer, pH 6.8, linear gradient in a total volume of 400ml (as denoted by the dotted line).*

The results from this run show a better separation between α-glucosidase and α-amylase than when using batch elution techniques (see Figure 4.14). The two major

activities have been separated and there appears to be some resolution between the different isoforms of the α-glucosidase, i.e. a shoulder is visible on the single, main α-glucosidase peak. Also, in this experiment, more than one α-amylase enzyme forms appear to have been separated. Enzyme recoveries for the α-glucosidase were low - approximately 30%, but α-amylase recoveries were quite good, 72% of the activity being recovered.

4.6.2.2 IEC using 0.2M HIC Fraction

The 0.2M HIC fraction (Figure 4.19), containing 12.6U of α-glucosidase activity and 4.9U of α-amylase activity, was adsorbed to a medium scale Q-Sepharose column, previously equilibrated with 0.01M phosphate buffer, pH 6.8. The enzymes were eluted using a linear gradient and all fractions were assayed for α-glucosidase activity, α-amylase activity and protein. The activity profiles are shown in Figure 4.20.

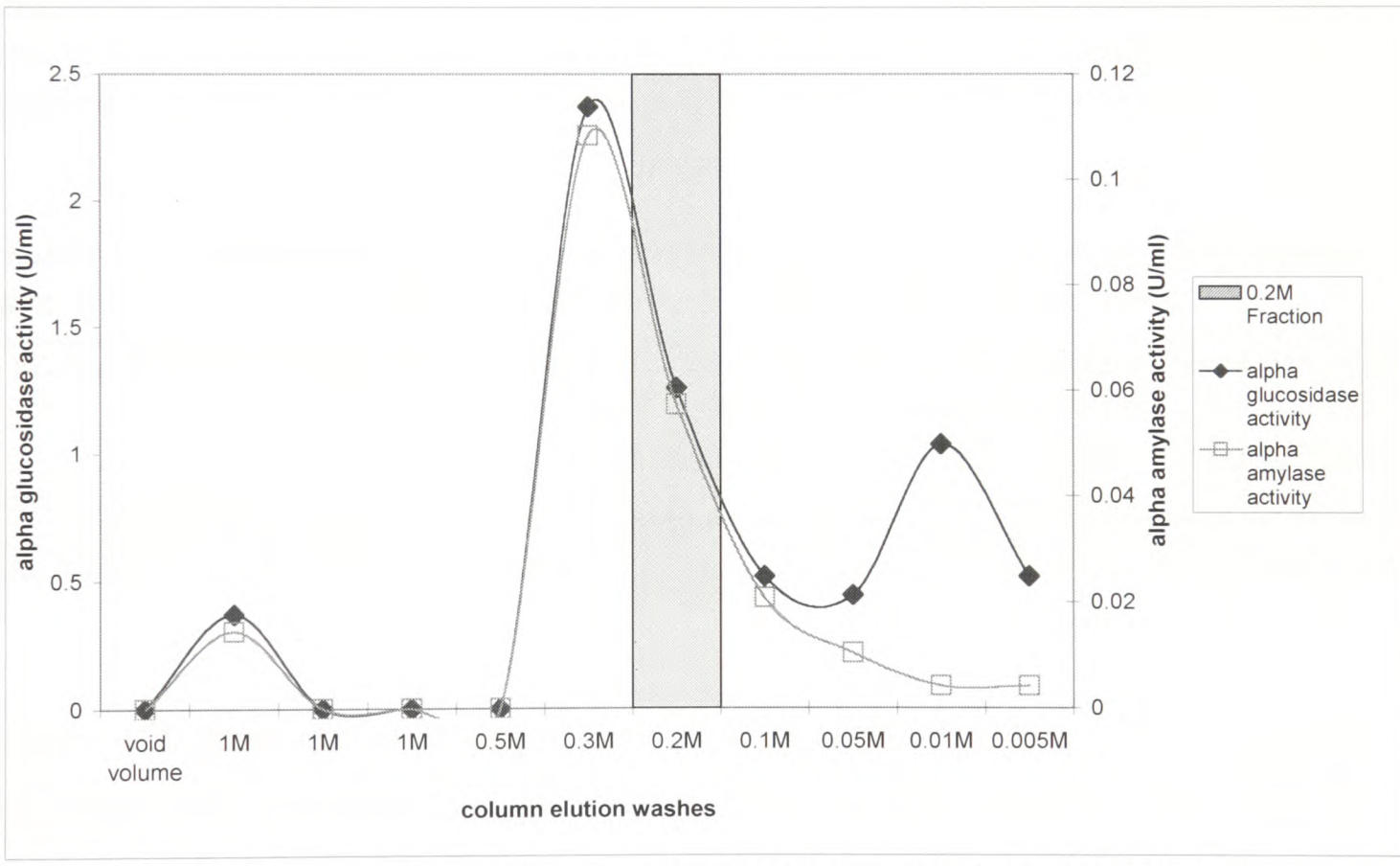


Figure 4.19: Chromatogram showing the separation of α-glucosidase and α-amylase enzyme activities using Phenyl Sepharose CL-4B. *The shaded area denotes the 0.2M HIC fraction, which was applied to a Q-Sepharose anion exchange column.*

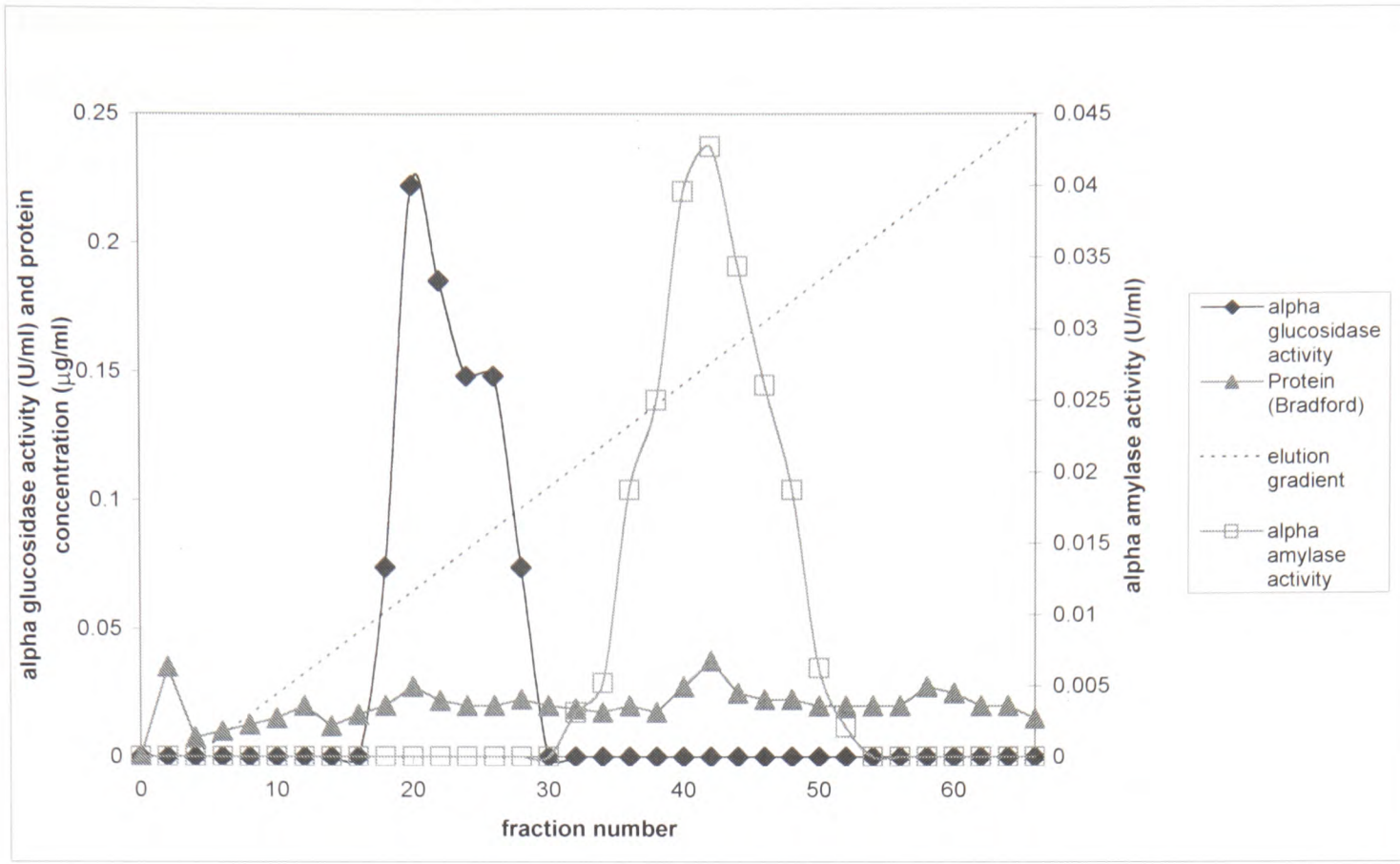


Figure 4.20: Chromatogram showing the elution of α-glucosidase and α-amylase enzyme activities using an 8ml bed volume Q-Sepharose column. *Enzymes were eluted from the column using a linear gradient of 0.01-0.5M sodium phosphate buffer, pH. 6.8 in 160ml total volume, (as denoted by the dotted line). 0.5mg/ml protein was applied containing 12.6U α-glucosidase activity and 4.9U α-amylase activity.*

Table 4.7: Purification table for α-glucosidase and α-amylase from 0.2M fraction previously eluted from Phenyl Sepharose CL-4B column (Figure 4.20).

Pooled fraction	Total Vol (ml)	Activity loaded (U)	Protein loaded (mg)	Total activity recovered (U)	Total protein (mg)	Specific activity U/mg	Reco-very (%)	Purific-ation factor
α-Glucosidase	45	12.6	0.5	8.3	0.07	112.6	66.2	3.6
α-Amylase	80	4.9	0.5	3.0	0.09	33.3	61.6	6.5

Figure 4.20 demonstrates that good resolution was achieved between the two enzyme activities with recoveries of approximately 60% for both enzymes when using Q-Sepharose. And due to the highly reproducible results achieved with Q-Sepharose it was chosen preferentially over the weak Whatman DEAE anionic media (Figure 4.12) for purifying all subsequent enzyme-rich HIC fractions.

The shoulder appearing in the α-glucosidase peak - also seen in the previous 0.3M fractionation - again indicates to the presence of more than one form of the enzyme. The α-amylase activity was eluted as a single, broad, fairly symmetrical peak

Despite the good resolution, there were some drawbacks: numerous dilute fractions were produced by the long, shallow elution gradient leading to a proportional increase in assay time, cost and expenditure. Also, the peaks were broad and the separation times long, (approximately six and an half hours to run the gradient at a flow rate of ~25ml/hour).

In a further experiment with the same 0.2M HIC sample, the effects of two modifications were assessed. Firstly, the elution gradient volume was reduced from 80 column volumes to a total of 20 times the column volume. Secondly, as both activities were observed to elute from the column after only 80% of the gradient had been run, (Figure 4.20), the ionic strength of the upper limit buffer was decreased, from 0.5M to 0.3M.

Repeating the experiment under these conditions (Figure 4.21) resulted in α-glucosidase eluting from the ion exchange column, while the α-amylase activity remained bound. α-Amylase was then desorbed from the column matrix by a single step-elution using 0.3M sodium phosphate buffer.

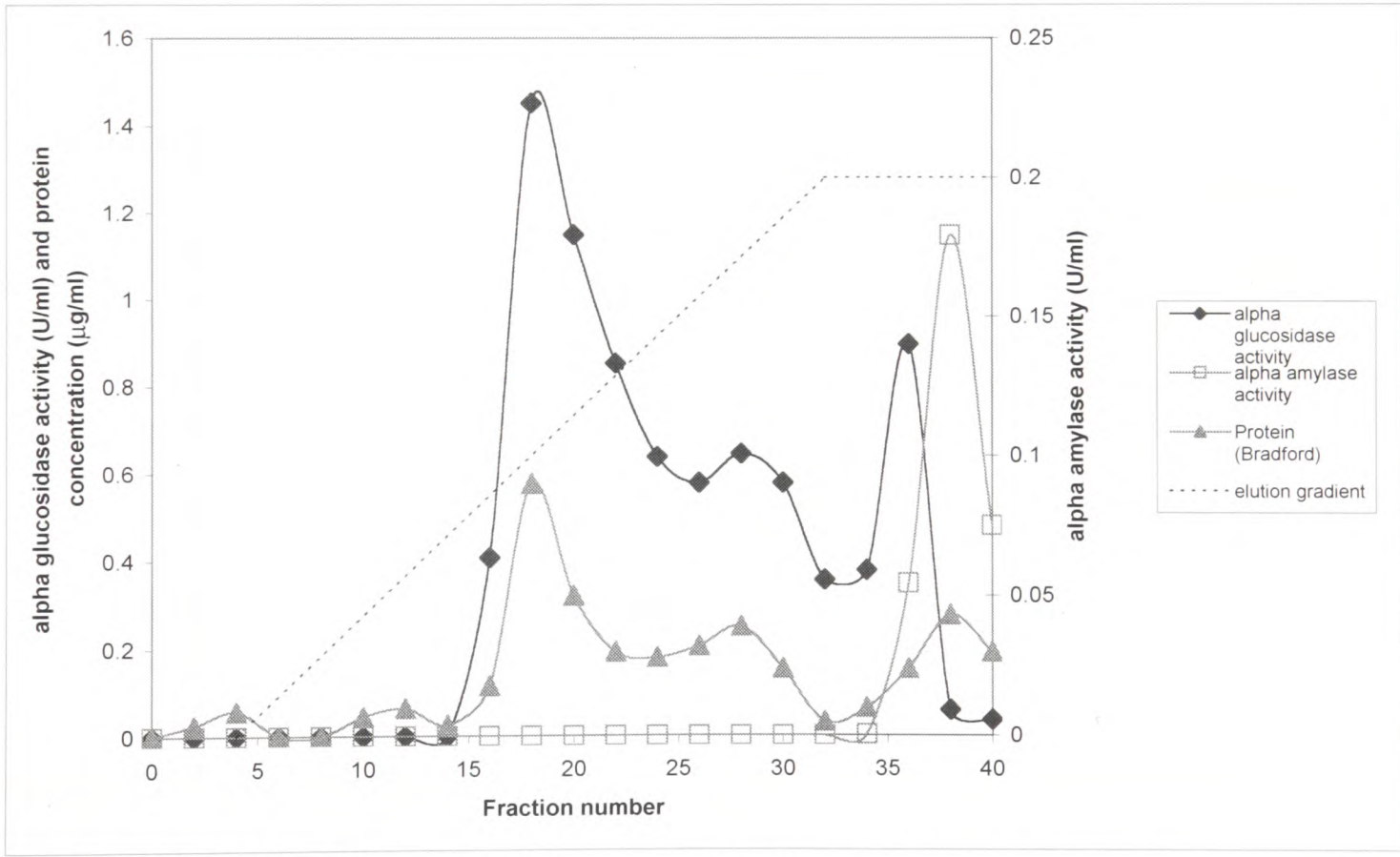


Figure 4.21: Purification of the 0.2M HIC fraction using Q-Sepharose. *α-Glucosidase and α-amylase activities were eluted using a combination of a reduced linear gradient 10-300mM phosphate buffer (80ml total volume), followed by batch elution using 300mM sodium phosphate buffer, pH 6.8. (The dotted line denotes the salt gradient).*

As Figure 4.21 shows, the separation when using a shorter, steeper gradient was faster and the peaks were sharper, but the resolution between the peaks was reduced. The broad α-glucosidase peak seen in Figure 4.20 was resolved into three distinct peaks when using the reduced gradient. Usefully, because of the reduced gradient volumes, both enzymes were eluted from the ion exchange column in a fairly concentrated form and therefore less time was required to prepare the fractions for further characterisation studies or for PAGE (Polyacrylamide Gel Electrophoresis).

4.6.2.3 IEC using 0.1M HIC Fraction

The 0.1M fraction (Figure 4.22) collected from a Phenyl Sepharose CL-4B column was dialysed and concentrated as described in sections 2.4.3.1 and 2.4.4, prior to adsorption to a Q-Sepharose column, pre-equilibrated in 0.005M sodium acetate buffer.

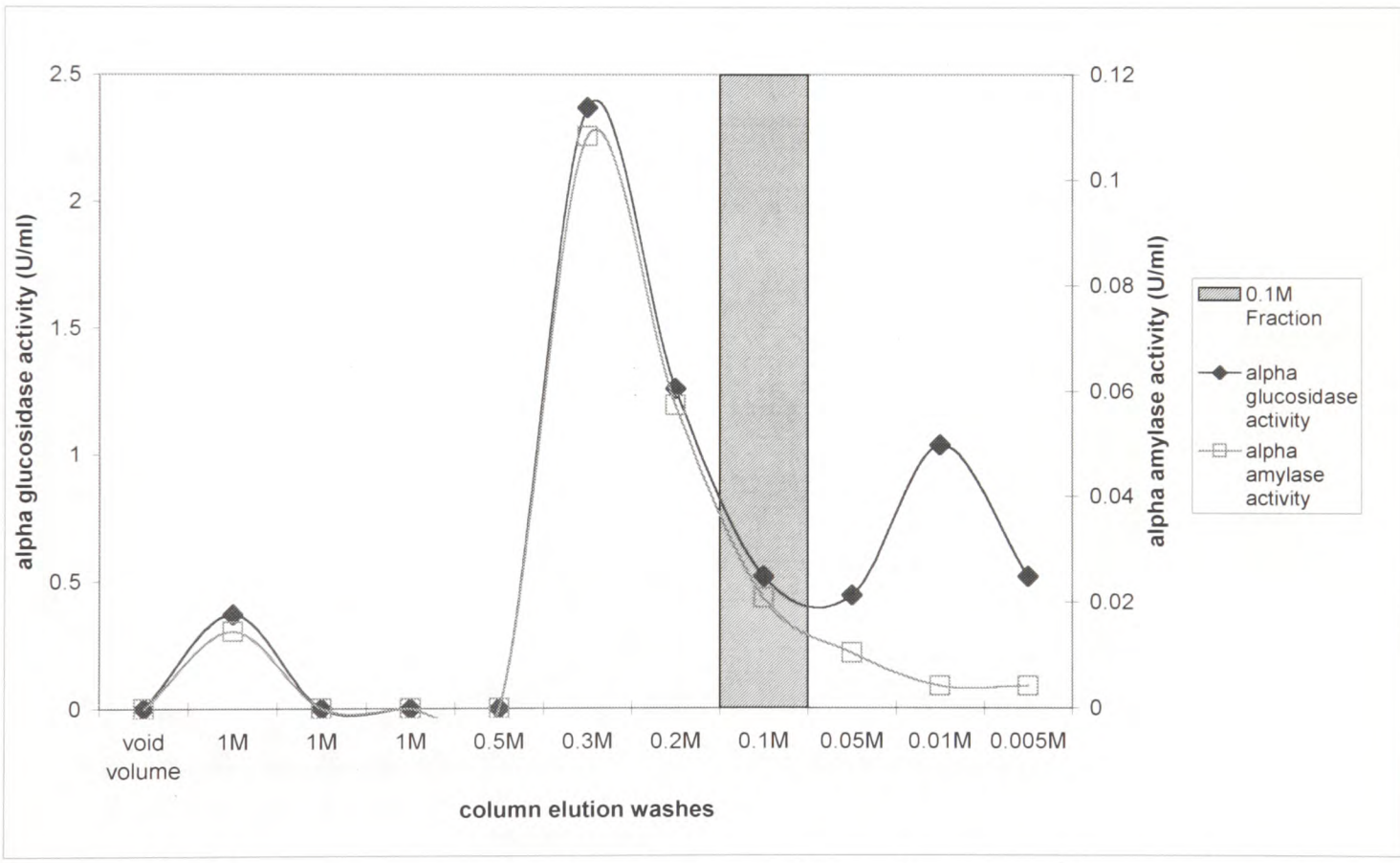


Figure 4.22: Chromatogram showing the separation of α-glucosidase and α-amylase enzyme activities using Phenyl Sepharose CL-4B. *The shaded area denotes the 0.1M HIC fraction, which was then further purified by anion exchange chromatography.*

The sample was run using a combination of gradient and isocratic elution but with a reduced gradient volume and an alternate sodium acetate buffer, pH 5. Once the sample was adsorbed, a linear gradient of 0.005-0.2M sodium acetate buffer, pH 5.0, in a total volume of 80ml, was applied. Fractions 4-25 were gradient fractions; fractions 26-34 were collected after a final step elution using 0.3M sodium acetate buffer, pH 5.0. Sodium acetate buffer was tried as an elution buffer because although phosphate buffers are important for enzyme stability, they are not the most appropriate choice for anionic exchange chromatography. This is because phosphate is itself is a counterion and neither form (HPO_4^{2-} and H_2PO^{4-}) is neutral and therefore could participate in the ionic exchange process, and affect the separation. Simple anions such as Cl^- or $acetate^-$ are recommended for anionic exchange chromatography (Scopes, 1994).

The IEC results are shown in Figure 4.23 and show that the reduced linear gradient was sufficient in eluting all the α-glucosidase activity from the column and that most of the α-amylase activity was eluted off the column using the 0.3M buffer.

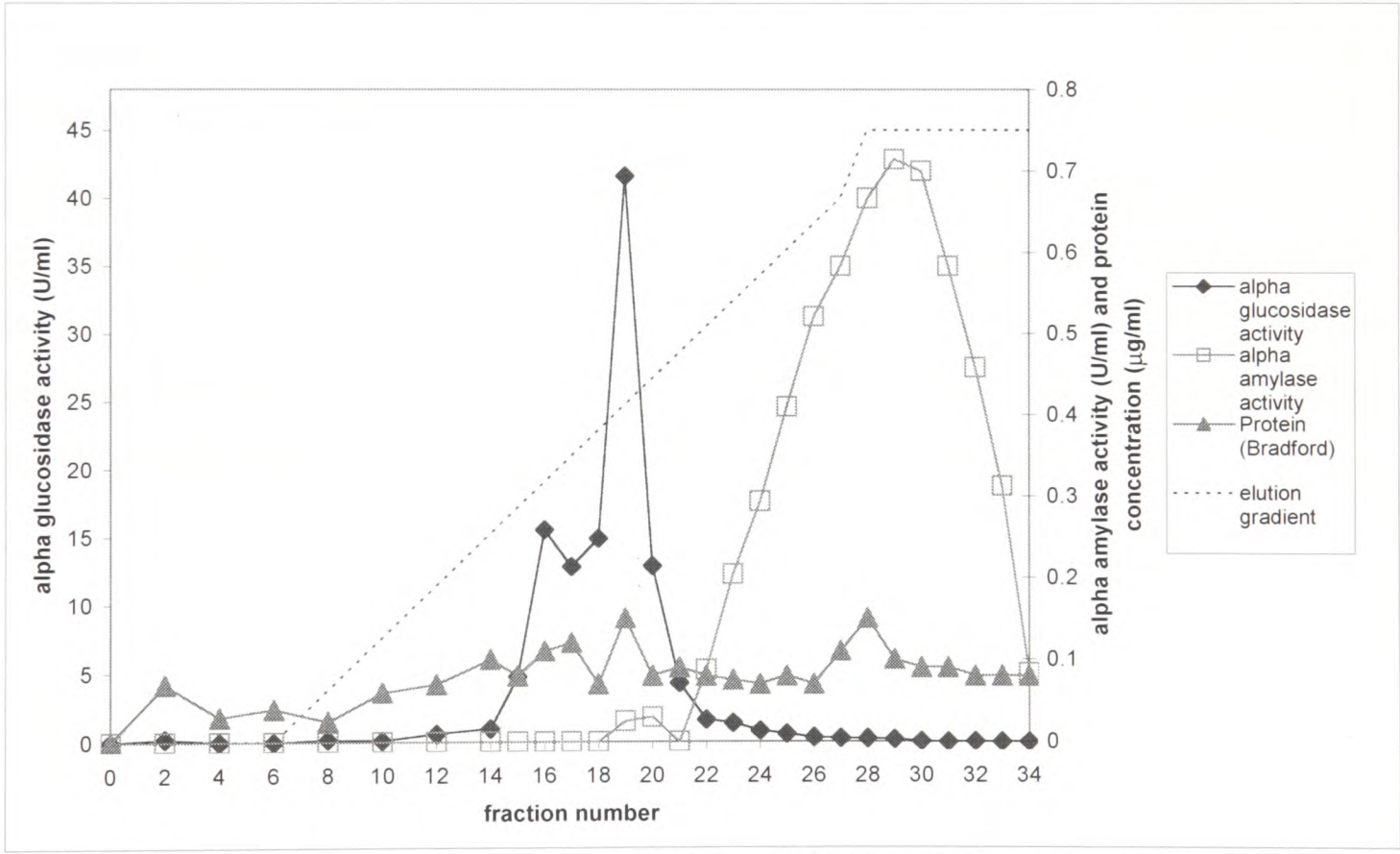


Figure 4.23: Chromatogram showing the elution of 0.1M HIC fraction using IEC. *α-Glucosidase and α-amylase activity was eluted from an 8ml Q-Sepharose column using an 80ml linear gradient of 0.005-0.2M sodium acetate buffer, pH 5.0, followed by step elution using 0.3M sodium acetate buffer, pH 5.0. (The dotted line denotes the salt gradient).*

Interestingly, by using a sodium acetate buffer to elute there appeared to be an increase in activity in both α-glucosidase and α-amylase activities. This was attributed to a pH affect on the enzyme activity buffer and is discussed in greater detail in section 5.4.1. There also appears to be different proportions of isozymes between the 0.2M and 0.1M HIC fractions when purified by anionic exchange. The 0.2M HIC fractions had 3 possible isozymes of α-glucosidase and 1 apparent major α-amylase species. Whereas the 0.1M HIC fraction was comprised of 2 possible isozymes of α-glucosidase and 1 major and 1 minor α-amylase species. Only by carrying out further experiments, such as running non-denaturing gels with specific enzyme activity staining would it possible to confirm the identity of all the different peaks.

4.6.2.4 IEC using 0.01M HIC Fraction

This HIC fraction had very little α-amylase activity. Using a short, reduced gradient (ten times the column volume) of sodium acetate buffer, pH 5.0, the sample was run to investigate whether the α-glucosidase could be purified further. A short elution gradient was used because the α-glucosidase activity elutes at a lower ionic strength compared to α-amylase activity and can therefore be eluted within a shorter gradient.
As with the previous run, the column was equilibrated in 0.005M sodium acetate buffer, the sample was loaded and a linear gradient applied from 0.005-0.2M. The gradient was exclusively collected in fractions 3-22, followed by step elution using 0.3M sodium acetate buffer, which was collected in fractions 23-36.

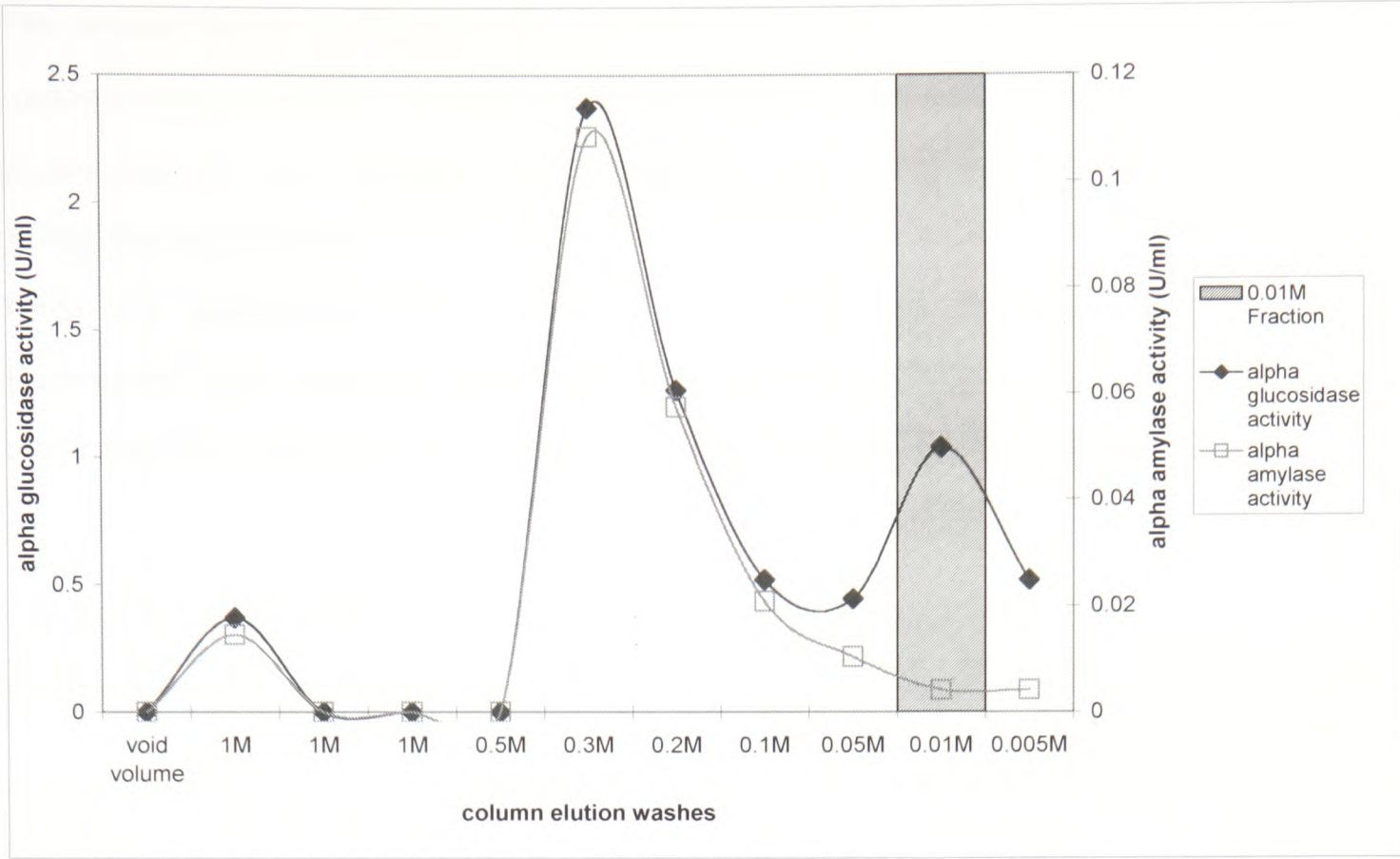


Figure 4.24: Chromatogram showing the separation of α-glucosidase and α-amylase enzyme activities using Phenyl Sepharose CL-4B. *The shaded area denotes the 0.01M HIC fraction, which was then further purified by anion exchange chromatography.*

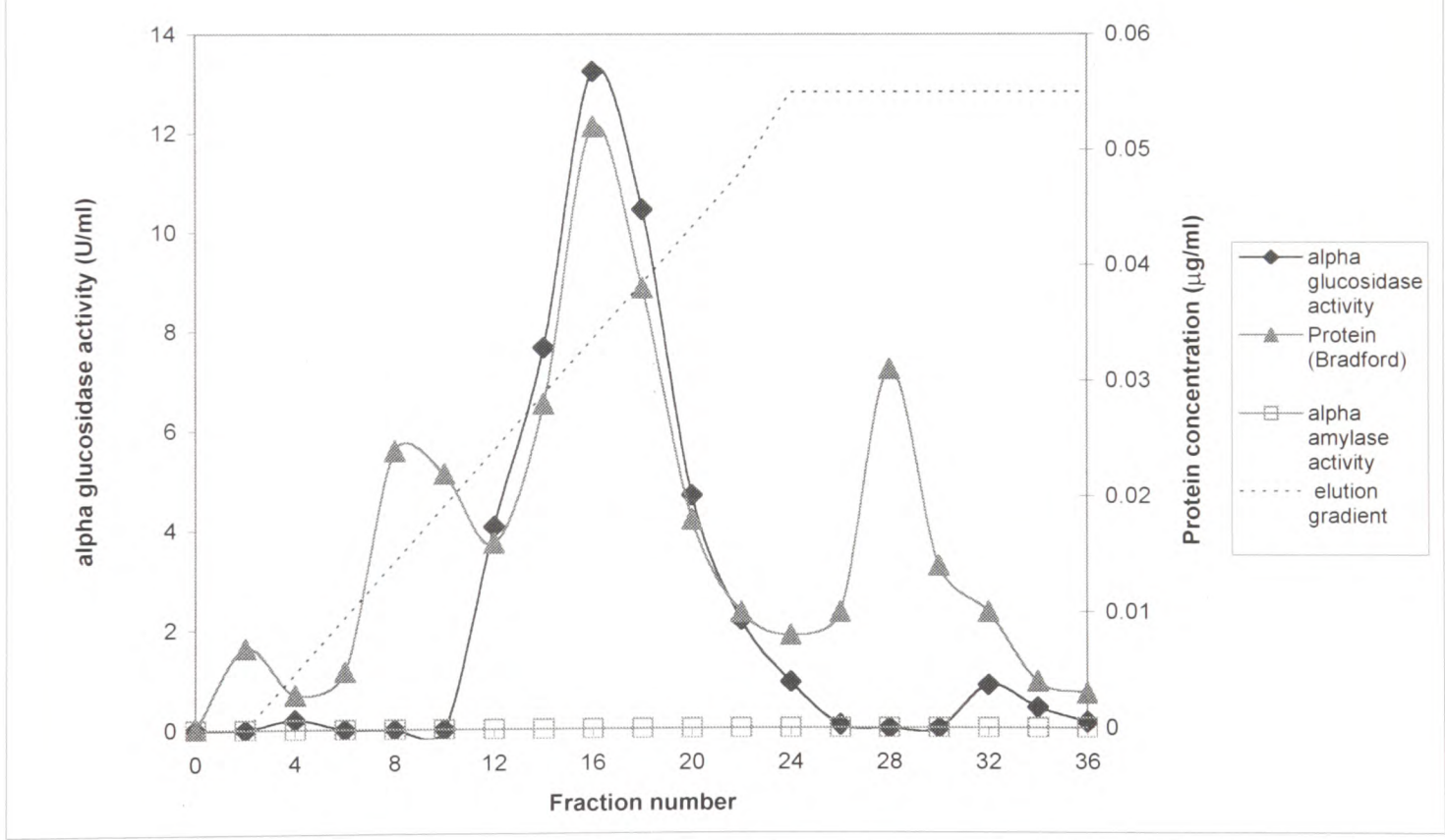


Figure 4.25: Chromatogram showing the elution of 0.01M HIC fraction using IEC. *α-Glucosidase and α-amylase activity was eluted from an 8ml bed volume Q-Sepharose column using linear gradient of 0.005-0.2M sodium acetate buffer, pH 5.0, followed by step elution using 0.3M sodium acetate buffer, pH 5.0 (as denoted by the dotted line)*

The results shown in Figure 4.25 illustrate the presence of impurities - presumably contaminating proteins - in the 0.01M HIC fraction that were separated from the α-glucosidase by ion exchange chromatography. No α-amylase activity was detected during the experiment.

Table 4.8 summarises the experiment and shows that for the pooled main α-glucosidase peak fractions, the purification factor was 24.6. In the case of the most active fraction, (fraction 16), a purification factor of 83.4 was calculated.

Table 4.8: Purification table of 0.01M HIC fraction using IEC

α-Glucosidase	Volume (ml)	Activity applied (U)	Protein loaded (mg)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	% Recovery in selected fractions	Purification factor
pooled fractions 10-26 inclusive	60	429.2	0.4	331.0	0.3	911.8	77	24.6
Individual fraction: 16	100µl	429.2	0.4	3.4	<0.01	3090.9	10.3	83.4

4.7 Data Summary of Chromatographic Experiments

The data presented in this table are derived from a particular purification experiment, in which the extracellular medium from a particular culture was subjected to ammonium sulphate precipitation followed by HIC and IEC, but it is representative of several similar experiments

Table 4.9: Purification Table and Specific Activity data of α-Glucosidase and α-Amylase

Step	α-Glucosidase	Total volume (ml)	Activity applied (U)	Total activity (U)	Total Protein (mg)	Specific Activity U/mg	(%) Recovery in selected fractions	Purific-ation Factor *
1	C.F.S	1000		210.2	9.6	21.9	100	1
2	$(NH_4)_2SO_4$ Pellet	30	210.2	161.0	9.0	18.0	71.5	0.8
3	HIC 0.3M	50	150.2	14.8	2.0	7.4	=59	0.4
	HIC 0.2M	30	150.2	33.3	1.1	31.8		1.8
	HIC 0.1M	50	150.2	22.2	1.8	12.7		0.7
	HIC 0.01M	50	150.2	18.5	0.5	37.1		2.1
4	IEC (0.3M)	27	6.8	2.0	0.08	25.0	29.2	3.4
	IEC (0.2M)	45	12.6	8.3	0.07	112.6	66.2	3.6
	IEC (0.1M)	53	19.6	2.0	0.5	4.0	10	0.3
	IEC (0.01M) Including Fraction 16	60	429.2	331.0	0.4	911.8	77	24.6
	Fraction 16	100µl		3.4	0.0011	3090.9	10.3	83.4
Step	**α-Amylase**	**Total volume (ml)**	**Activity applied (U)**	**Total activity (U)**	**Total Protein (mg)**	**Specific Activity U/mg**	**(%) Recovery in selected fractions**	**Purific-ation Factor ***
1	C.F.S	1000		20.3	9.6	2.1	100	1
2	$(NH_4)_2SO_4$	30	20.3	18.0	9.0	2.0	88.7	1.0
3	HIC 0.3M	50	16.8	2.5	2.0	1.3	=72	0.6
	HIC 0.2M	30	16.8	5.4	1.1	5.1		2.6
	HIC 0.1M	50	16.8	4.2	1.8	2.2		1.1
	HIC 0.01M	50	16.8	0	0.5	0	0	0
4	IEC (0.3M)	34	0.8	0.6	0.06	9.5	72.7	7.6
	IEC (0.2M)	80	4.9	3.0	0.09	33.3	61.6	6.5
	IEC (0.1M)	86	4.17	4.08	0.66	6.2	97.8	2.8
	IEC (0.01M)		0	0	0	0	0	0

** The purification factor of each individual fraction was calculated by reference to the specific activity determined of the immediately preceding step in the purification process.*

Table 4.10: Summarised Protein Recovery Data after Ion Exchange Chromatography

Fraction	protein applied to HIC (mg)	protein recovered from selected HIC fractions (mg)	protein applied to IEC (mg)	protein recovered from selected IEC fractions (mg)	% protein recovered in selected fractions post HIC and IEC
α-Glucosidase					
0.3M	9.00	2.00	0.99	0.08	0.9%
0.2M		1.05	0.51	0.074	0.8%
0.1M		1.75	1.20	0.49	5.47%
0.01M		0.5	0.363	0.29	3.2%
α Amylase					
0.3M	9.00	2.00	0.99	0.06	0.7%
0.2M		1.05	0.51	0.09	1.0%
0.1M		1.75	1.20	0.66	7.4%

The collated specific activity and protein data summarised in tables 4.9 and 4.10 track the α-glucosidase and α-amylase activities purified on a two column chromatographic system. The results clearly indicate considerable purification of both enzymes with the specific activity of 911.8U/mg calculated for the purified α-glucosidase and 33.3 U/mg for the α-amylase, as determined after completing the second chromatography step.

4.8 Discussion: Purification of α-Glucosidase and α-Amylase Activity using a Two Column Chromatography System

An important aspect of developing a complete purification scheme is to keep the number of steps to a minimum - essentially to maintain enzyme stability and prevent loss of activity through denaturation or proteolytic degradation. Here, the use of hydrophobic interactive chromatography was especially useful in conjunction with ion exchange chromatography - combining two techniques based on different principles, and thereby exploiting different surface properties of the different proteins to be separated. Logically, HIC fits naturally after an ammonium sulphate precipitation, where the sample already has high ionic strength. Also the high salt provides a stabilizing effect on the protein fraction, beneficial to the long-term stability of the active fractions. The column was effective in removing a large proportion of contaminating material, and was also partially successful in separating the bulk of the α-glucosidase from the main α-amylase peak. The HIC medium chosen was Phenyl Sepharose CL-4B because it has a high binding capacity, allowing large amounts of protein to be processed on smaller (quicker) columns, and enzyme recoveries were good (Table 4.4).

The overall performance of HIC, for example, when using Phenyl Sepharose CL-4B, can be affected by factors such as temperature. By performing all hydrophobic chromatography at room temperature (~21°C), and essentially standardizing all the other experimental conditions - such as buffer composition, run-to-run variability was minimized which allowed for the development of a reproducible method for purifying the α-glucosidase.

α-Glucosidase activity was detected in more than one peak and it could be possible to improve on the resolution of the minor peaks by either selecting a different medium to one with a greater or lower degree of ligand substitution – however, the initial screening experiments of different hydrophobic ligands (Octyl or Butyl Sepharose), did not show a significant increase in resolution. (This could be due in part, to the complex nature of the protein precipitate, which may contain a significant number of contaminating proteins). Currently, HIC ligand manufacturers do not produce a

hydrophobic ligand, with a lower degree of ligand substitution than Phenyl Sepharose CL-4B.

Another possible option was to elute the enzymes from a Phenyl Sepharose CL-4B column with a pH gradient instead of a decreasing salt gradient. This may have been a much more selective method in resolving all the α-glucosidase-containing-peaks, present in the resuspended ammonium sulphate pellet.

Choosing a suitable ion exchange medium as the second chromatographic column and selecting suitable eluting conditions was not straightforward. Ultimately, the IEC matrix, Q-Sepharose was chosen in preference to DE52, as DE52 was found to be inconsistent, with considerable run-to-run variability. While, α-glucosidase and α-amylase activities showed some selectivity for the DE52 ligand – (the main peaks were separated), the DE52 adsorbent showed poor capacity for the enzymes under the chosen experimental conditions. DEAE DE52, as a weak anionic exchanger, is susceptible to changes in pH and ionic strength. For a robust, reproducible assay, the column matrix must be better able to maintain charge over a wide range of experimental conditions and withstand pH fluctuations and ionic strength changes of buffer. Q-Sepharose is a strong exchanger designed to provide the essential properties for reproducible anion exchange chromatography: a fast flow rate with strong binding, is better able to maintain its charge over a wide range of experimental conditions. Q-Sepharose has a ligand with more highly substituted ionic groups, and therefore any unwanted hydrophobic interactions should be eliminated.

Practically, Q-Sepharose displayed a number of advantages over DE52, such as requiring a minimum number of pre-treatment steps prior to use. There was no problem with fines disturbing the column matrix. There was also no need to eliminate the irreversible binding sites on the matrix, which had previously resulted in a loss in recovery of α-glucosidase activity from DE52 (as encountered at the early stage in the purification process (section 4.3.1)). Experiments carried out using both matrices demonstrated consistent performance and improved binding capacity when using Q-Sepharose.

Selecting the correct elution conditions was an important factor in achieving the maximum resolution between the two enzyme activities by anionic exchange. A variety of column conditions were assessed, with a combination of gradient and isocratic elution techniques. A long shallow gradient separated the two activities but

some problems still persisted, such as the appearance of broad peaks eluting across many fractions. Assaying numerous, dilute fractions was also time-consuming and costly. However, the fractionations were largely improved upon by reducing the total gradient volume, and by reducing the concentration of the upper limit buffer. The most effective use of IEC achieved here comprised of a linear gradient followed by step-elution, using a higher concentration buffer. Thus, as shown in Figure 4.25, α-glucosidase eluted from the column with the linear gradient while the α-amylase eluted off the column in a tight, concentrated peak with the higher ionic strength buffer (care was taken to avoid co-elution of any other strongly bound enzymes by not washing the column with buffers with an ionic strength greater than 0.5M).

Overall, the two-column system was effective in purifying α-glucosidase and α-amylase activity. Individual HIC fractions purified by ion exchange showed between a 0.3 to 24.6 fold increase in purity for α-glucosidase and between a 2.8 to 7.6 increase in purity for α-amylase, and by selecting the most active α-glucosidase fraction, isolated after running the 0.01M HIC fraction on ion exchange, the overall purification factor rose to 83.4.

To achieve a higher enzyme yield, the HIC step could be scaled-up, or alternatively, several medium-scale hydrophobic columns could be set up in parallel, with all the subsequent 0.01M HIC fractions pooled, concentrated, and then purified in one step by ion exchange chromatography. The α-glucosidase enzyme thus purified, and at concentrations approaching milligram levels, would be suitable for much further characterisation work.

Chapter 5: Electrophoretic Analyses of Protein Fractions and Physicochemical Properties of Purified α-Glucosidase and Enzyme Activity Staining

5.1 Introduction

The results described in this chapter include gel electrophoretic analyses of chromatographic fractions produced by various carbohydrase purification experiments (see Chapter 4) - and concomitant estimation of molecular weights of prominent protein bands (putative α-glucosidases/carbohydrases).

The molecular weights of yeast α-glucosidases have been determined by a number of groups, with estimates varying between 36,000 for the α-glucosidase purified from *Lipomyces starkeyi* CBS 1809 (Kelly *et al.*, 1985), and 270,000 Daltons for an α-glucosidase purified from *Saccharomyces logos* by Chiba *et al.*, (1973).

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) is very widely used to analyse protein mixtures, simultaneously providing molecular weight information, and it was the electrophoretic technique mainly used here. However, native (non-denaturing) PAGE gels were also used to assess molecular weights, and, because they do not necessarily inactivate enzymes, they were used in experiments designed to identify the enzyme activity corresponding to protein bands made visible by Coomassie Blue staining.

In other experiments reported here, purified α-glucosidase was analysed by isoelectric focusing, the effects of pH and temperature on α-glucosidase were investigated, and the enzyme's carbohydrate content was estimated.

5.2 Results and Discussion

5.2.1 Monitoring Carbohydrase Purification by SDS Gel Electrophoresis and Estimating Molecular Weights

Many carbohydrases purification experiments were monitored by electrophoretically analysing samples at different purification stages: after ammonium sulphate precipitation (section 4.2.1), post-hydrophobic interactive chromatography (HIC) (section 4.4.2) and post-ion exchange chromatography (IEC) (section 4.6).

5.2.1.1 Monitoring of Fractions from HIC

HIC using Phenyl Sepharose CL-4B was selected to fractionate the proteins from the initial ammonium sulphate pellet. Figure 5.1 shows the results of analysing representative and selected fractions collected from a typical HIC column, by SDS-PAGE.

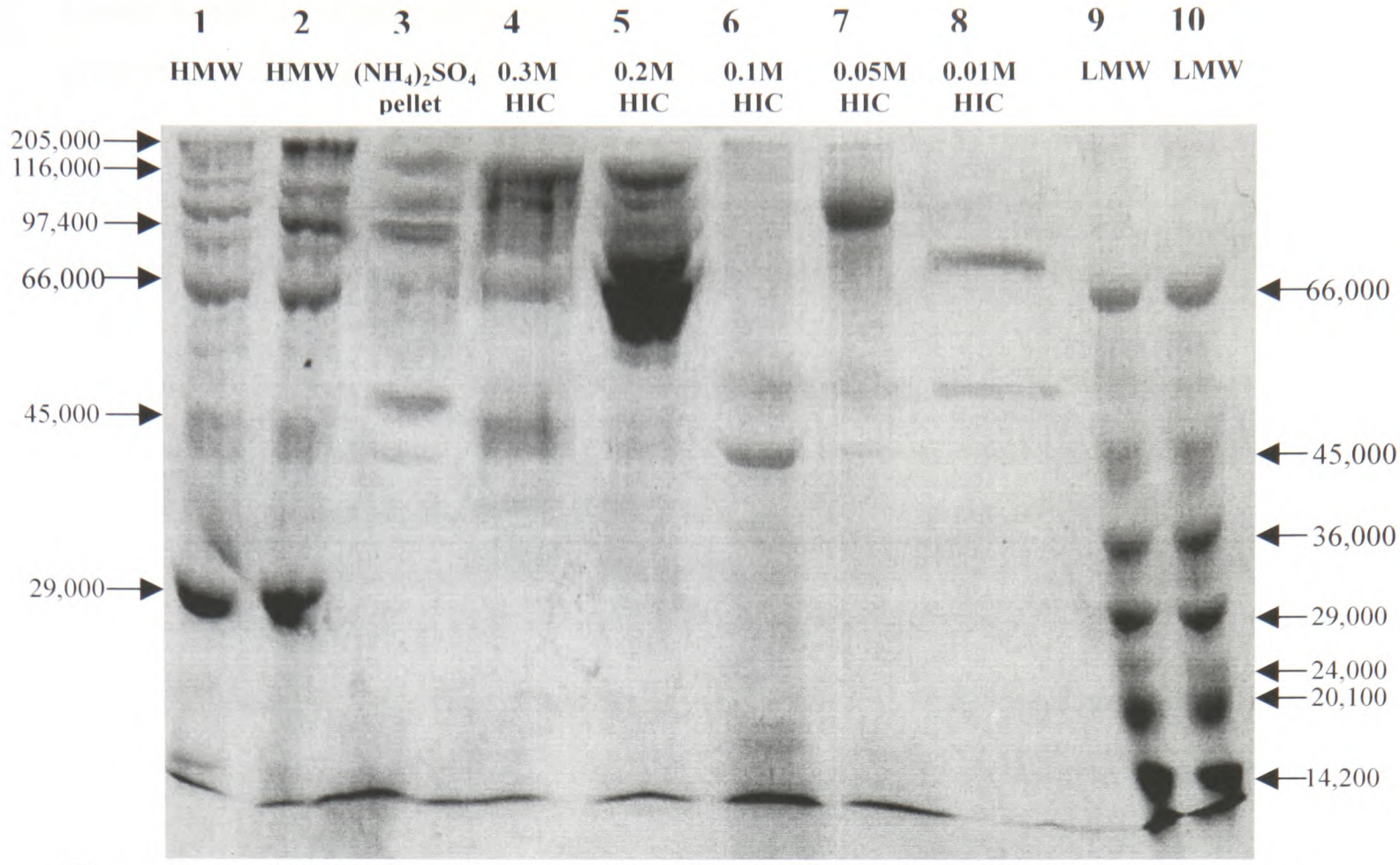


Figure 5.1 A 9% SDS-polyacrylamide gel of HIC fractions (Section 4.4.4, Figure 4.6); *lane 3: ammonium sulphate precipitate containing 10μg protein (Section 4.2.1); lane 4: 0.3M HIC fraction containing 20μg protein; lane 5: 0.2M HIC fraction containing ~ 25μg protein; lane 6: 0.1M HIC fraction containing ~10μg protein; lane 7: 0.05M HIC fraction containing ~20μg protein; lane 8: 0.01M HIC containing ~10μg protein; lanes 1, 2: (HMW) high molecular weight markers (Sigma); lanes 9,10: (LMW) low molecular weight markers (Sigma).*

The gel results in Figure 5.1 are representative of those obtained by other SDS gel analyses of comparable HIC experiments and fractions collected.

Although 8 bands were detected in the re-dissolved ammonium sulphate pellet (lane 3), the adjacent lanes, 4 to 9, (which contain fractions eluted from Phenyl Sepharose CL-4B column), appear to contain a number of additional bands not visible in lane 3. This is possibly due to either under-loading the lane and therefore some bands were below the limit of detection of Coomassie Blue, or the fact that HIC effectively concentrated the enzyme fractions, and that minor bands were now visible. The salt precipitation step was effective in extracting the dilute α-glucosidase present in the cell-free supernatant but other contaminating proteins present in the extracellular medium were also precipitated. This is consistent with the earlier observation that ammonium sulphate precipitation effected little purification relative to the cell-free supernatant (see Table 4.9).

Lanes 4 and 5, which represent fractions eluted in high salt buffer, have a high proportion of different proteins, which is consistent with protein assays conducted on this material – which demonstrated a high protein content (section 4.4.4). Lane 6 contains a number of low molecular weight bands, not detected in the ammonium sulphate resuspended pellet. Lanes 7 and 8, which represent fractions eluted with a lower ionic strength buffer, contained relatively few bands and indicated that HIC was effective in fractionating the ammonium sulphate pellet. Overall, the different band patterns in the different column washes indicate that some significant protein fractionation occurred, as is also indicated by the different carbohydrase specific activities of these fractions.

Molecular weight estimates of all major and minor bands were determined from a calibration curve constructed using the marker proteins, (Appendix 2), and are summarised in Table 5.1.

Table 5.1: Molecular weight estimates of protein bands seen in Figure 5.1.
α-Glucosidase and α-amylase active fractions after batch elution from a typical Phenyl Sepharose CL-4B HIC column, (section 4.4.2). Figures in bold depict main, prominent bands.

LANES									
1	2	3	4	5	6	7	8	9	10
HMW	HMW	$(NH_4)_2SO_4$ pellet	0.3M	0.2M	0.1M	0.05M	0.01M	LMW	LMW
205,000	205,000	149,000							
		130,000	**125,000**	125,000	125,000	125,000			
116,000	116,000								
		114,000	112,000	114,000		**110,000**			
		107,000		105,000					
97,400	97,400						**98,000**		
						97,000			
		92,000	**89,000**	88,000		85,000			
				77,000	73,000				
66,000	66,000		67,000	70,000				66,000	66,000
		63,000		63,000					
							60,000		
		56,000		52,000	56,000	57,000			
45,000	45,000	46,000	**48,000**	47,000	**45,000**	46,000		45,000	45,000
			39,000	38,000	35,000			36,000	36,000
29,000	29,000				30,000			29,000	29,000
					24,000			24,000	24,000
					21,000			20,100	20,100
					16,000				
					15,000			14,200	14,200

In summary, this gel shows that the resuspended ammonium sulphate pellet is a complex mixture containing approximately 19 different proteins - summing all the apparently different species in all the different fractions. However, allowing for a 5% margin of error for molecular weight determinations, it is possible to identify specific bands in different lanes as being the same protein, but eluting in different HIC fractions – and showing slightly different mobility. This cross-identification reduces

the number of proteins detected from 19 to approximately 12 main species, of molecular weights: of 149,000, 125,000, 110,000; 107,000; 97,000; 89,000; 77,000; 70,000; 63,000; 60,000; 57,000 and 48,000. Several lower molecular weight bands (<29,000) seen in lane 7 could conceivably be proteolytic degradation products, and/or medium-derived products (yeast extract and peptone).

As lane 8 contains one significant band – 98,000, and it has substantial α-glucosidase activity, but very little α-amylase, the 98,000 species could be speculatively identified as an α-glucosidase. Lane 4 has a sample of the fraction containing the highest α-glucosidase and α-amylase activities. Though not all bands are clearly resolved, there appears to be three prominent bands, of 125,000, 89,000 and 48,000, which could either be α-glucosidase or α-amylase or contaminant species.

5.2.1.2 SDS-PAGE of Fractions Eluted from IEC

Initially, fractions after HIC containing α-glucosidase and α-amylase activity were pooled separately and dialysed against 10mM sodium phosphate buffer, pH 6.8 and freeze-dried prior to analysis by ion exchange chromatography (Figure 4.14). After IEC, active fractions were electrophoresed under denaturing conditions. In some experiments, the gel was first stained by Coomassie Blue and then, to detect any minor species, the gel was silver-stained to increase sensitivity and detect all the minor species in each fraction.

Figure 5.2 shows a typical result for α-glucosidase and α-amylase-rich fractions collected after DE52 ion exchange chromatography, and then electrophoresed.

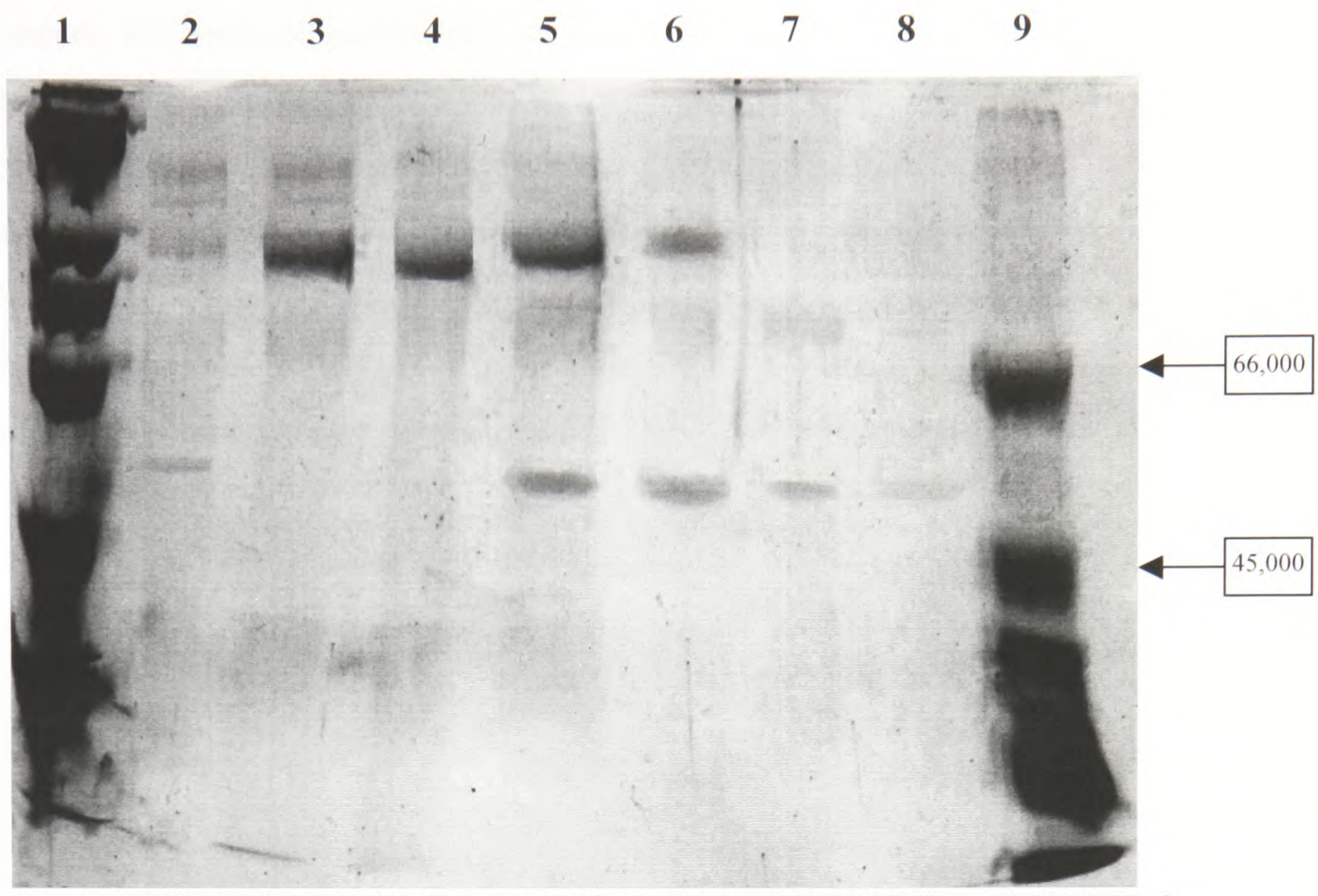


Figure 5.2: 9% SDS-PAGE gel of fractions batch-eluted from a DE52 IEC column (Section 4, Figure 4.14). *Lane 1: HMW markers; Lane 2: pooled α-glucosidase and α-amylase -rich fraction HIC fraction – which was applied to the IEC column: Lane 3: 0.1M IEC fraction; Lane 4: 0.2M IEC fraction; Lane 5: 0.3M IEC fraction, Lane 6: 0.4M IEC fraction; Lane 7: 0.5M IEC fraction; Lane 8: 0.5M IEC fraction; Lane 9: LMW markers.*

The gel shows that the IEC fractions have distinctive protein band profiles, indicating that significant fractionation had been achieved, more so than with HIC (Figure 5.1). Although the DE52 column (Figure 4.14) did not entirely separate α-glucosidase and α-amylase activities, the peak fraction for α-glucosidase ("0.1M") lacked detectable α-amylase, whereas the peak α-amylase fraction ("0.3M") had about half the peak level of α-glucosidase activity. A prominent protein band fitting the α-glucosidase elution pattern has a molecular weight of about 98,000, whereas two protein bands, of 78,000 and 48,000, fit the α-amylase elution pattern, the 48,000 band being stronger.

5.2.1.3 SDS-PAGE of α-Glucosidase-rich HIC fractions purified by IEC

However, the finalised purification scheme exploited the resolution achieved at each of the HIC and IEC steps, by minimising the pooling of active fractions, and the relevant electrophoretic analyses are described next.

When individual HIC fractions were run separately on ion exchange columns (section 4.6), substantial purification was seen in terms of increased specific activity (Table 4.9) and this was reflected in simplified band patterns of SDS-PAGE analysis of samples. Figure 5.3 shows a typical result for an HIC 0.01M salt fraction.

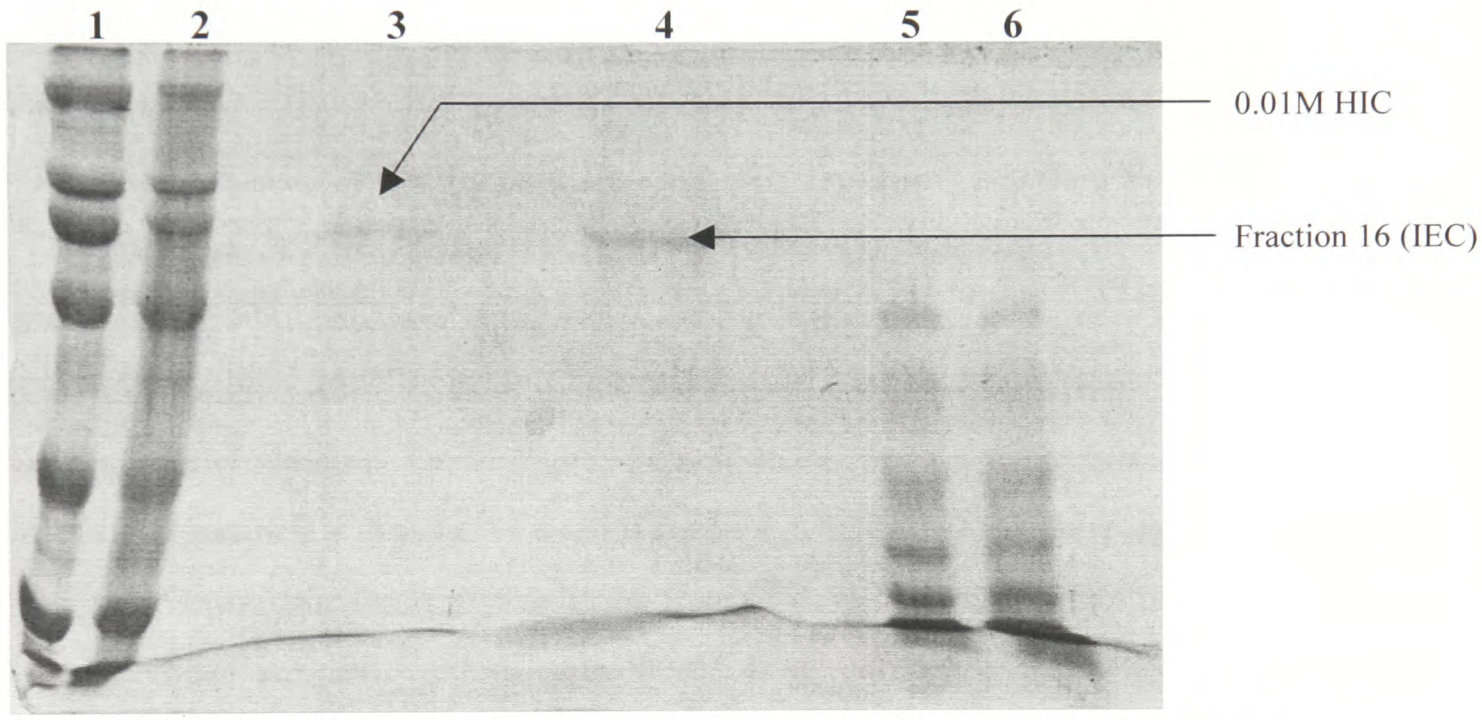


Figure 5.3: 7.5% SDS-PAGE gel of 0.01M HIC fraction purified by IEC.
Lane 3: 0.01M HIC fraction (Figure 4.24, Section 4.6.2.4), lane 4: fraction 16 collected after IEC (Figure 4.25); lanes 1-2, HMW markers; lanes 5-6: LMW markers

Track 3 of Figure 5.3 shows that the 0.01M HIC fraction, which had little detectable α-amylase activity when assayed using the Phadebas α-amylase assay (section 2.10.2), contains a single detectable α-glucosidase species of ~93,000. Track 4 shows that the purest α-glucosidase fraction (16) obtained by IEC of the 0.01M HIC fraction (and which also had no detectable α-amylase activity (Figure 4.25)) contained only a single detectable α-glucosidase species, apparently identical to that of track 3.

The 0.3M, 0.2M and 0.1M HIC fractions - containing both α-glucosidase and α-amylase activity were also purified by IEC and fractions were analysed by SDS-

PAGE. Figure 5.4 shows the results obtained after a 0.2M HIC fraction was applied to a Q-Sepharose column (Figure 4.21).

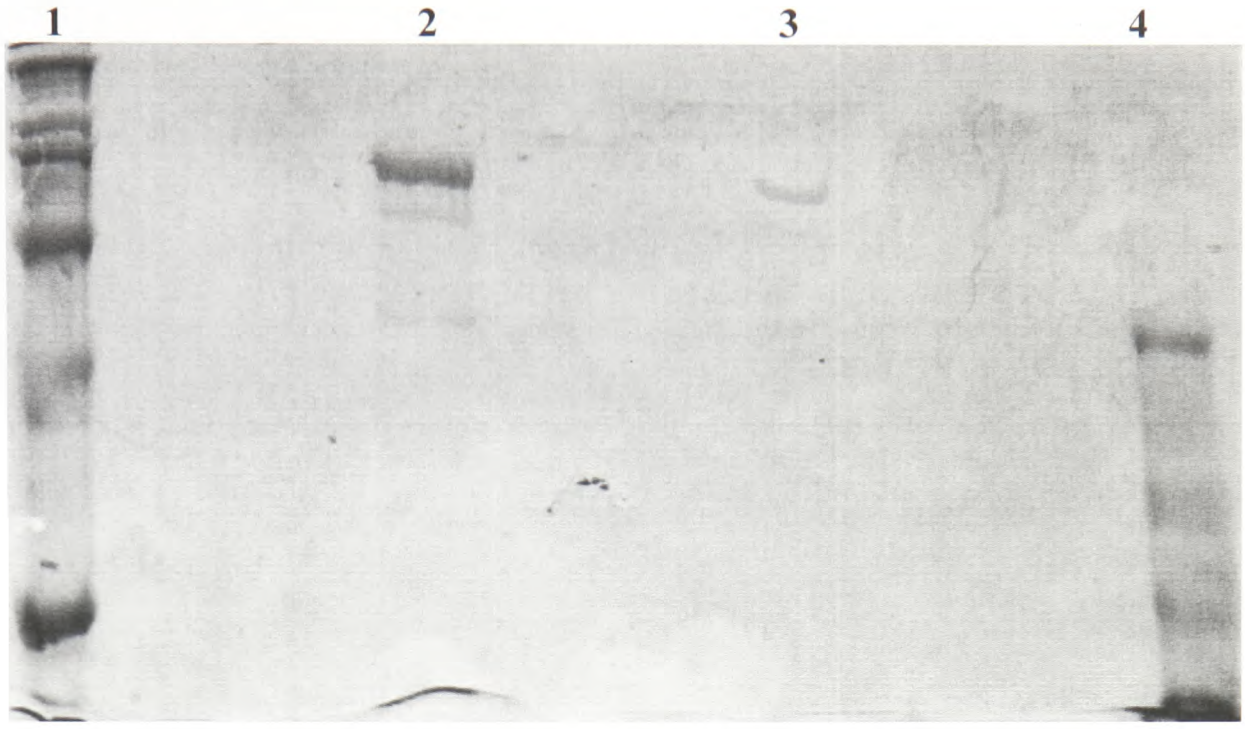


Figure 5.4. A 7.5% polyacrylamide gel of α-glucosidase-rich IEC fraction, purified using Q-Sepharose (Figure 4.21). *Lane 1: HMW marker; Lane 2: IEC fractions 22-24; Lane 3, individual fraction 17 collected after IEC; Lane 4: LMW marker.*

Tracks 2 contained a sample of pooled fractions 22-24, which were collected from the Q-Sepharose anionic exchange column. Lane 3 was loaded with the individual fraction 17 (the most active α-glucosidase fraction in this run). Track 3 has only one discernible band, apparently identical to the most prominent band in track 2 – a species of ~93,000 - 97,000. Track 2 also shows two fainter bands of molecular weights of ~85,000 and 58,000 respectively.

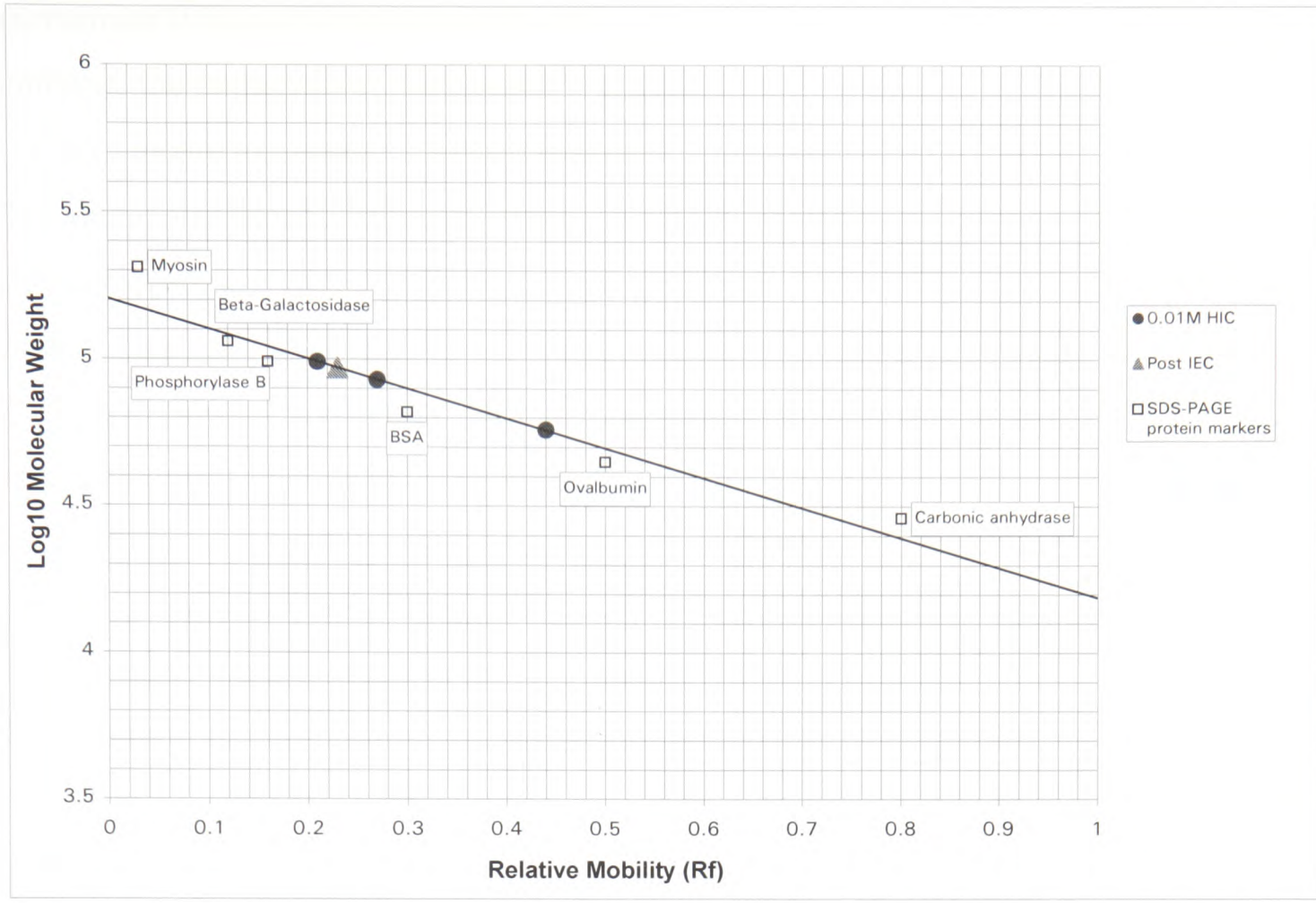


Figure 5.5: Calibration curve constructed to determine the molecular weight of α-glucosidase after anionic exchange chromatography.

5.2.1.4 Molecular Weight Estimates of α-Glucosidase and α-Amylase Activities using SDS-PAGE

In summary, the SDS-PAGE analyses of the various chromatographic fractions produced by the various carbohydrase experiments shows that some degree of purification between the two enzymes has been achieved. The concomitant molecular weight determinations of the α-glucosidase and α-amylase after each stage of the purification were estimated (within 5% margin of error), and indicated that a putative α-glucosidase of 93,000-98,000 and 4 possible α-amylase activities ranging from between 48,000 – 125,000, have been purified.

The molecular weight estimates for the *L. starkeyi* α-glucosidase purified here is within the range of the published molecular weight determinations of other yeast α-glucosidases. Chiba *et al.* (1973b) purified an intracellular α-glucosidase purified from *Saccharomyces logos.* They discovered that the α-glucosidase was similar to that found in moulds, when they examined various structural and kinetic properties. From these results they determined that the purified α-glucosidase was a glycoprotein -

containing 50% (w/w) carbohydrate, and estimated the molecular weight at 270,000. In contrast, the extracellular thermostable α-glucosidase purified by Kelly *et al.,* in 1985 – from *Lipomyces starkeyi* - had an estimated molecular weight of 35,000.

The molecular weight estimates of other yeast α-glucosidases lie within this 35,000 to 270,000 Dalton range. Halvorson and Ellias (1958) purified an intracellular α-glucosidase from the yeast, *Saccharomyces italicus* (Y1225). The purified enzyme had a pH optimum of between 6.6 and 6.8, an estimated molecular weight of 85,000 and appeared to be sensitive to heavy metals.

In 1967, Khan and Eaton reported that the α-glucosidase purified from a strain of *Saccharomyces cerevisiae* (1412-4D) could be separated into two enzymatically active components, maltase and isomaltase. The purified maltase preparation gave a single band of molecular weight of 68,500, while preparations of isomaltase was found using PAGE to contain two bands (a major and minor band). It was assumed the major band represented the enzyme, and the molecular weight was estimated at 64,700.

Needleman *et al.,* (1978) purified an α-glucosidase from the yeast *Saccharomyces carlsbergensis*. They estimated the molecular weight at 66,000 and pH optimum between 6.7-6.8. Matsusaka *et al.,* (1977) identified three types of glucosidase activity from a strain of *Saccharomyces cerevisiae* and designated them simply as I, II and III. They explored the substrate specificity of the three enzyme activities, but they did not determine the molecular weights of any of the three fractions.

Oda *et al.*; (1993), partially purified an intracellular α-glucosidase from the yeast *Torulaspora pretoriensis YK1*. Preparative PAGE identified a single band with an estimated molecular weight of 69,000, though with gel filtration, the molecular weight of the protein was estimated as 60,000. From the similar values obtained by both methods, they concluded that the enzyme was monomeric.

5.2.2 Analysis, Molecular Weight Estimation and Activity Detection using Native Gel Electrophoresis

After purifying α-glucosidase by HIC and then by IEC and visualizing a single band by SDS-PAGE, there still remained the possibility that a contaminant protein had been purified and therefore there was a need for a specific activity stain to confirm the identity of the band. Clearly, native (non-denaturing) gel electrophoresis would need to be employed prior to applying an activity stain. A typical result of native gel electrophoresis analysis of IEC-purified carbohydrase samples (bands detected by staining with Commassie Blue), is shown in Figure 5.6.

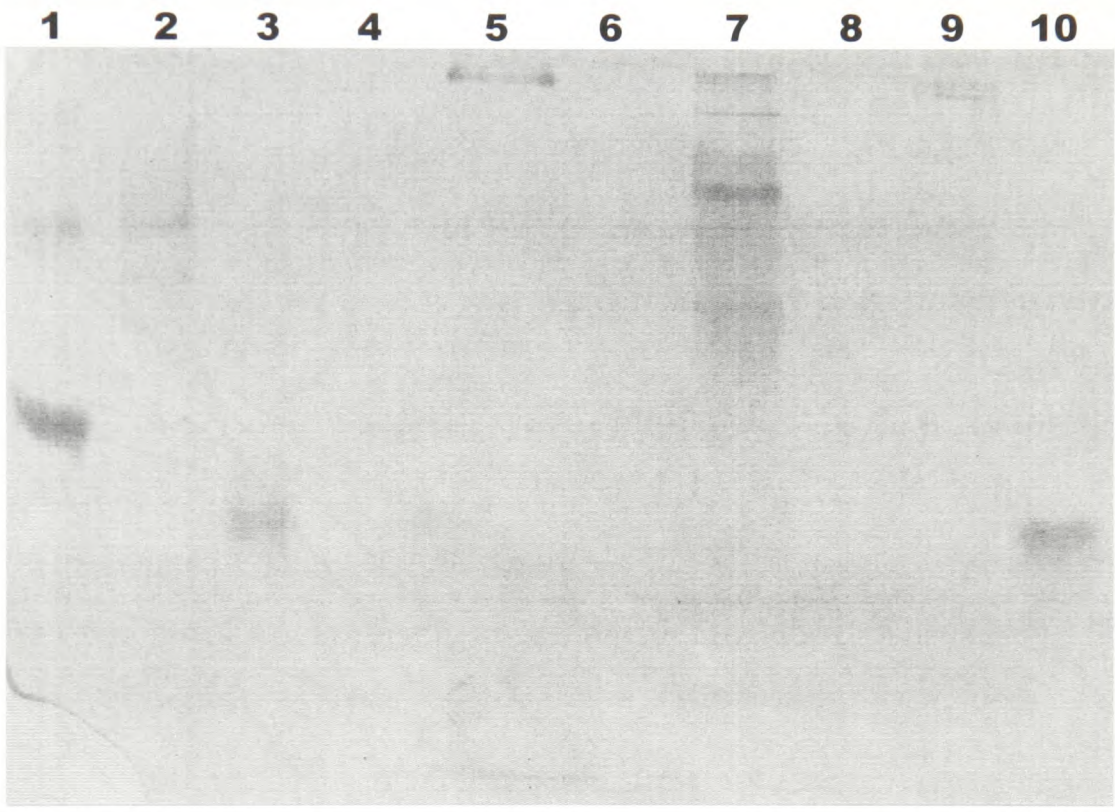


Figure 5.6: 7.5% Native polyacrylamide gel of samples collected after IEC (Section 4.6.2.4). *Lane 1: BSA, Lane 2: Carbonic anhydrase, lane 3: chicken egg albumin, Lane 5: L. starkeyi α-glucosidase (10μg/ml), Lane 7: L. starkeyi α-amylase, (10μg/ml, Section 4.6.2.2), Lane 9: Urease, Lane 10: α-Lactalbumin*

Lane 5, which contained purified α-glucosidase, apparently contains only a single high molecular weight band, whereas, lane 7, which contains α-amylase activity collected after ion exchange chromatography, appears as a heterogeneous sample with several bands visible, though one band is very predominant.

5.2.2.1 Molecular Weight Estimation of Purified α-Glucosidase using Native PAGE

The molecular weight of the putative α-glucosidase was estimated by further non-denaturing PAGE analysis: the enzyme was electrophoresed alongside standard marker proteins using a variety of different acrylamide gel concentrations, ranging from 7.5% to 12% (section 2.3).

The relative mobility Rf of each of the standard markers was calculated, and then 100[Log (Rf x 100)] was plotted against the gel concentration as a percent for each protein standard (Figure 5.7). The negative slopes from these curves were then plotted against the known molecular weights of the standards on a 2-cycle log-log curve (Figure 5.8). The molecular weight of the unknown protein was then extrapolated from this graph.

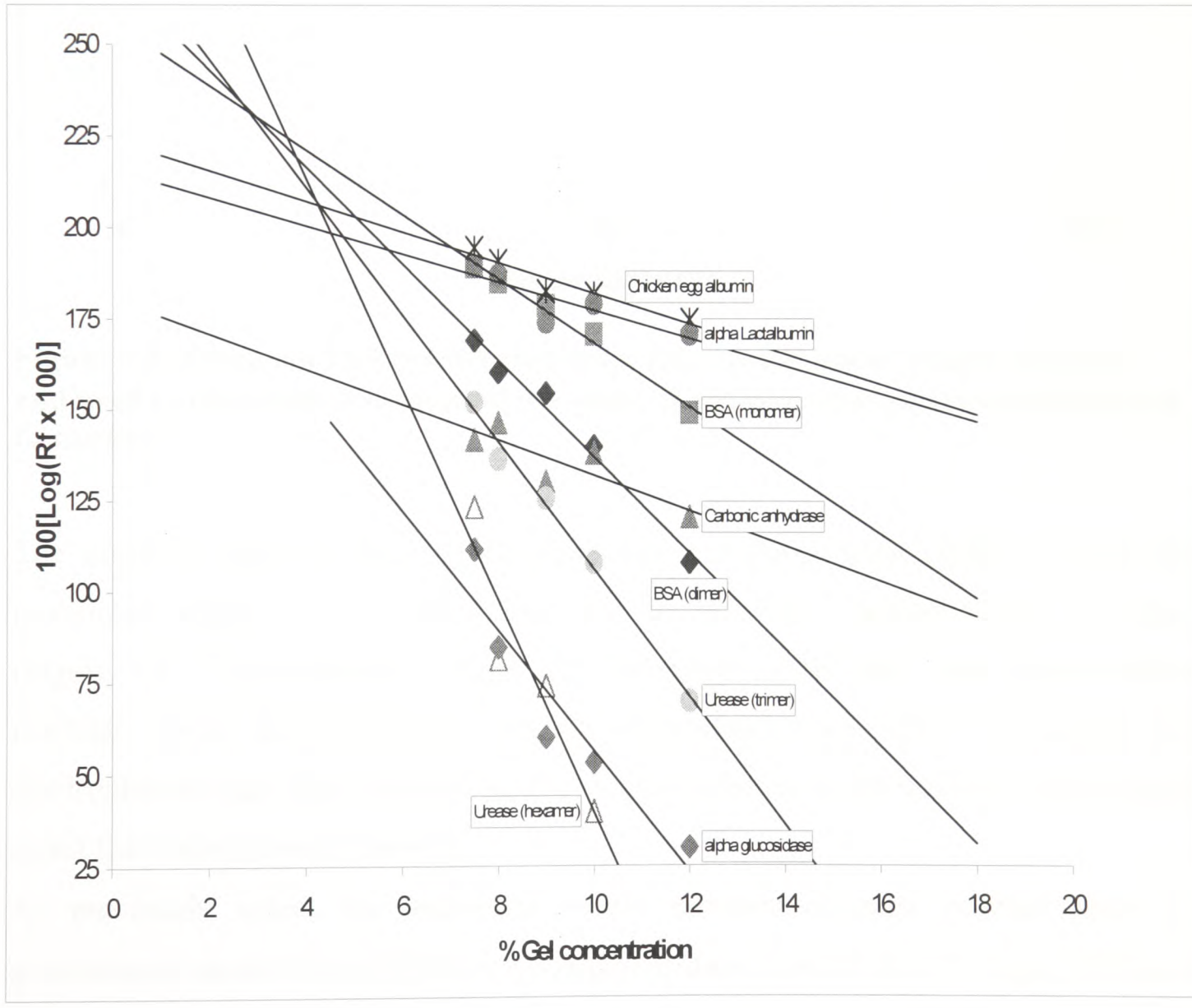


Figure 5.7: Determination of molecular weight of α-glucosidase using a calibration curve of known molecular weight markers by non-denaturing PAGE.

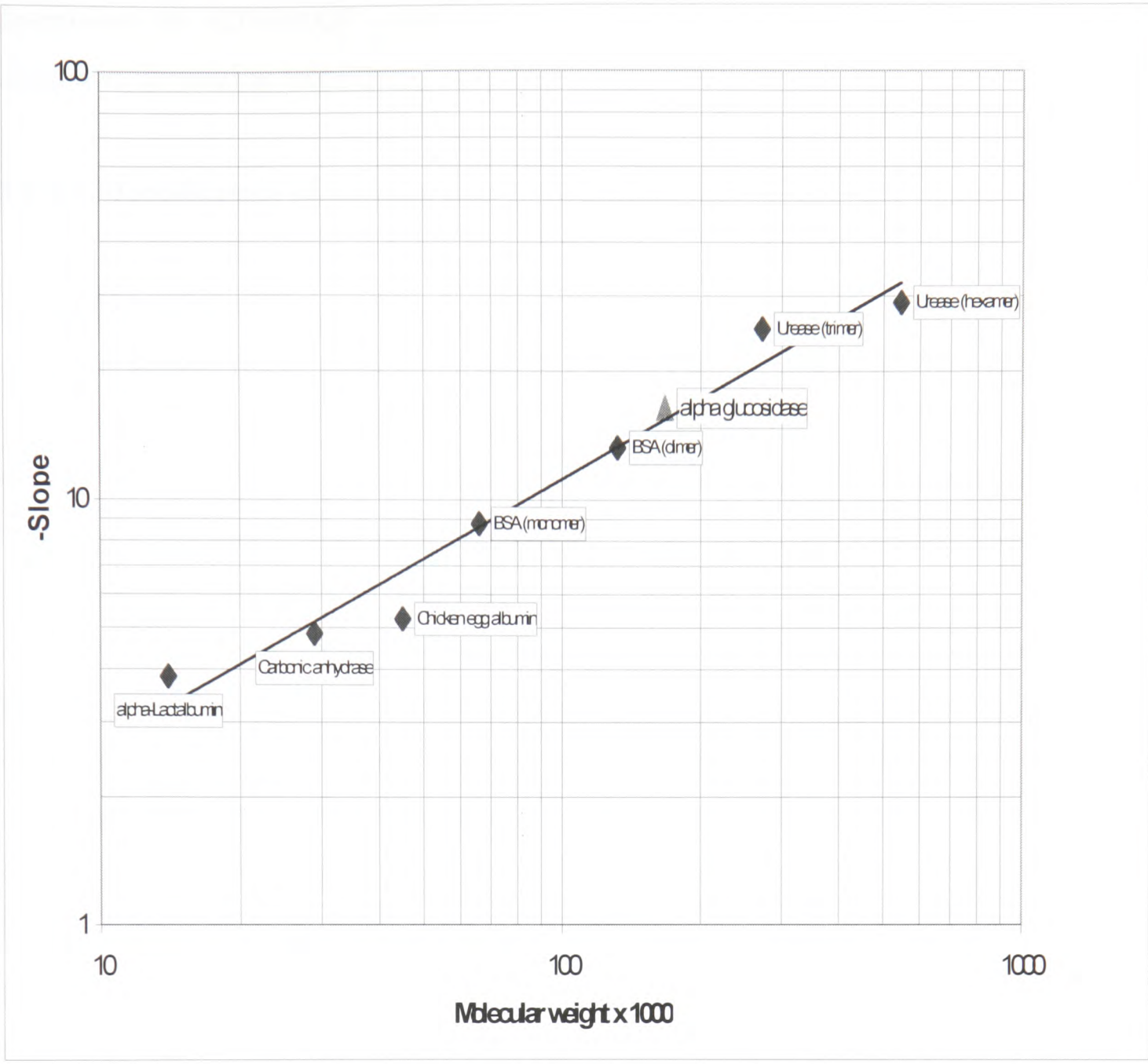


Figure 5.8: Ferguson Plot constructed from known molecular weight markers and used to determine the molecular weight of native α-glucosidase purified from *L. starkeyi.*

The negative slope of the α-glucosidase was calculated from Figure 5.7 and the molecular weight was calculated from the curve of the standard marker proteins (Figure 5.8). The molecular weight was estimated as 167,000. This figure differs markedly from the estimate of 98,000 determined from SDS (denaturing) gel electrophoresis and may possibly be due to glycosylation of the enzyme, which could affect the electrophoretic mobility.

As previously noted, the molecular weight estimate of other purified yeast α-glucosidases varies from 270,000 for a α-glucosidase purified from *S. logos* (Chiba *et al.*, 1973), to an estimated 36,000 for an α-glucosidase purified by Kelly *et al.*, (1985) from *L. starkeyi,* (Section 1, Table 1.1). Most molecular weight estimates have been

determined by SDS-PAGE with little published on the molecular weight estimates using non-denaturing techniques.

5.2.2.2 Identification of α-Glucosidase Activity in Gels

Typically, α-glucosidase activity in gels is measured by using the enzyme's action on chemical derivatives of glucose, to produce a fluorescent or coloured product, which can then be used directly to generate a chromophore (Swallow *et al.*, 1975; Kopetzki *et al.*, 1989). A disadvantage of this type of method is that the glucose derivatives are not specific for α-glucosidases, since they can also be substrates for enzymes that have α-1,6 enzyme activity - such as isomaltase.

Alternatively, normal α-glucosidase substrates such as maltose and starch are used, and the glucose released is coupled to the generation of a coloured product by the use of auxiliary enzymes. A disadvantage to using this type of method is that hazardous, carcinogenic, fluorescent dyes are used.

In 1990, Finlayson *et al.* reported two new staining procedures, (Method A and Method B), which were capable of differentiating between α-1,4- and α-1,6-glucosidase activities and did not inactivate the enzymes, which could then be subsequently recovered from the gel. In Method A, an agarose overlay, which contains substrate (maltose or isomaltase), glucose oxidase, peroxidase, 2,4-dichlorophenol and 4-L-aminophenazone, is cast over the PAGE gel, and a purple colour is produced at the site of enzyme activity. An advantage of this method is that non-hazardous chemicals are used; unfortunately the stain is not permanent.

Method B gives a permanent stain but uses hazardous chemicals. This time the polyacrylamide gel is overlaid with agarose containing substrate (maltose or isomaltose), glucose oxidase, phenazine methosulphate and nitroblue tetrazolium. Glucose production results in the nitroblue tetrazolium being oxidised to an insoluble formazan with a dark-blue colour.

Method A was used in an attempt to measure the *Lipomyces starkeyi* α-glucosidase activity after PAGE, but problems arose in the sulphonation of the 2,4-dichlorophenol and in the subsequent negative staining of the original PAGE gel and to a lesser degree the agarose overlay. The stain was faint, possibly due to the low concentration of

enzyme loaded or insufficient blotting of the gel, and disappeared within a few minutes. Such a non-permanent stain was impractical (Method A), and as the use of toxic chemicals was undesirable and expensive (Method B), other methods for identifying α-glucosidase activity in gels were explored.

5.2.2.2.1 Assay of α-Glucosidase Activity after PAGE by Elution from Gel Slices

Extraction of protein from acrylamide gels can be accomplished by passive diffusion, (Bhown and Bennett, 1984), so an attempt was made to assay α-glucosidase extracted from gel slices. A 10 track 7.5% native polyacrylamide gel was prepared (section 2.3.14), and active fractions collected after IEC (Figure 4.25) were applied (lanes 3-10), alongside non-denatured BSA molecular weight markers (lanes 1, 2). The gel was then divided into two portions. One portion (lanes 1-8), was stained with Coomassie Blue (section 2.3.9) while the other portion of the gel (lanes 9, 10), was immersed in 10mM sodium citrate buffer (pH 5.0) for one hour, to remove the electrophoresis running buffer.

The unstained gel portion was then cut into strips corresponding to the sample lanes and these strips were then sectioned at right angles into equal segments. Each segment was placed into separate 1.5ml microfuge tubes, crushed using a sterile spatula and 1ml of 10mM sodium acetate buffer (pH 5.0) was added. Tubes were then incubated at 37°C for 24 hours, centrifuged at 12,000 rpm, (7,200 x *g*) for 5 minutes. The supernatant was removed and subsequently assayed for α-glucosidase activity and α-amylase activity as described in sections 2.10.1 and 2.10.2.

The Coomassie Blue stained portion of the gel was used to look for matches between visible protein bands and enzymatically active slices.

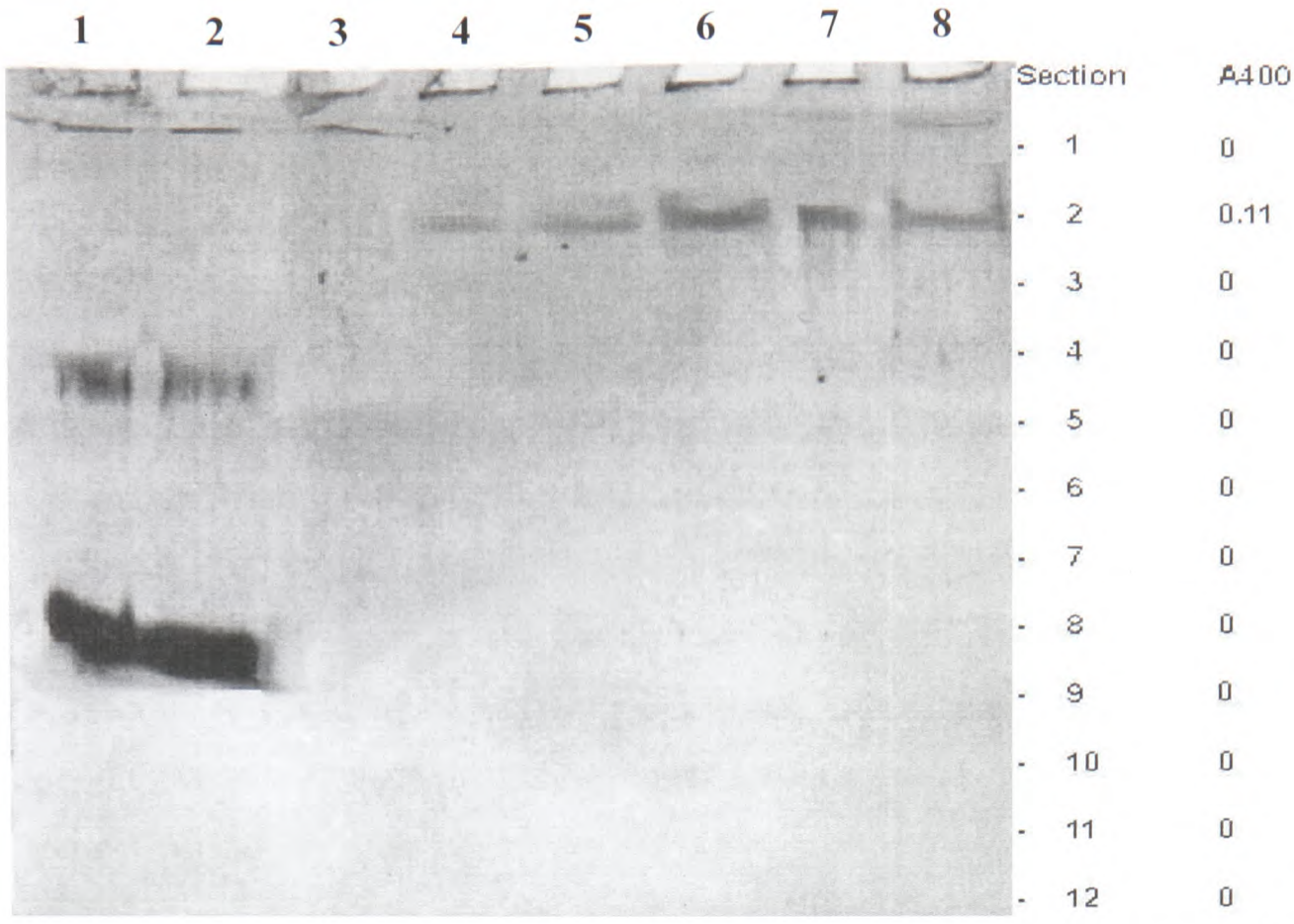


Figure 5.9: A 7.5% native polyacrylamide gel of α-glucosidase samples from *L. starkeyi* after IEC. *Lanes 1-2: BSA non-denatured dimer marker; lanes 3-8: α-glucosidase-rich fractions collected after IEC; (Section 4.6.2.4, Figure 4.25) samples applied were fractions 12-17 respectively. Lanes 9 and 10 were duplicate loadings of post-ion exchange fraction 16 and were sectioned and assayed as described in section 5.3.2 with the average α-glucosidase activity value of the two lanes given alongside the gel in the "A_{400}" column.*

The section of gel labelled 2 showed strong α-glucosidase activity and matched up with the major bands detected in lane 4, 5, 6, 7 and 8, on the Coomassie Blue stained portion of the gel. The gel slices were tested for α-amylase, but no activity was detected in the gel confirming that it was possible to separate the *L. starkeyi* α-glucosidase activity from the α-amylase activity using HIC, followed by ion exchange chromatography.

However, assaying for α-glucosidase activity extracted from gel sections was not very reproducible, as the protein concentrations were low and long incubation periods (>24 hours), were required to allow all the enzyme activity to leach out of the gel and into the buffer solution. A second, more precise method for measuring enzyme activity was attempted.

5.2.2.2.2 Determination of α-Glucosidase Enzyme activity after PAGE using Agarose Underlay

A 10% native polyacrylamide gel loaded with two identical sets of samples, (so that when cut, the two gel halves were exact duplicates), was run at 100mV for 2 hours. The gel was then cut into two halves, one half stained in the normal way using Coomassie Blue (section 2.3.9); and the other half being left to equilibrate in 10mM sodium acetate buffer, pH 5, for two hours. The latter gel half was then laid onto a thin (2mm), 1% (w/v) agarose gel containing 1mM PNPG, covered with a sponge pre-soaked in sodium acetate buffer, pH 5.0 and then a glass plate bearing a 50-100g weight was placed on top of the sponge. This gel "sandwich" was left for 2 hours at room temperature after which the polyacrylamide gel was removed and the agarose underlay was stained by flooding with 0.2M borax (disodium tetraborate). The activity stain developed within a few minutes and active bands stained yellow. The gel was examined and/or photographed within one hour of development, as the colour dissipated and completely diffused away after two hours.

The results of this activity stain can be seen in Figure 5.10.

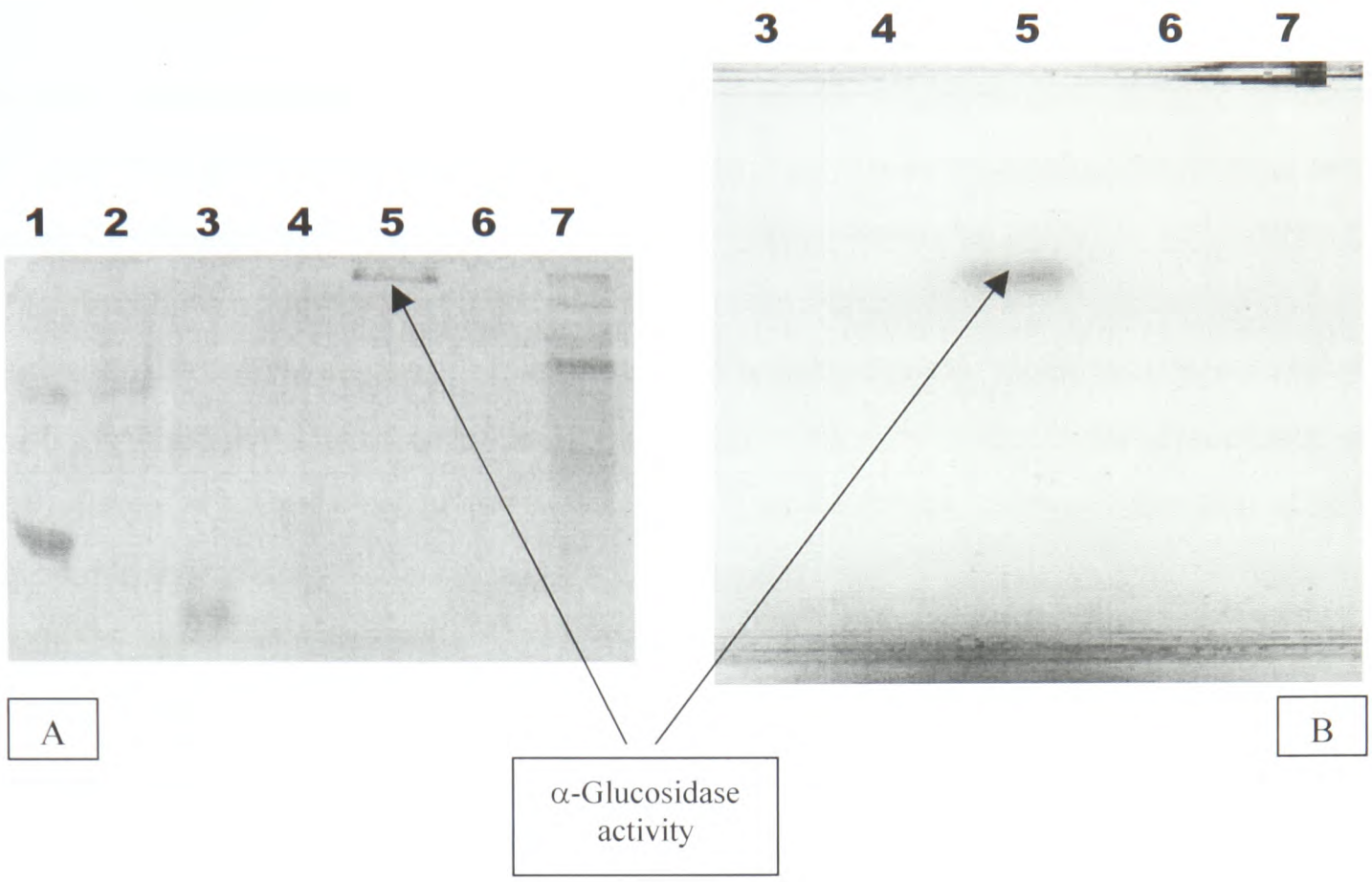


Figure 5.10: Plate A shows a 7.5% native gel with α-glucosidase (lane 5) and α-amylase activity (lane 7) electrophoresed alongside native marker proteins (lanes 1, 2 and 3). Plate B shows the agarose overlay containing the substrate, PNPG. *Only a single band was detected on the agarose underlay, which corresponded exactly to the single stained protein band in Lane 5, which contained α-glucosidase activity. Enzyme activity was detected by the appearance of a yellow band. No other activity was detected in the agarose underlay (Plate B) that corresponded to the protein markers or α-amylase detected in Plate A.*

This method for testing for α-glucosidase activity was sensitive (detecting 10μg protein), and specific (α-amylase activity was not detected), and allowed for a relatively quick estimate of enzyme activity in native gels. The gel result confirms that a single α-glucosidase activity was purified by HIC and then IEC, and that this activity corresponds to a specific Coomassie-blue stained protein band, and that this band was the only protein detectable in the purified fraction analysed (Figures 5.4 and 5.6).

5.2.2.3 Activity Staining for α-Amylase using an Agarose Underlay

A 10% non-denaturing gel was run as described for α-glucosidase activity (section 5.3.3.1). The gel was then cut into two portions, one portion stained in the normal way using Coomassie Blue (section 2.3.9); and the other portion being left to equilibrate in 10mM sodium acetate buffer, pH 5, for two hours. This portion of the gel was then laid onto a thin 1% (w/v) agarose gel containing 1% (w/v) soluble starch and then covered with a sponge pre-soaked in buffer, a glass plate with a 50-100g weight placed onto of the sponge. This gel "sandwich" was left for 2 hours at room temperature after which the polyacrylamide gel was removed and the agarose underlay was stained, by flushing with 5% (w/v) iodine solution.

Active α-amylase bands were immediately visible as clear bands against a dark blue background. The stained agarose gel was stable, and could be kept for several days, allowing a ready comparison between the agarose underlay bands and Coomassie Blue-stained bands of the original native gel.

Figure 5.11: Plate A shows a 10% native polyacrylamide gel with post-IEC α-amylase activity. *Lanes 1-4 were loaded with active fractions collected from intermediate Q-Sepharose column (Figure 4.20) at ~10 μg protein per lane. Lane 6: native molecular weight marker: Urease. Plate B shows the agarose underlay containing starch. Three active bands were detected when flushed with iodine in lanes 1-4. No α-glucosidase activity was detected when the agarose underlay containing starch, was flushed with iodine.*

Of the four Coomassie Blue-stained bands detected in the α-amylase fractions (approximate molecular weights of 120,000, 82,000, 68,000 and 48,500), the faint

band corresponding to a molecular weight of 82,000 appears to be the most active while the 68,000 and 45,000 bands were also clearly active. The band corresponding to a molecular weight of 120,000 appeared to show no activity with the starch underlay and could possibly be a contaminant or another amylolytic enzyme activity. These data correlate well with the previous native gel analyses of the post-IEC material (Figure 5.6), in that the activity corresponds to three out of the four Coomassie Blue stained protein bands, whereas only two bands were visible on SDS-PAGE analysis of the post-IEC α-amylase-rich samples (Figure 5.2).

Overall, the results of the specific α-amylase enzyme staining of the native gel, suggest that when *L. starkeyi* (NCYC 1436) is grown on a starch medium, it can secrete three distinct α-amylase activities, in addition to α-glucosidase activity, into the growth medium.

5.3 Isoelectric Focusing of α-Glucosidase

Purified α-glucosidase samples collected after ion exchange chromatography (Figure 4.25) and which had previously been analysed by SDS-PAGE and native gels (Figures 5.4 and 5.10), were applied to an Ampholine PAGplate, (pH range 3.5 to 9.5), precast polyacrylamide gel (Pharmacia), as described in Section 2.9.

Three bands of clearly similar pI were observed – consistent with three isoforms of the α-glucosidase and no significant contaminants. Thus, the α-glucosidase purified and resolved by SDS-PAGE to a single band (Figure 5.4) was in fact composed of several isoforms when analyzed by isoelectric focussing, (Figure 5.12).

The precise isoelectric points were estimated with the help of marker proteins, which were run alongside the α-glucosidase. After focusing and staining, the migration distances from the cathodic edge of the gel to the different marker protein bands were plotted on the Y-axis and the corresponding pIs of the marker proteins plotted on the X-axis. The isoelectric point of the α-glucosidase was then extrapolated from the calibration curve (Figure 5.13).

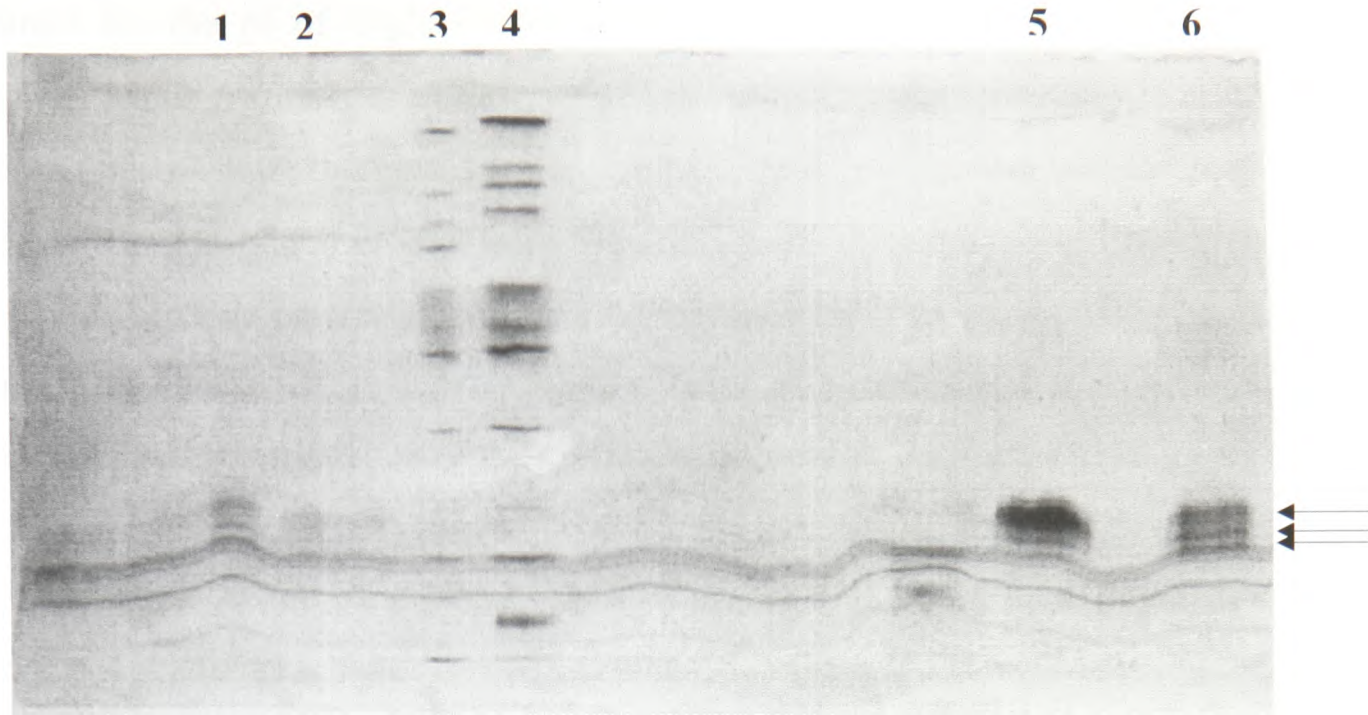


Figure 5.12: Isoelectric focusing gel using a broad pI gradient. *Lanes 3-4: pI markers; lane 1: post IEC α-glucosidase sample (10μg); lane 2: post IEC α-glucosidase sample (5μg); lane 5: post IEC α-glucosidase sample-fraction 16 (30μg); lane 6: post IEC α-glucosidase sample-fraction 16 (20μg).*

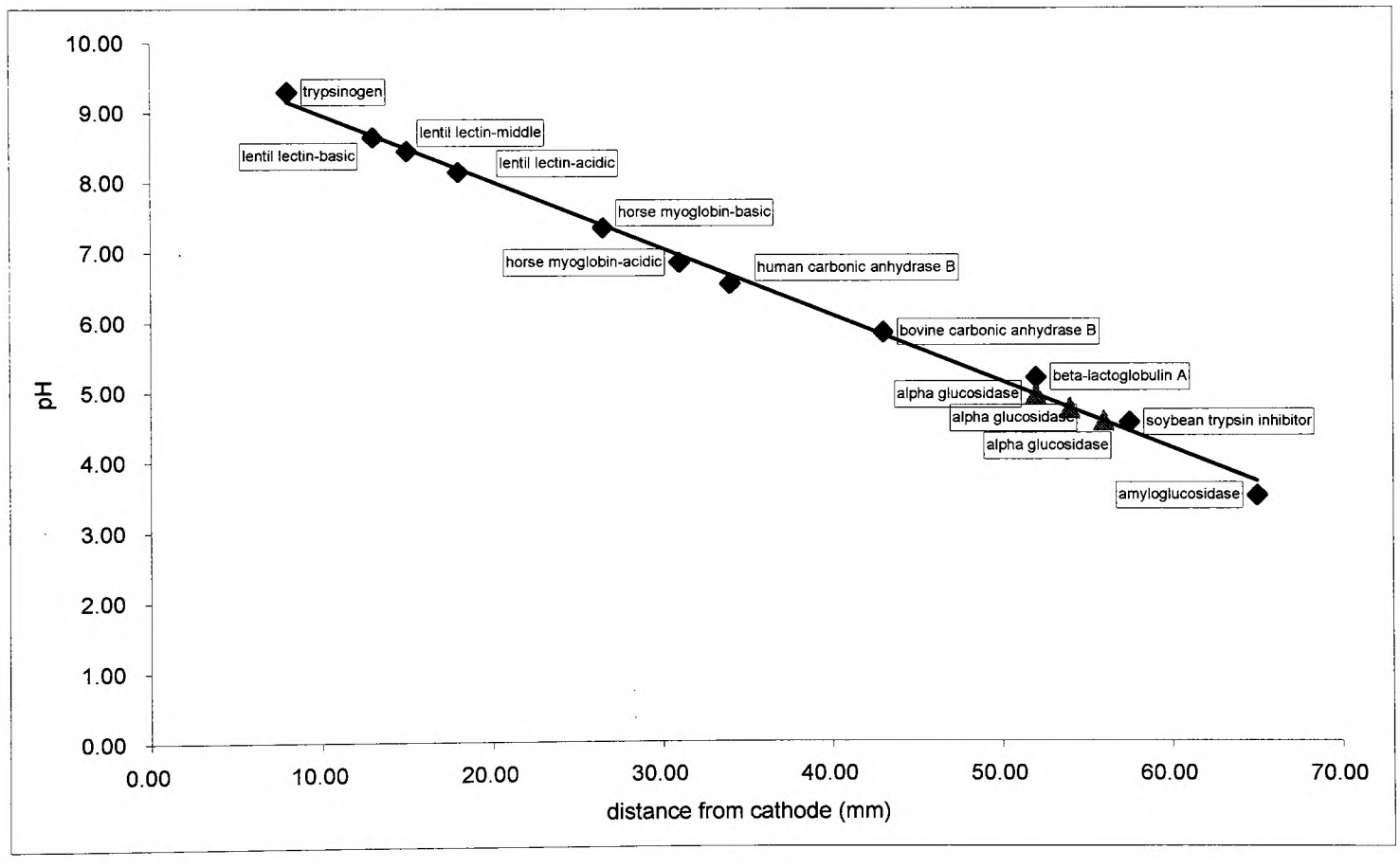


Figure 5.13: Calibration curve to determine the pI of α-glucosidase from *Lipomyces starkeyi* using the broad pI kit, pH 3-10.

The isoelectric points of the three individual bands were estimated as 4.6, 4.8 and 5.0. Very little has been reported on the pI of yeast α-glucosidases, however, the results

obtained for the pI of α-glucosidase from *L. starkeyi* are typical for extracellular enzymes, which naturally exist in lower pH environments (Scopes, 1994).

The presence of more than one band in the purified α-glucosidase samples may be due to the interaction of the protein with the ampholytes of very similar isoelectric points and could therefore be artefacts. If an ampholyte mixes with the protein a conjugate of intermediate isoelectric point can form. Carrier ampholytes can co-migrate with the protein of interest and may alter the migration of proteins (Scopes, 1994).

The diffuseness of the band pattern seen in Figure 5.12 could be due to a number of factors. pH gradients are susceptible to cathodic drifts, a phenomenon in which there is a gradual decrease in pH at the cathodic end of the gel and a flattening out of the pH at the anodic end (Hoeffer, 1994).

This is apparent in Figure 5.12, where the wavy bands are seen at the cathodic end. If the gel is focussed at a high voltage for too long then fuzzy bands are evident and cathodic drift is a problem due to the lability of the ampholytes. The pH gradient can breakdown as the electric field is removed which can also lead to the incomplete focusing of the protein.

Alternatively, the wavy band could be attributed to high salt. IEF requires low ionic strength, preferably below 50mM salt concentration. The smearing seen in lanes 5 and 6 could indicate high salt in the applied sample, which could lead to a poor estimate of pI, and therefore dialysis of the samples prior to IEF may have increased the resolution. Other possibilities are that the fuzzy bands could be due to a variable carbohydrate component of the enzyme, which could mask some of the charges of the enzyme, or proteolysis of the sample, also resulting in diffuse, poorly, resolved bands.

5.4 Further Characterisation of α-Glucosidase

Some physicochemical properties of the *Lipomyces starkeyi* α-glucosidase isolated after ion exchange chromatography were examined.

5.4.1 pH Study

Post-IEC α-glucosidase-rich fractions (Figure 4.25) containing approximately 15μg protein were incubated with 5mM PNPG prepared by dilution in buffers at pH values ranging from 2.5 to 9.0. All samples were assayed as described in section 2.10.1.

Table 5.2: Summary of pH Buffers Tested.

Buffer	pH range tested
Citric acid-Na_2HPO4 (McIlvaine)	2.5-3.0
Citric acid-sodium citrate buffer	3.0-4.0
Sodium acetate-acetic acid	4.0-5.5
Sodium phosphate	6.0-7.5
Tris (hydroxymethyl) aminomethane-HCl	8.0-9.0

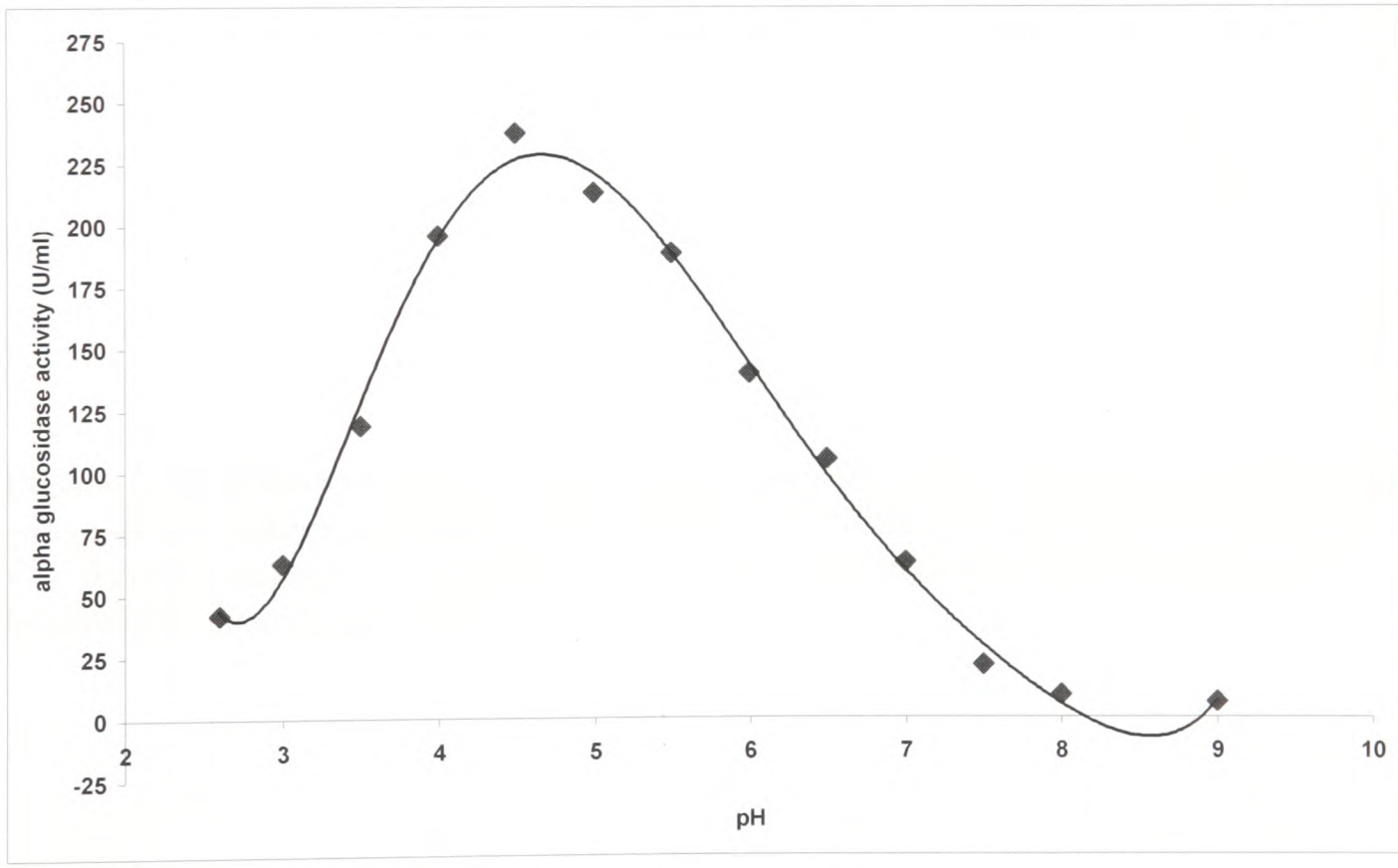


Figure 5.14 Effect of pH on α-Glucosidase Activity.

From the data obtained in Figure 5.14, it can be seen that the α-glucosidase purified from *Lipomyces starkeyi* has a pH optimum of around 4.5. A low optimum pH may be related to the fact that most fungi acidify the medium during growth.

The curve is characteristically bell-shaped, which indicates that only one of two possible ionization states of the relevant amino acid side chains is effective in enzyme catalysis.

Enzyme activity on either side of the optimum is reduced and this can be due to the effect of pH on the stability of the enzyme or affect the affinity of the enzyme for its substrate.

To determine if the enzyme was irreversibly denatured at certain pH values, the α-glucosidase enzyme was exposed for 15 minutes to the same range of pH values as before and then assayed for α-glucosidase activity (Figure 5.15).

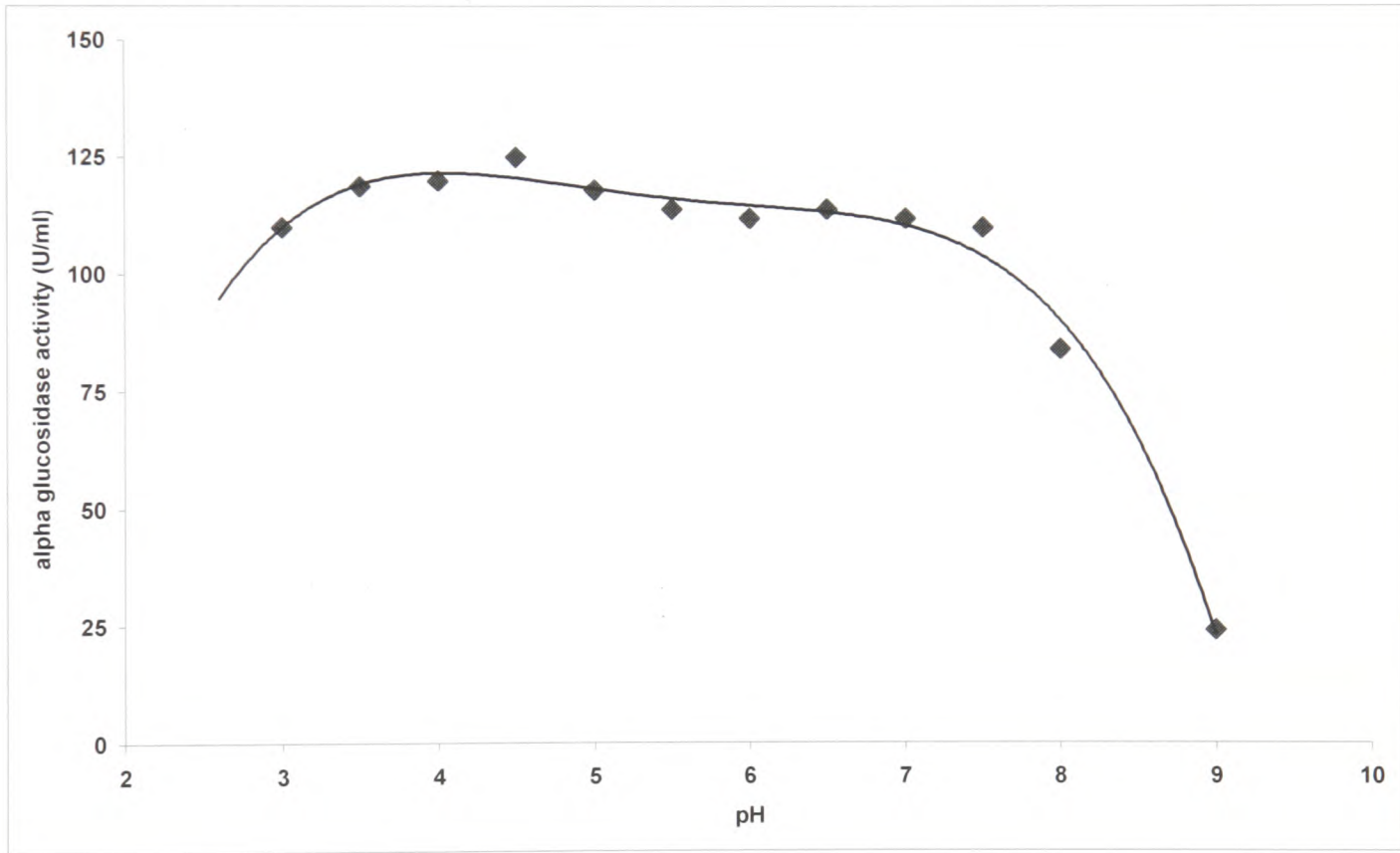


Figure 5.15: Effect of pH on α-glucosidase stability after enzyme was exposed to a range of pH values and then activity was tested after pH adjustment to pH 6.8
The decrease in activity is progressive and the curve indicates that the enzyme is irreversibly denatured at pH's above 8.0.

5.4.2 Thermostability of α-Glucosidase

The effect of temperature on α-glucosidase enzyme activity was investigated by asssaying post-IEC α-glucosidase-rich fractions (Figure 4.25), containing approximately 15μg protein, at different temperatures (20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70 and 75°C), in sodium acetate buffer, pH 5.5.

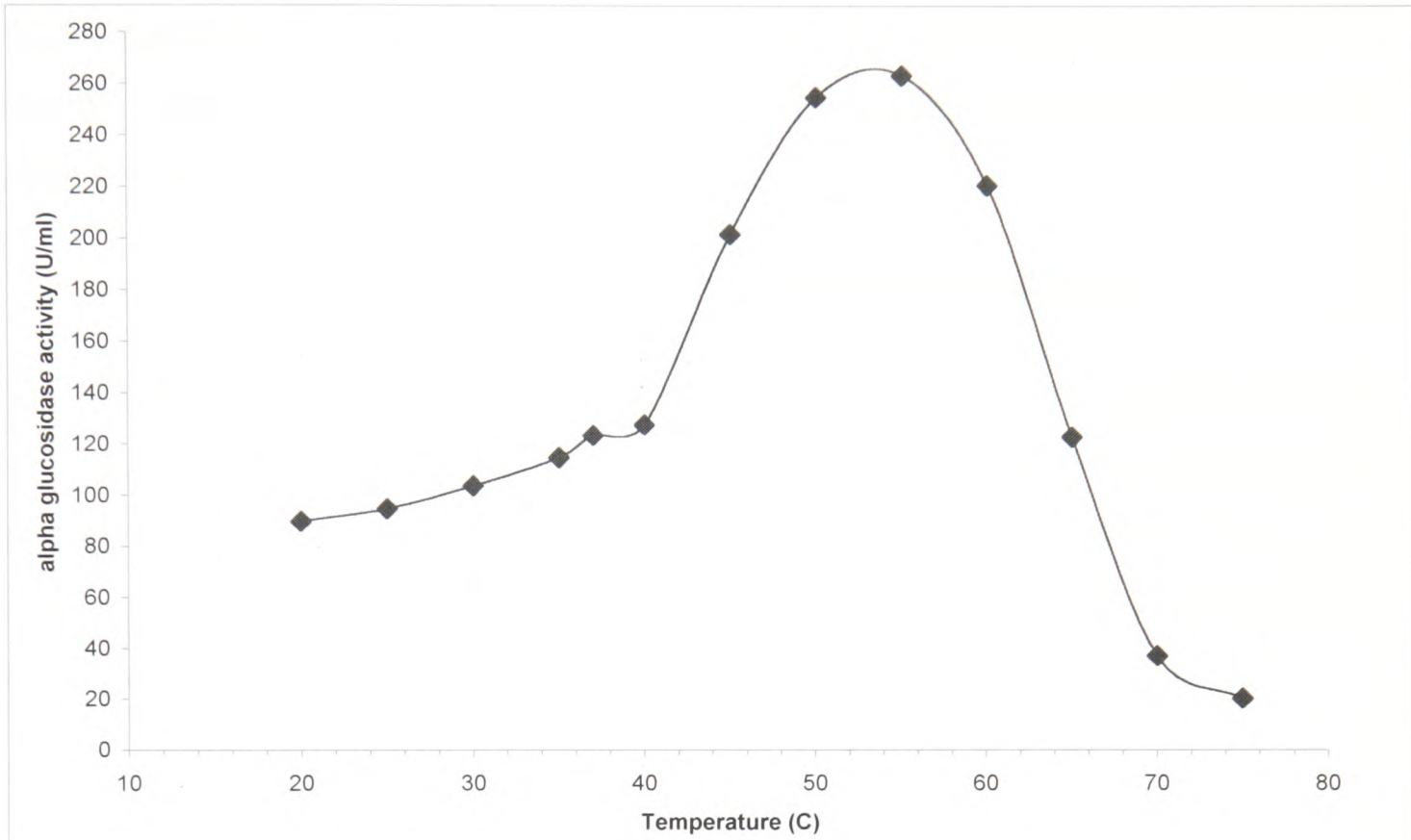


Figure 5.16: Effect of temperature on α-glucosidase activity from *Lipomyces starkeyi* NCYC 1436.

From the graph shown (Figure 5.16), the α-glucosidase shows maximum activity at 55°C. As the temperature increases from 20 to 55°C, the rate of reaction increases outweighing the effects of denaturation. As the temperature increases above 55°C, the activity falls due to enzyme denaturation and outweighs the kinetic effect of the temperature rise.

These estimates are comparable to the α-glucosidase purified by Kelly *et al.*, (1985) who estimated the temperature optimum at 60°C under their particular assay conditions. Oda *et al.*, (1993), estimated the temperature optimum for the α-glucosidase extracted from *Torulaspora pretoriensis YK-1* at 35°C, under their specific assay conditions. Matsusaka *et al.*, (1977) purified three types of α-glucosidase from

the yeast, *Saccharomyces cerevisiae,* with optimum temperatures calculated at 42, 42 and 36°C respectively.

The temperature optimum calculated for the *L. starkeyi* (NCYC 1436) α-glucosidase under these assay conditions is considerably higher than other reported yeast glucosidases and more closely resembles bacterial or fungal maltases (Table 1.1). The thermostability of the enzyme maybe due in part to the carbohydrate component of the enzyme. De-glycosylation has been known to lead to reduced thermostability in some yeast glucoamylases (Yamashita *et al.,* 1985b).

5.4.3 Measurement of Neutral Carbohydrate Content

The amount of neutral carbohydrate covalently attached to the α-glucosidase enzyme was determined by the phenol-sulphuric acid method described by Dubois *et al.*, (1956). Samples and standards were assayed as described in section 2.10.3 and a calibration curve of glucose standards containing between 10-80μg of glucose was constructed (Figure 5.17).

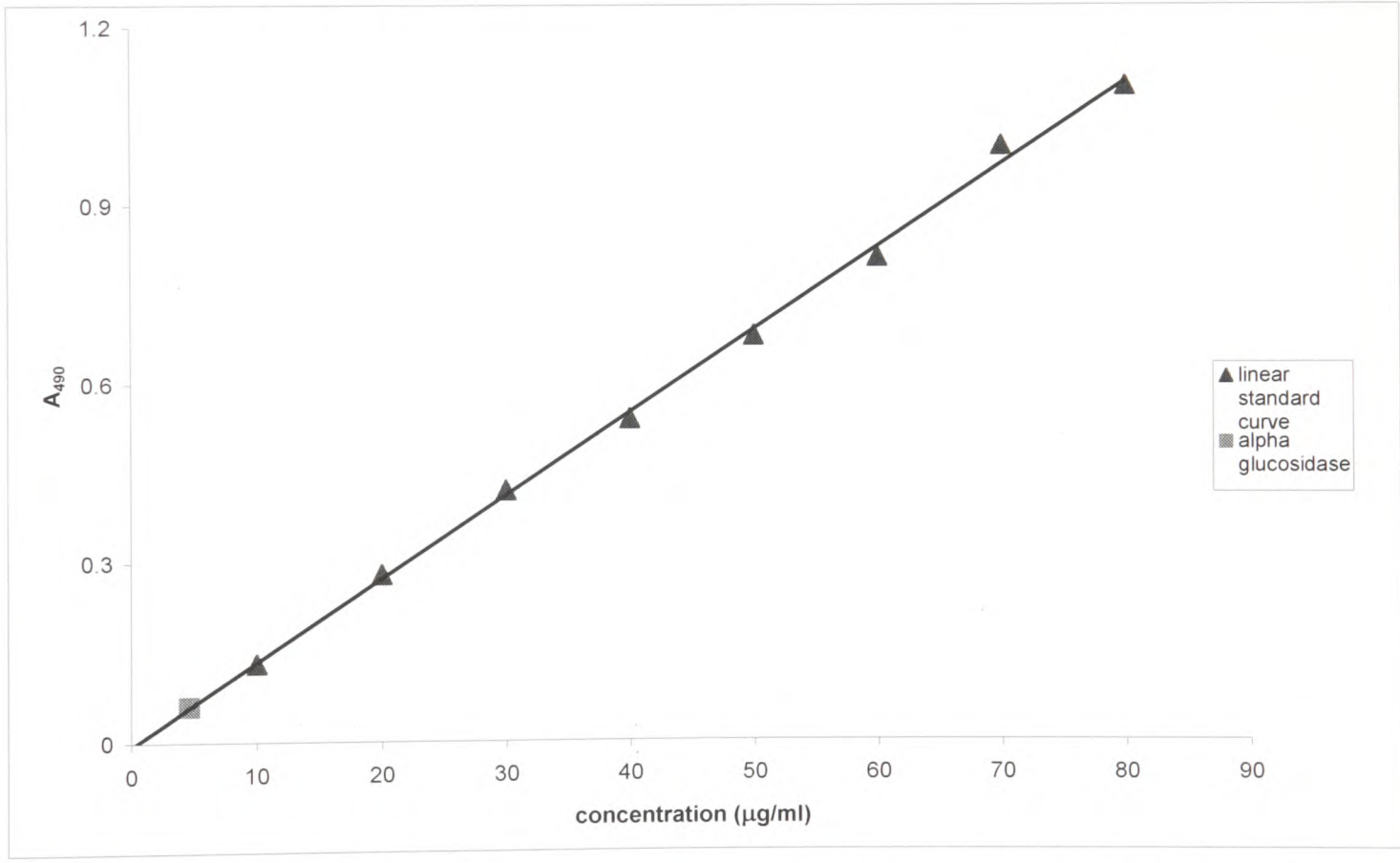


Figure 5.17: Calibration curve for the determination of the carbohydrate content of α-glucosidase from *Lipomyces starkeyi* NCYC 1436 using the Phenol-Sulphuric acid method.

10μg/ml of post IEC α-glucosidase was assayed alongside glucose standards. The carbohydrate content was then estimated by using the standard curve shown in Figure 5.17. The carbohydrate content for the α-glucosidase from *Lipomyces starkeyi* NCYC 1436 was estimated at approximately 4.6μg/ml of carbohydrate per 10μg/ml protein, which was equivalent to 32% (w/w).

The carbohydrate content has been determined in a number of *Lipomyces* carbohydrases - with estimates varying from between 6.5 to 80%. Gallagher *et al.*, (1991) determined the carbohydrate content of the extracellular carbohydrase (α-glucosidase) purified from *Lipomyces tetrasporus* to between 9.0-15.8%. Chun *et al.*, (1995), purified two glucoamylase isozymes (GI and GII), from *Lipomyces kononenkoae*, and estimated the carbohydrate content as 7.5% and 6.5% respectively. Prieto *et al.*, (1995), also estimated a low sugar content (10%) for the extracellular α-amylase they purified from a strain of *L. kononenkoae.* Whereas, the characterisation of the extracellular glucoamylase purified from *Saccharomyces cerevisiae* (Kleinman *et al.*, 1994), revealed that 80% of the enzyme was carbohydrate.

Chapter 6: Catalytic Properties of α-Glucosidase

6.1 Introduction

It has been commonly acknowledged that α-glucosidases possess a broad substrate specificity (Phillips, 1959). In fact, virtually all of the literature published on α-glucosidases, regardless of whether the enzyme was extracted from a microbial or bacterial source, plant or animal tissue, examines the kinetic behaviour of the purified enzyme with different substrates.

The enzyme activity with different substrates of the α-glucosidase purified from *Lipomyces starkeyi* (NCYC 1436) was assessed in the work described in this chapter.

Many of the substrates chosen for evaluation, such as maltose, produced glucose as an end-product, and therefore the continuous linked hexokinase assay was appropriate to use to monitor the rate of glucose release. It was essential to add coupling enzyme and substrate in sufficient quantities to ensure the overall measured rate was the rate of the α-glucosidase acting on this substrate, and that the glucose assay reactions were not rate-limiting. An equivalent amount of substrate was also added to each control to account for possible glucose contamination of substrates. Prior to any analysis, the α-glucosidase enzyme preparation was tested to ascertain that non-enzyme catalysed reactions were not taking place, by inactivating the enzyme preparation by heating it to 100°C for ten minutes, and then assaying the preparation as described in section 2.7.

Standard assays were then carried out using 5μg of the concentrated post - ion exchange enzyme preparation (section 4.6.2.4), which was then incubated with each substrate. This material was pure and determined to be electrophoretically homogeneous (section 5, Figure 5.4). All progress curves used for determining initial rates were linear for the first 5 minutes.

The kinetic data generated for the α-glucosidase could be analysed in a number of ways, with each method having different merits. The Lineweaver-Burk plot shows the variables V and [S] plotted on separate axis, however, it exaggerates the error at low substrate concentration levels where measurement is often less accurate as the

velocities are slower. The error distribution is more uniform in other plots, such as the Hanes plot, and therefore, Hanes plots were constructed for all the substrates investigated in this study, to estimate *V*max and *K*m for the α-glucosidase purified from *L. starkeyi.*

Due to the limited availability of some costly substrates, (which were considered essential for a thorough assessment), a variable number of datum points were reported for each of the substrates that were tested. In this study, a minimum of 5 datum points was considered a prerequisite, to produce an accurate and representative progress curve for each substrate.

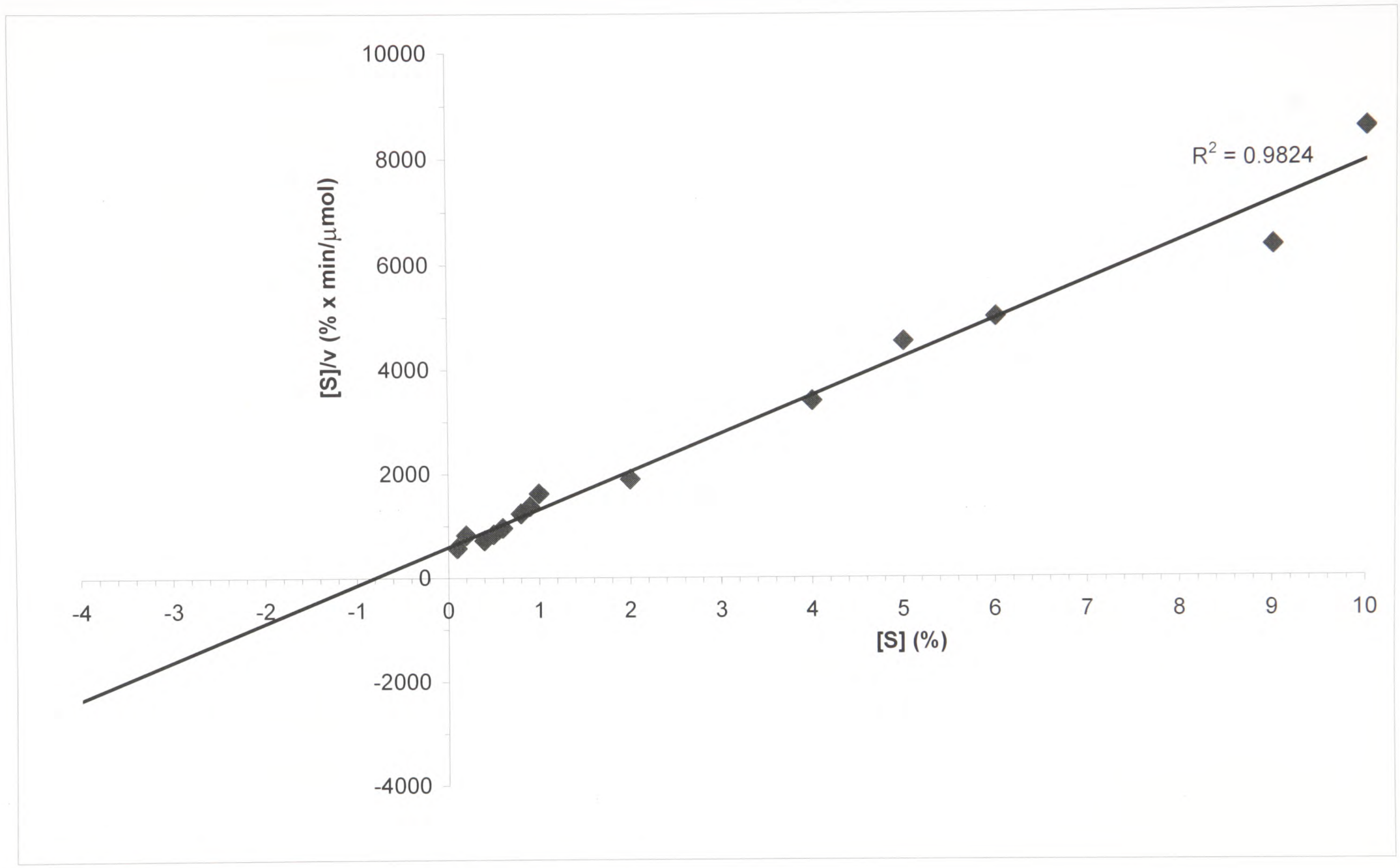


Figure 6.1 Hanes plot of α-glucosidase using soluble starch

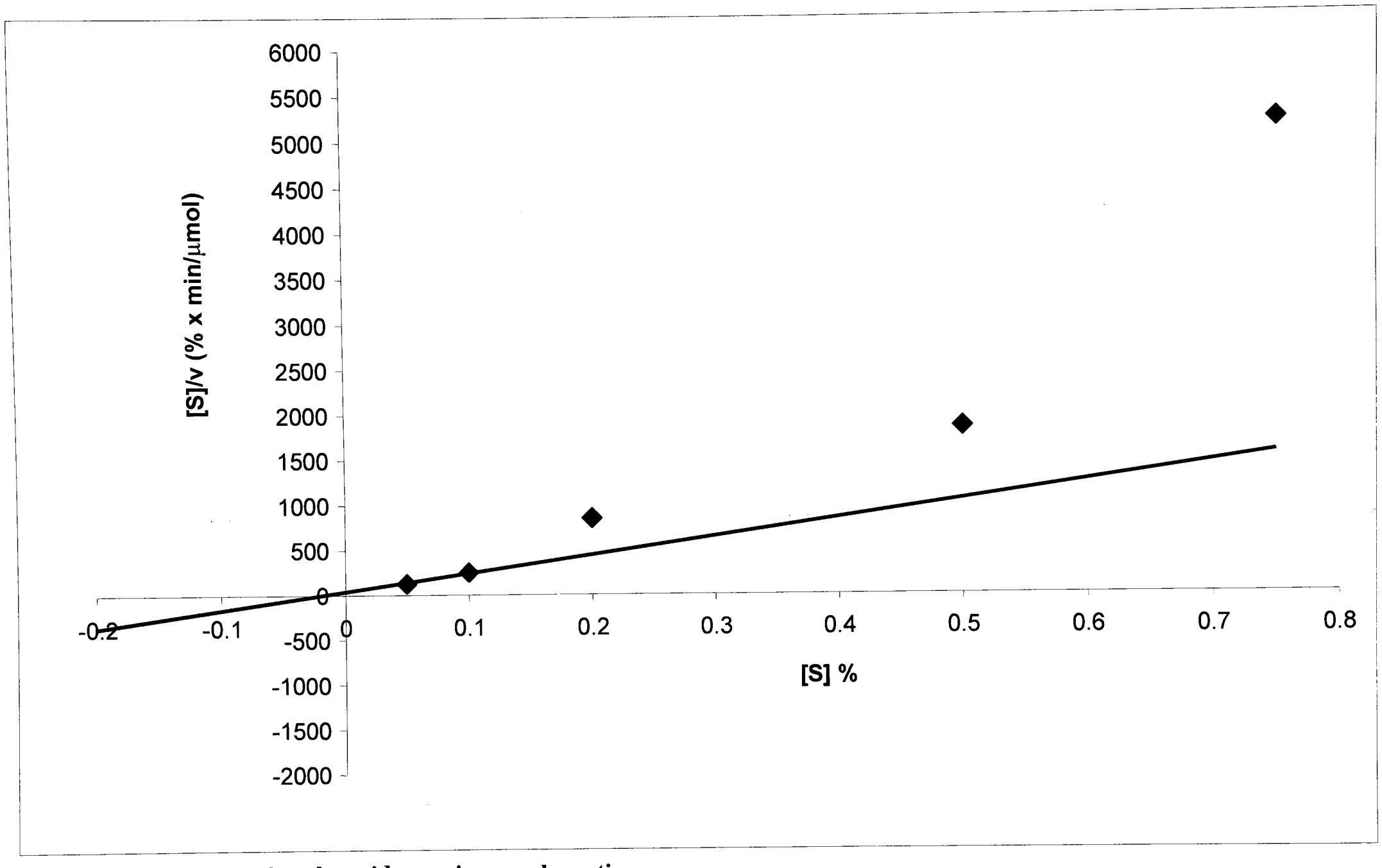


Figure 6.2 Hanes plot of α-glucosidase using amylopectin

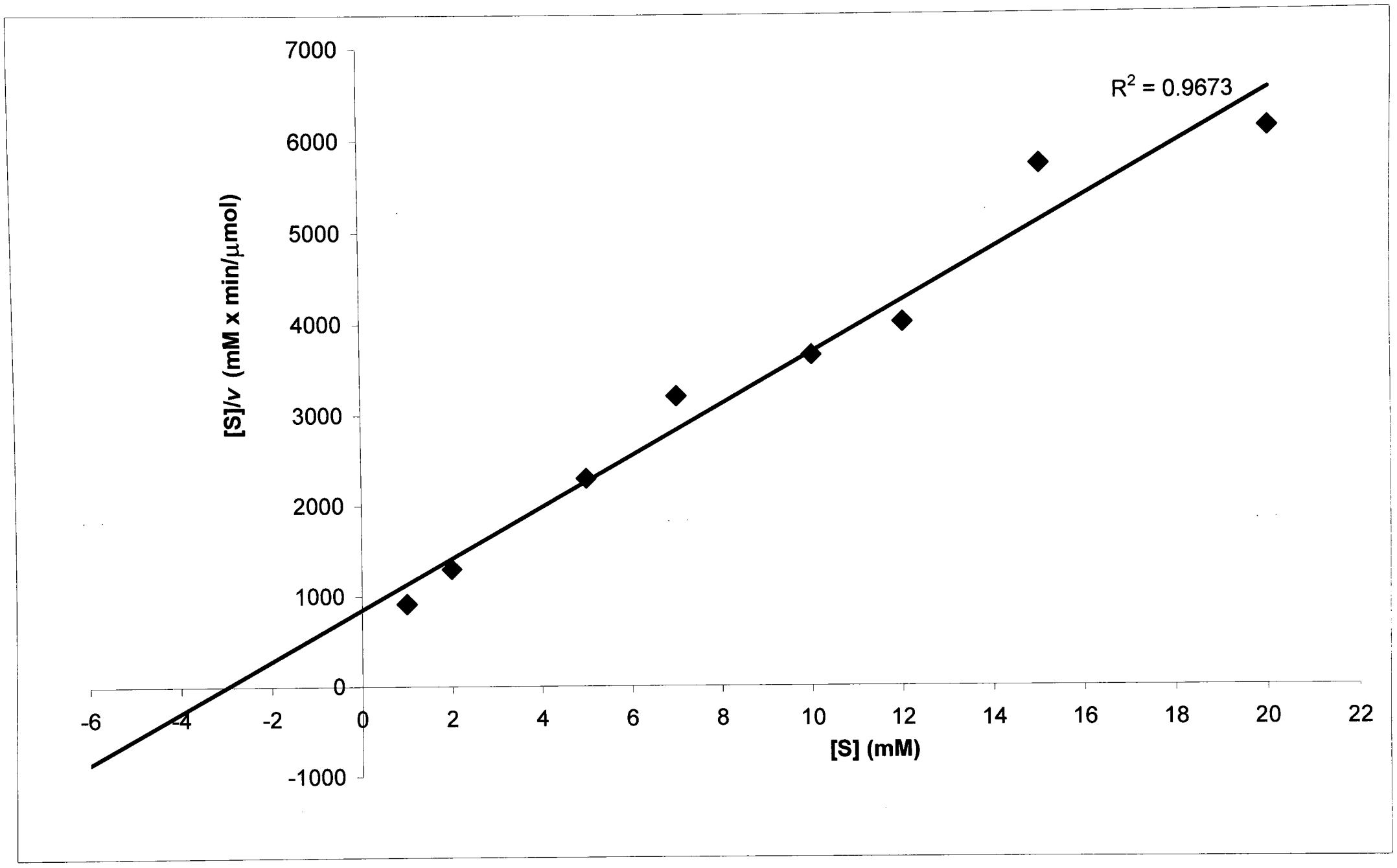


Figure 6.3 Hanes plot of α-glucosidase using p-Nitrophenyl Glucopyranoside (PNPG)

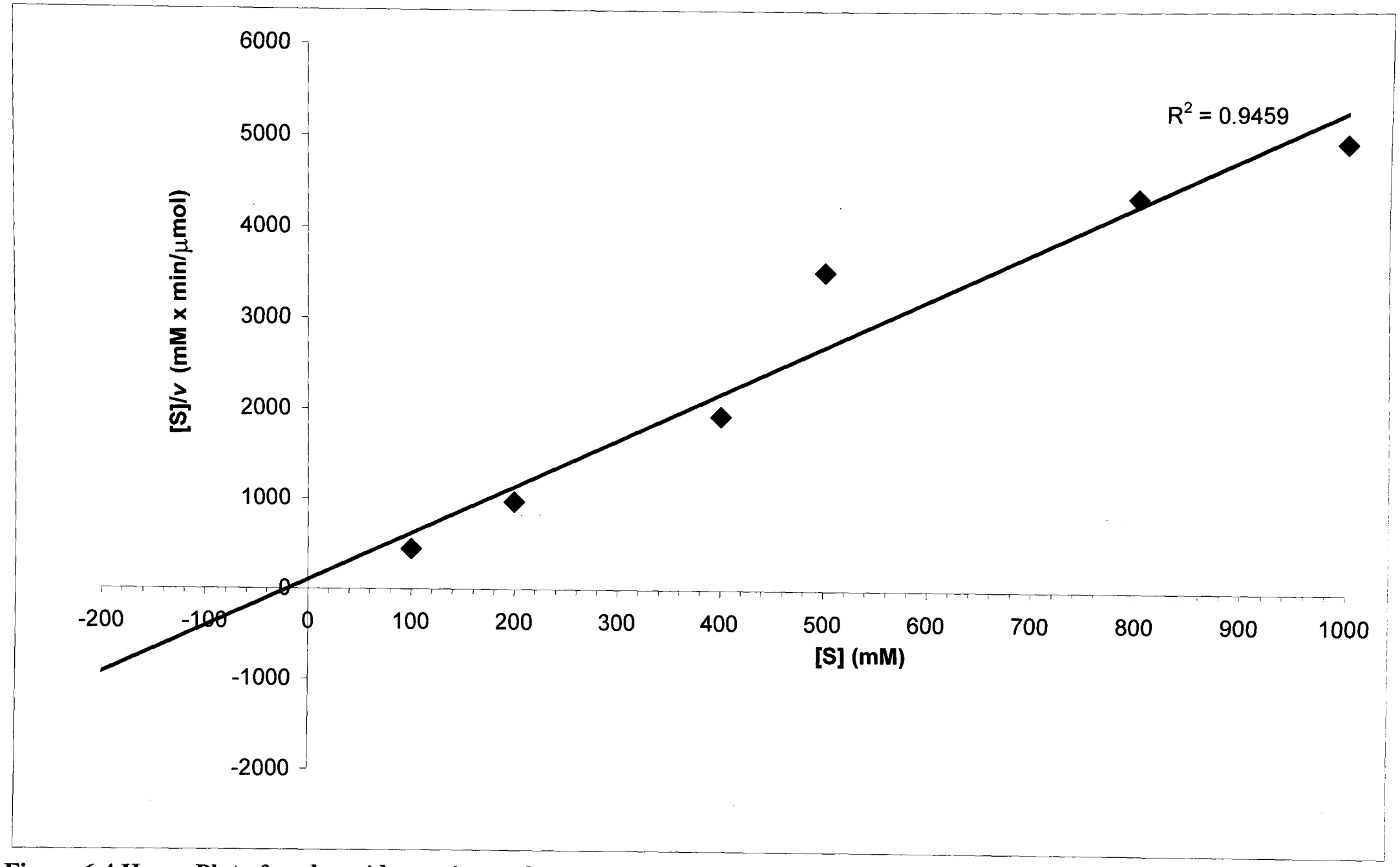


Figure 6.4 Hanes Plot of α-glucosidase using maltose

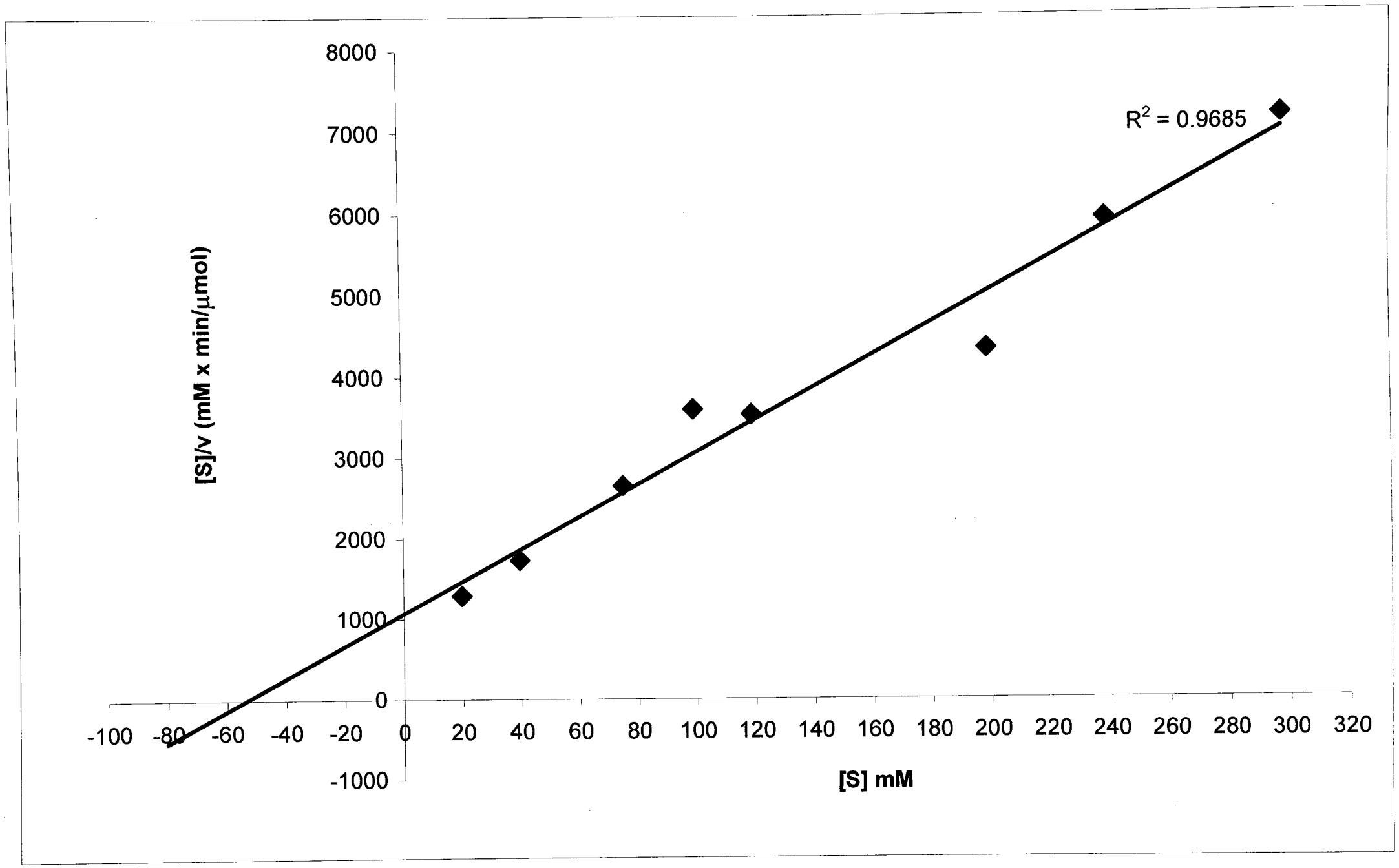


Figure 6.5 Hanes plot of α-glucosidase using maltotriose

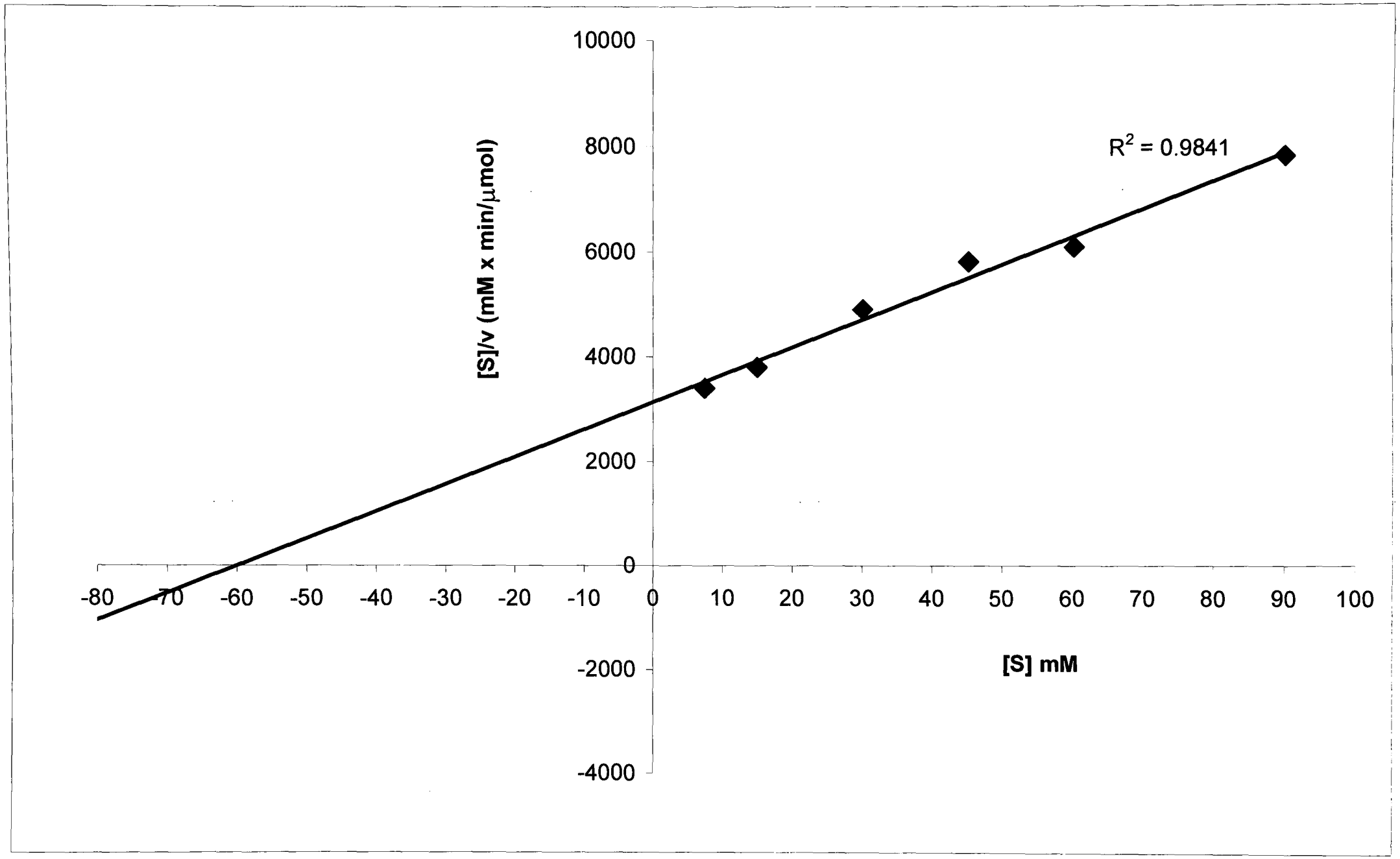


Figure 6.6 Hanes plot of α-glucosidase using maltotetraose

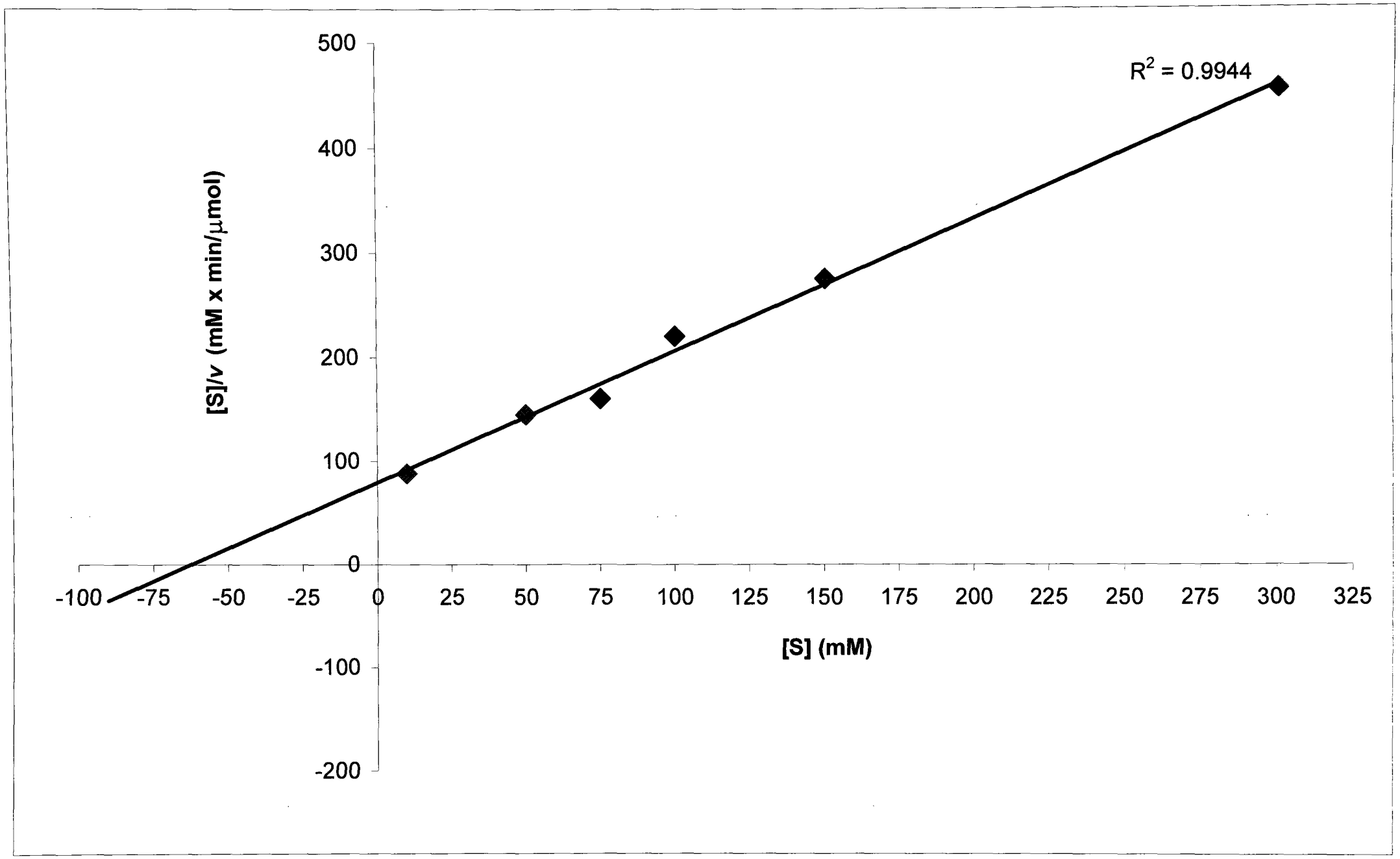


Figure 6.7 Hanes plot of α-glucosidase using maltopentaose

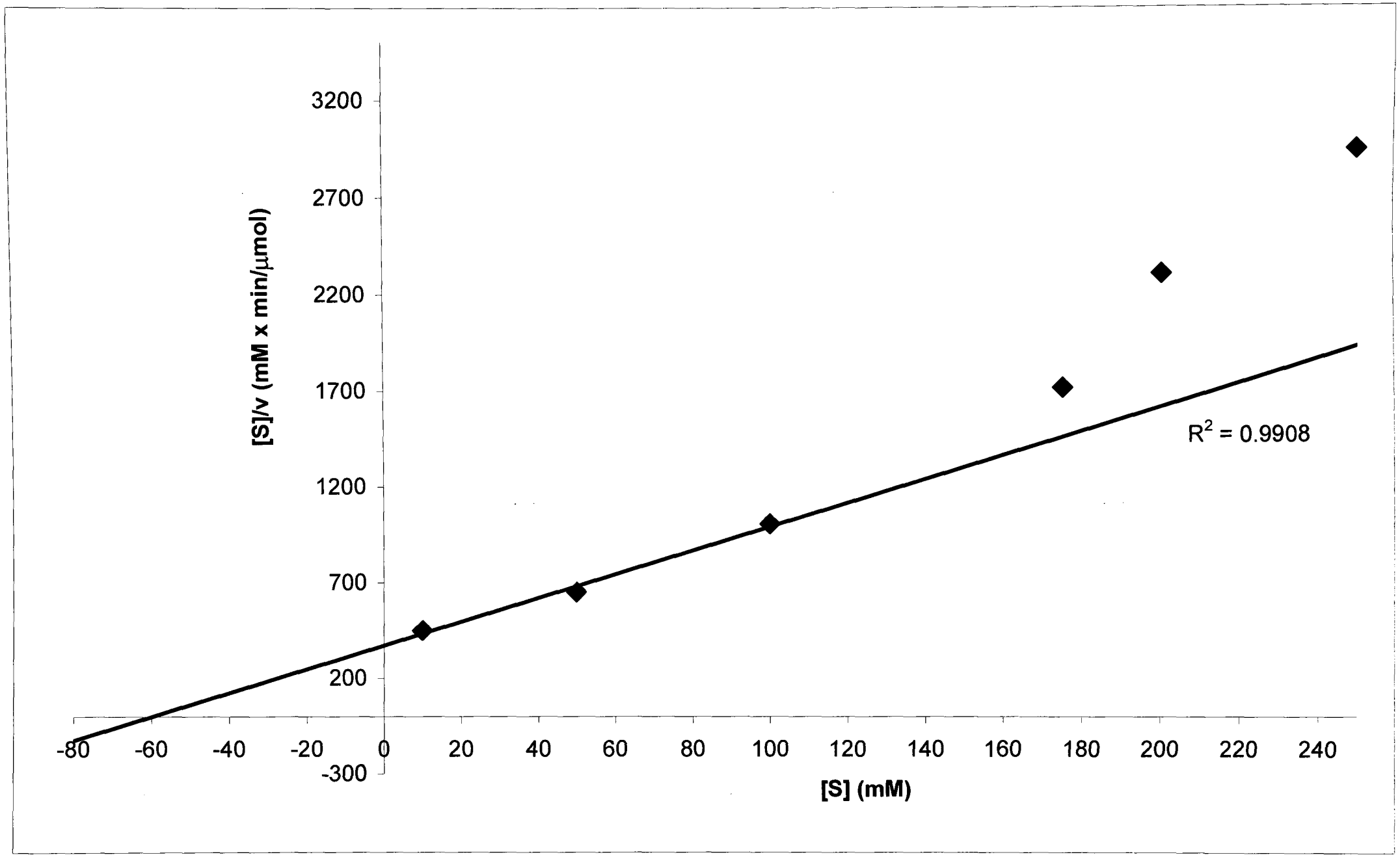


Figure 6.8 Hanes plot of α-glucosidase using maltohexaose

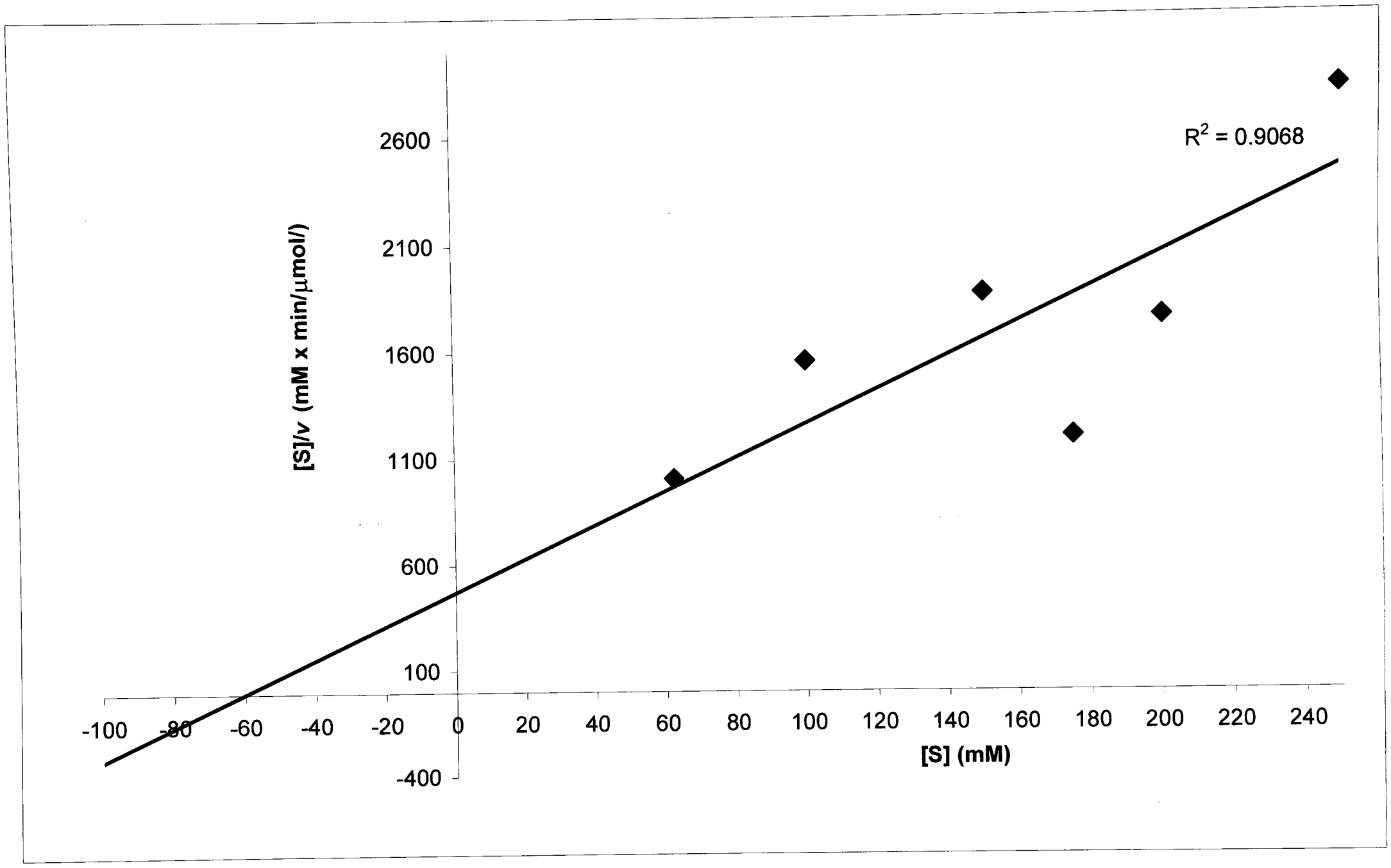


Figure 6.9 Hanes plot of α-glucosidase using maltoheptaose

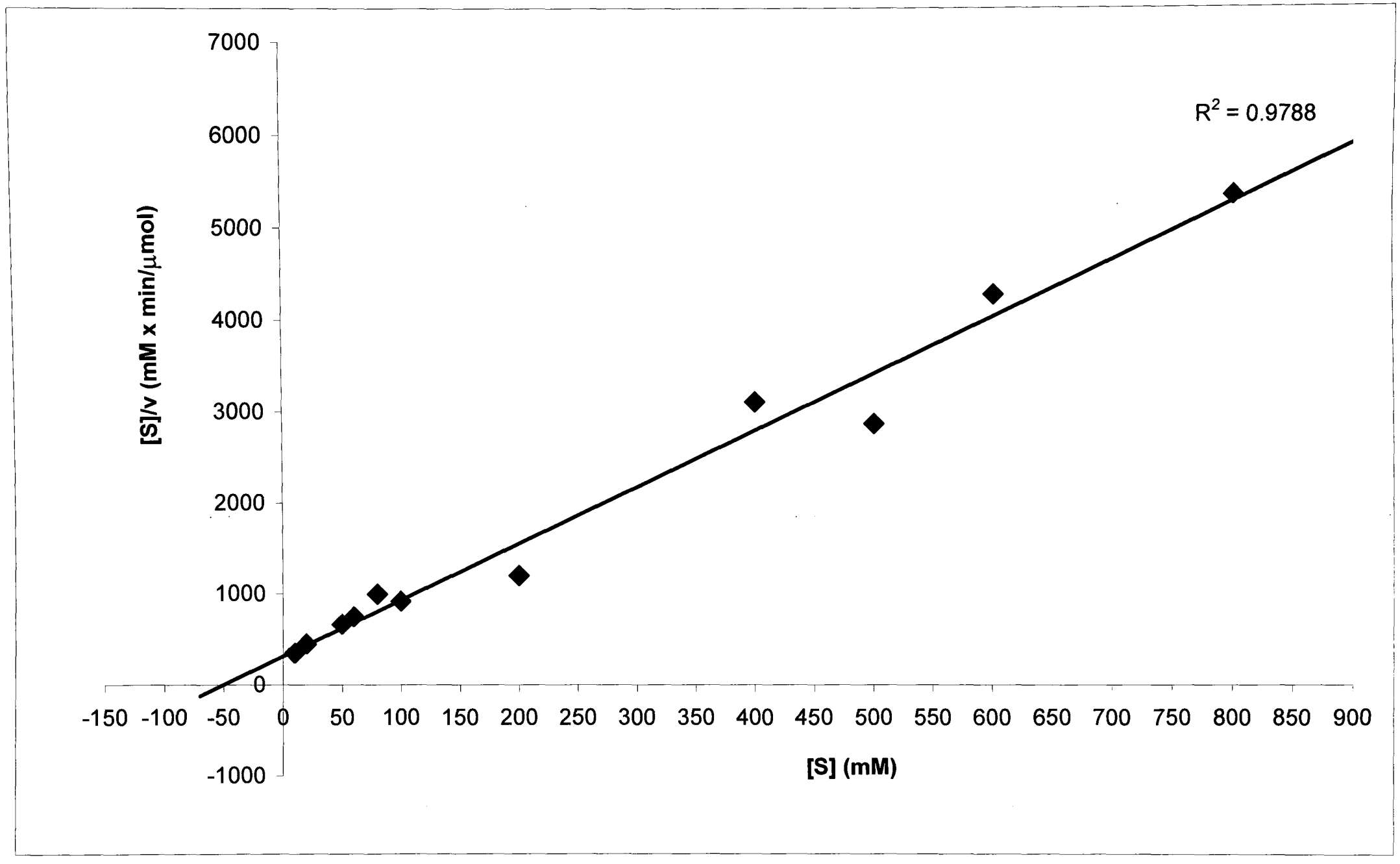


Figure 6.10 Hanes plot of α-glucosidase using sucrose

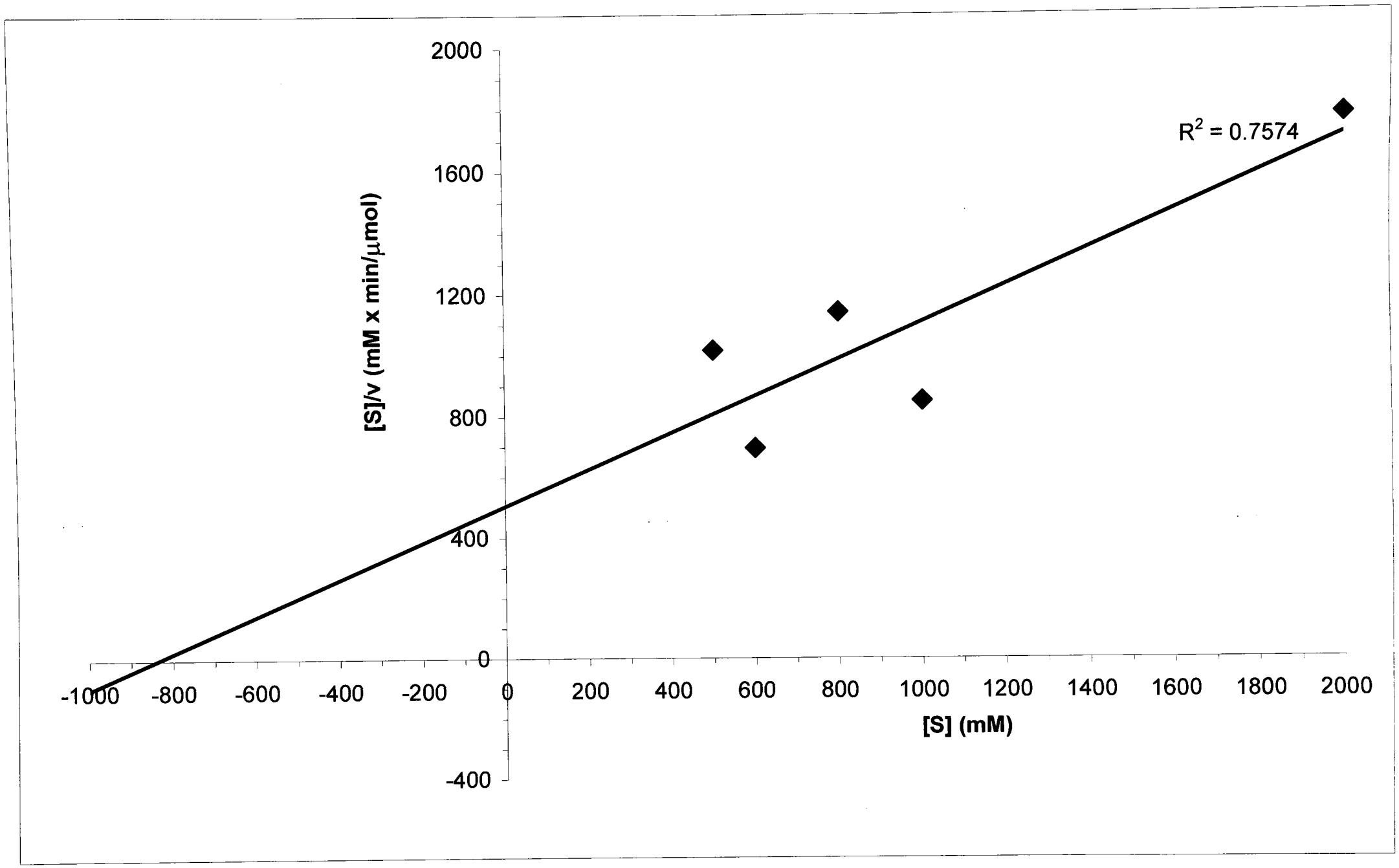


Figure 6.11 Hanes plot of α-glucosidase using β-lactose

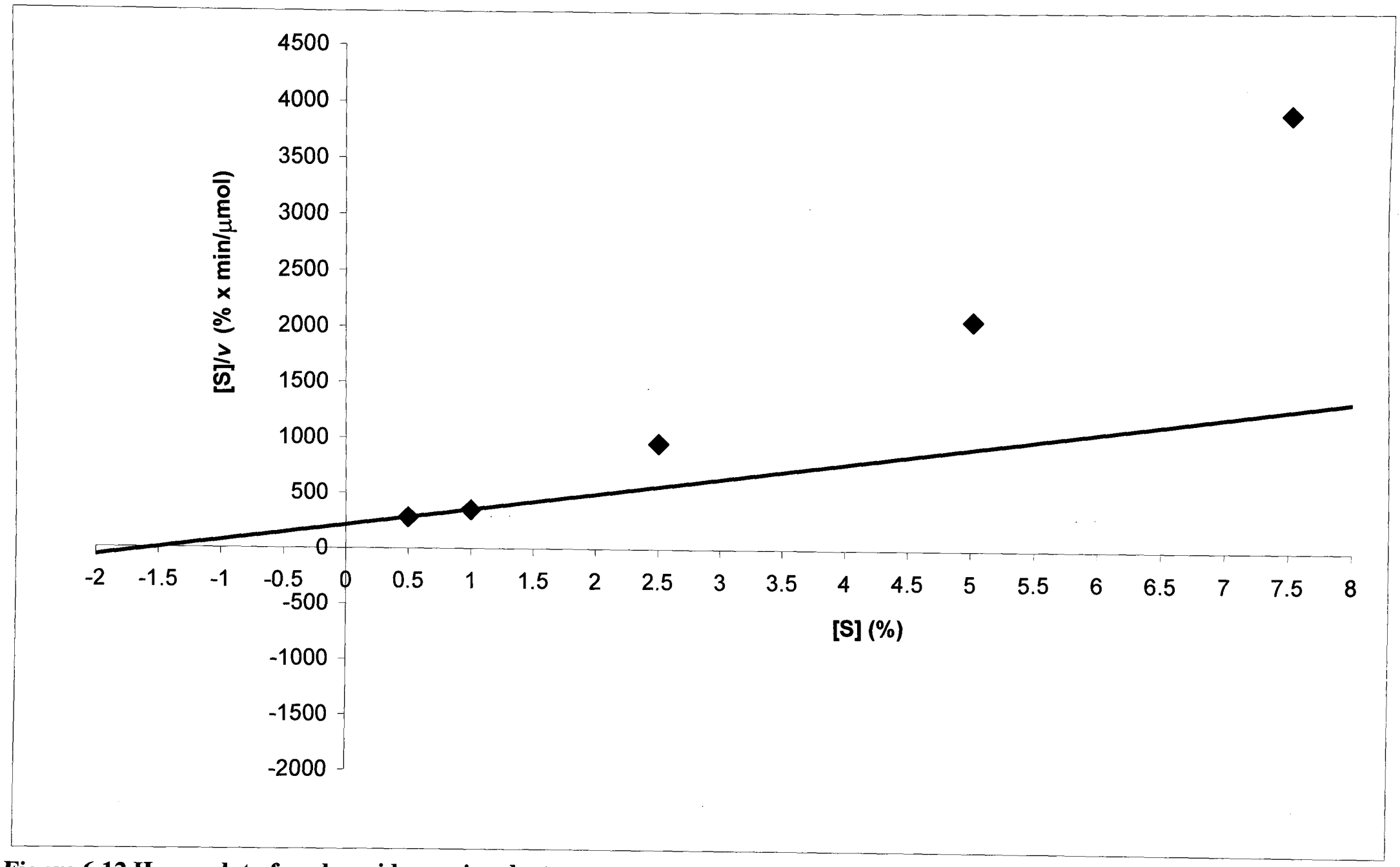


Figure 6.12 Hanes plot of α-glucosidase using dextran

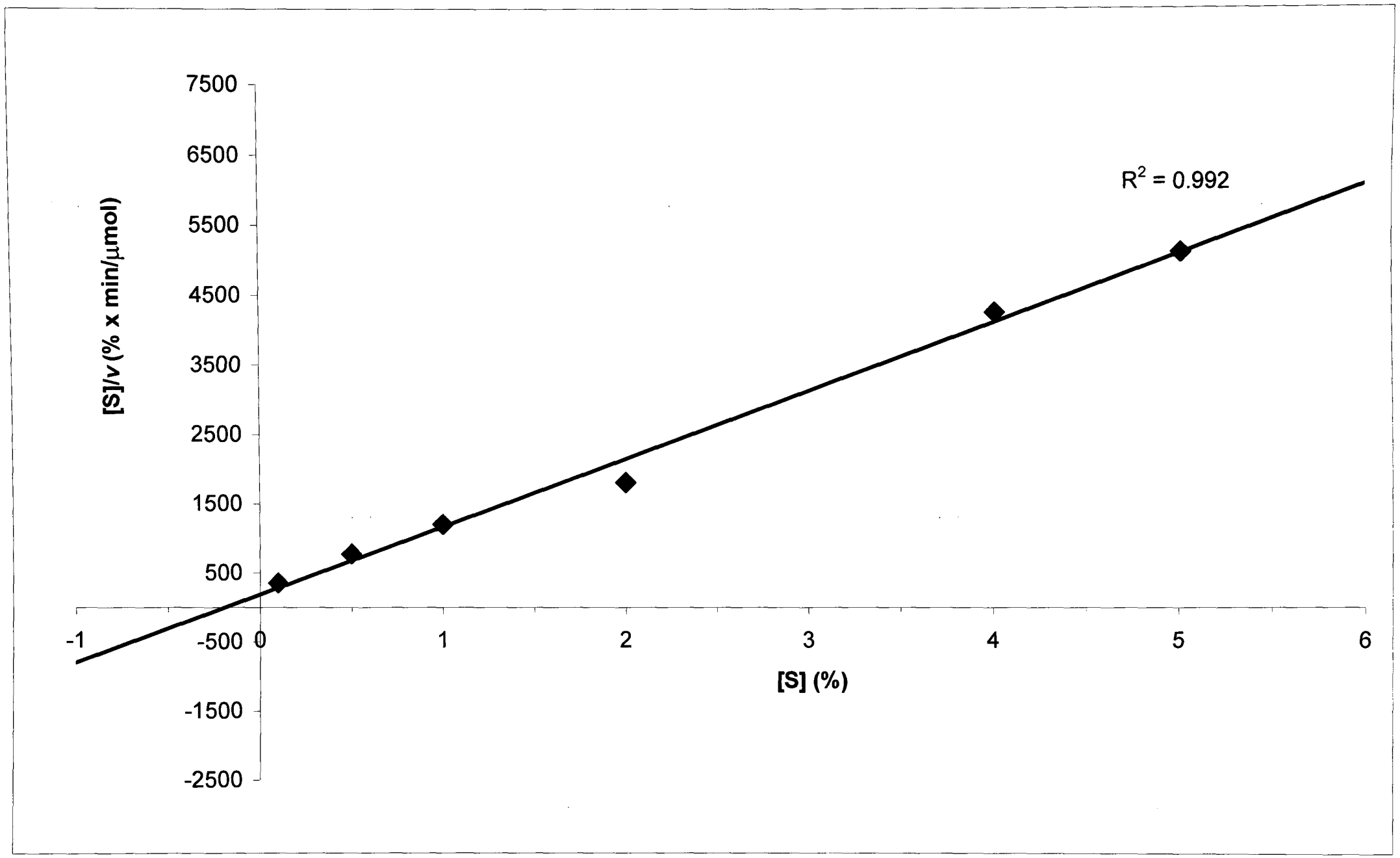


Figure 6.13 Hanes plot of α-glucosidase using pullulan

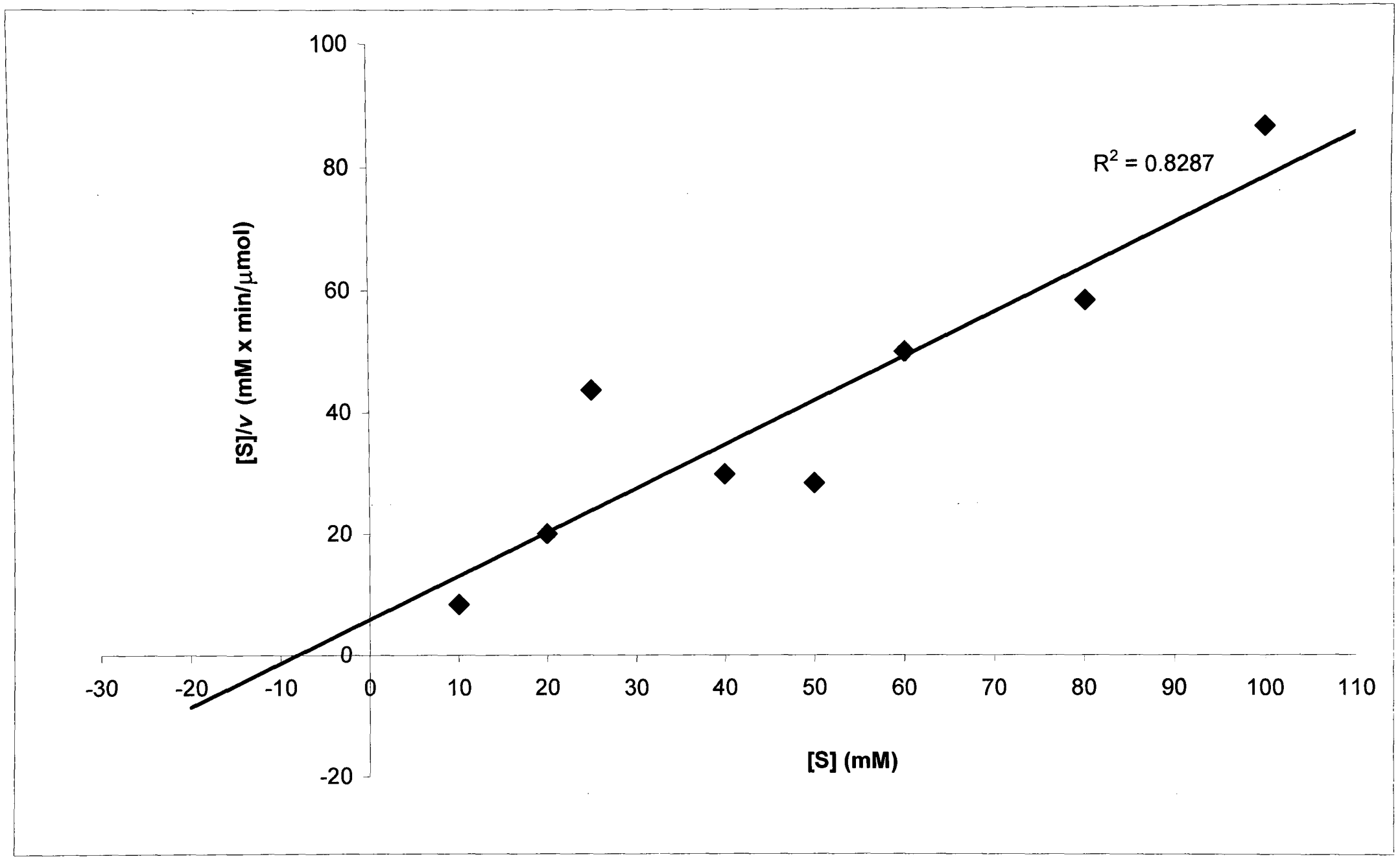


Figure 6.14 Hanes plot of α-glucosidase using raffinose

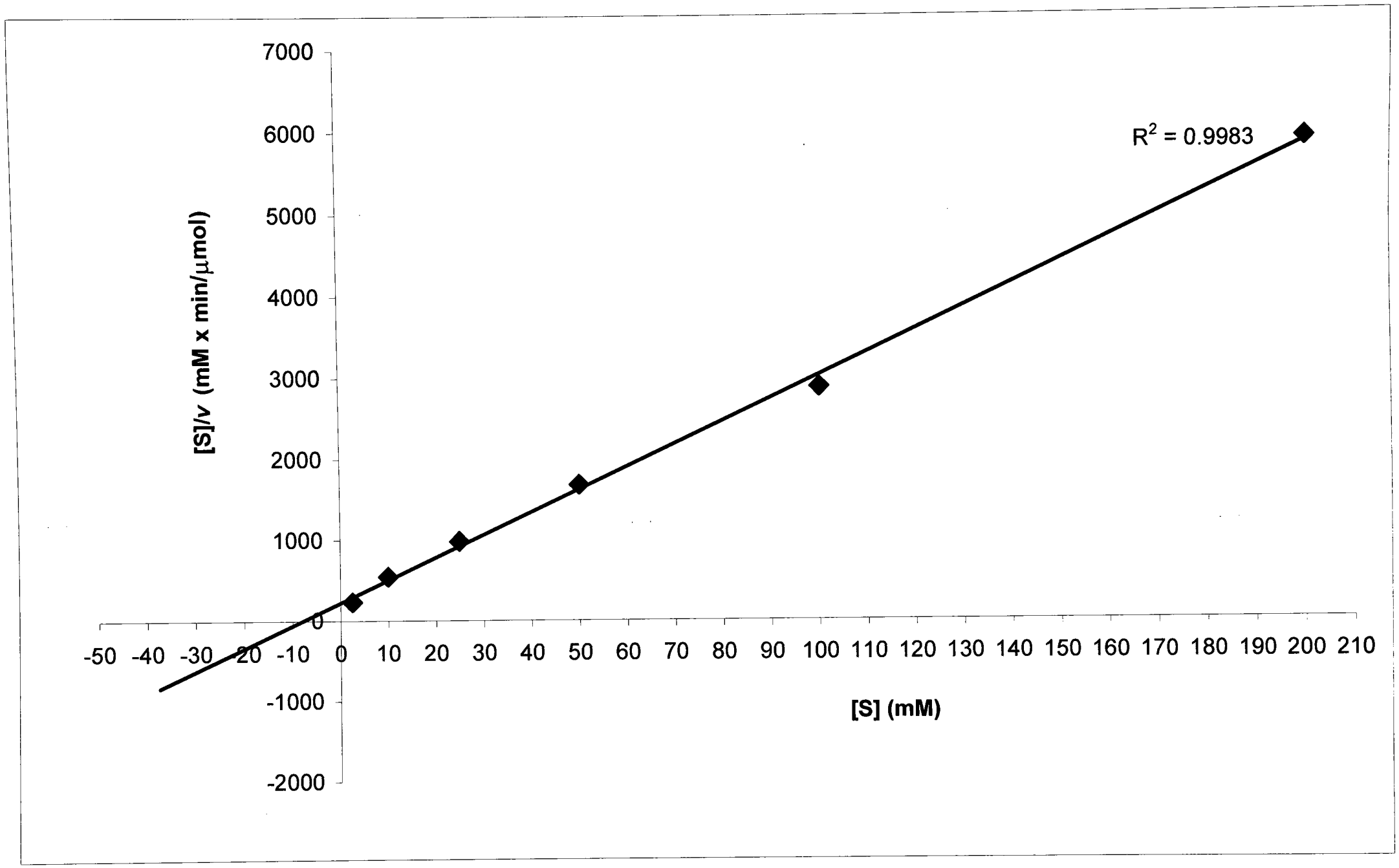


Figure 6.15 Hanes plot of α-glucosidase using nigerose

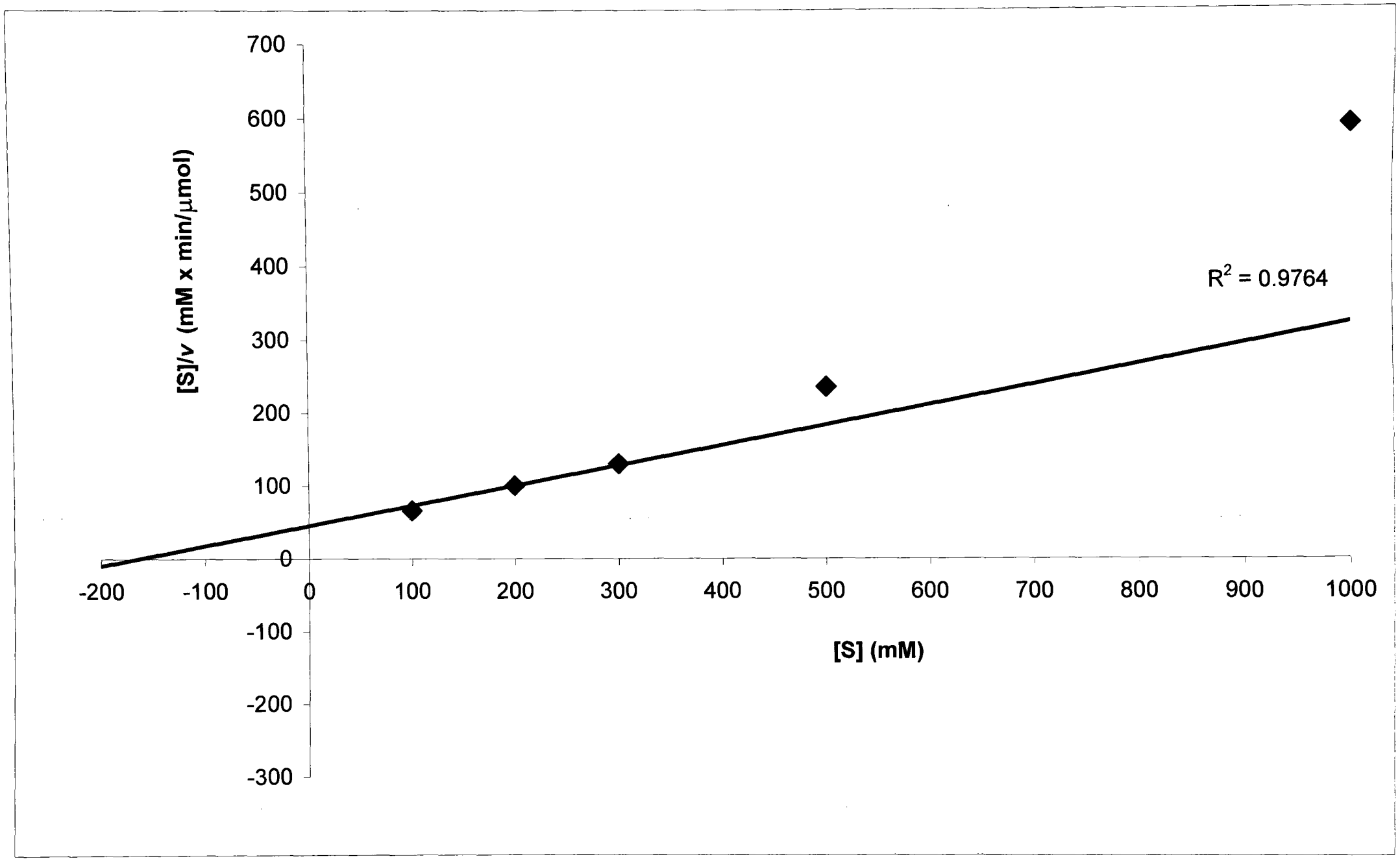


Figure 6.16 Hanes plot of α-glucosidase using trehalose

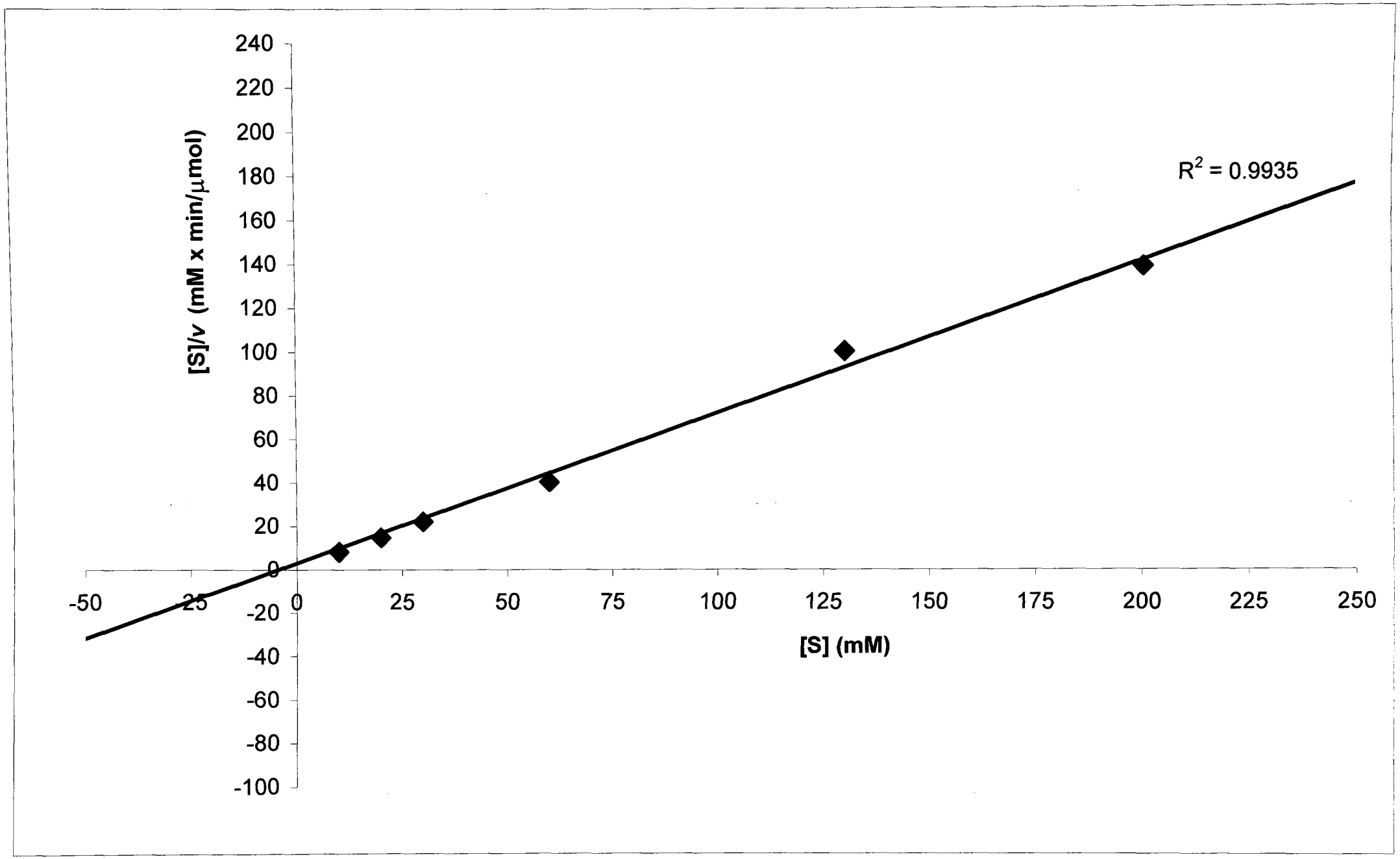


Figure 6.17 Hanes plot of α-glucosidase using stachyose

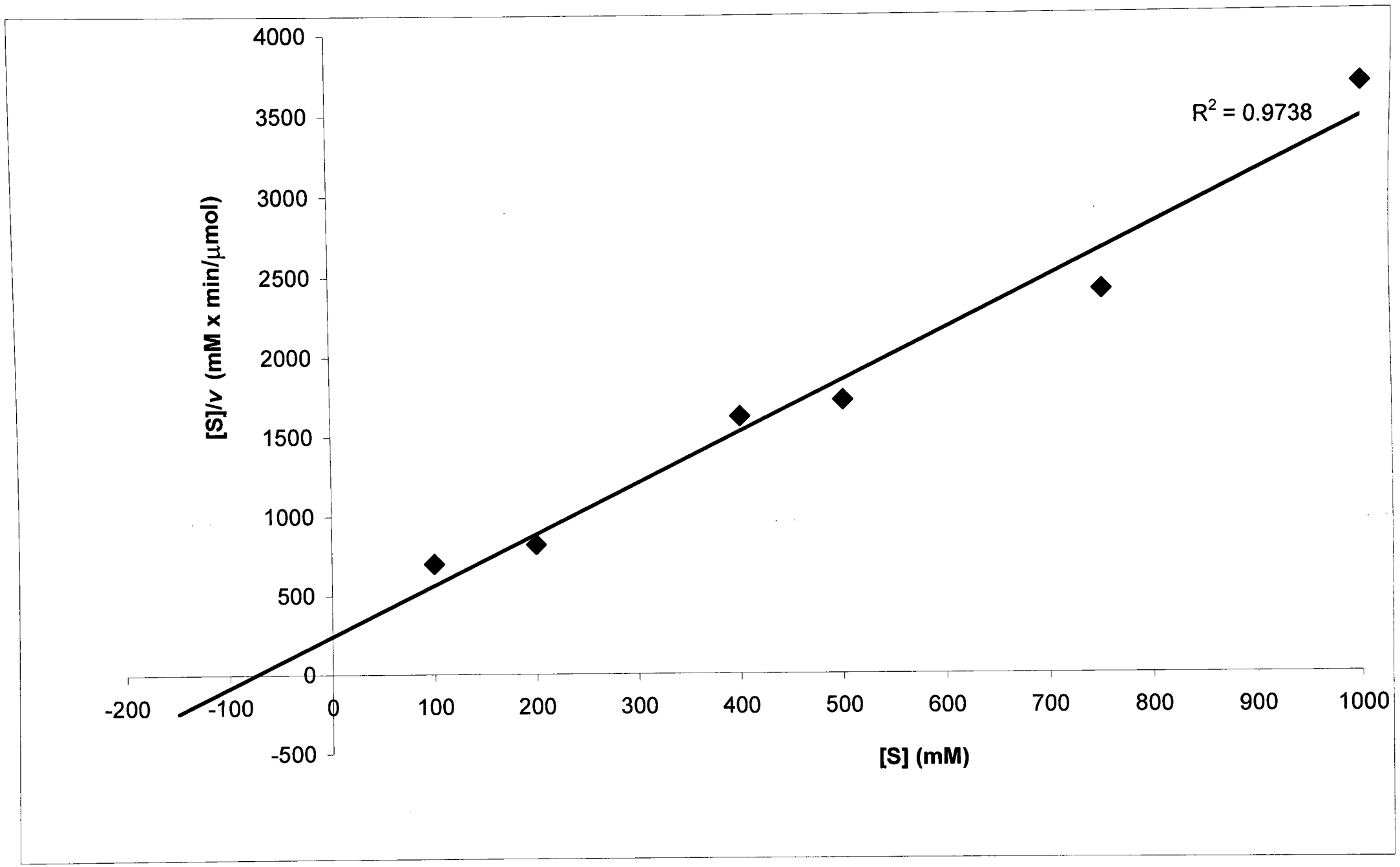


Figure 6.18 Hanes Plot of α-glucosidase using isomaltose

Table 6.1 Summary of *K*m, *V*max and correlation coefficients for the *Lipomyces starkeyi* (NCYC 1436) α-glucosidase.

Substrate	*K*m (%)	*K*m (mM)	*V*max (μmol/min/mg)	Correlation factor	Sugar-linkage type
Soluble starch	0.07	n/a	5.50	0.9824	Polysaccharide containing a mixture of α-1,4 and α-1,6 bonds
Amylopectin	4.00	n/a	0.16	N/A	Branched polymer of amylose chains joined by α-1,6 bonds
PNPG		0.30	14.10	0.9673	Aryl-α-D-glucoside
Maltose (4-O-α-D-Glucopyranosyl-D-glucose)		2.00	7.74	0.9459	α-D-Glc-(1→4)-α-D-Glc
Maltotriose		5.50	7.10	0.9685	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc
Maltotetraose		6.00	7.40	0.9841	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc
Maltopentaose		6.25	7.30	0.9944	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4) -α-D-Glc
Maltohexaose		6.00	6.20	0.9908	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc
Maltoheptaose		6.00	5.28	0.9068	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4) -α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc
Sucrose (1-*O*-α-D-Glucopyranosyl-β-D-fructofuranoside)		5.00	1.61	0.9788	α-D-Glc-(1↔2)-β-D-Fruc

Substrate	*K*m (%)	*K*m (mM)	*V*max (μmol/min/mg)	Correlation factor	Sugar-linkage type
β-Lactose		85	0.0066	0.7574	β-D-Glc-(1→4)- β-D-Glc
Dextran	15.0		2.86	N/A	Linear chains of mainly α-1,6 bonds but 1,2, 1,3 and 1,4 possible
Pullulan	20.0		4.08	0.992	α-D-Glc-(1→4), α-D-Glc-(1→6)-α-D-Glc
Raffinose (O-α-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside		80	0.014	0.8287	α-D-Gal-(1→6)-α-D-Glc-(1↔2)-β-D-Fru
Nigerose (3-O-α-D-Glucopyranosyl-D-glucopyranose)		0.80	14.30	0.9983	α-D-Glc-(1→3)-α-D-Glc
Trehalose (1-*O*-α-D-Glucopyranosyl-α-D-Glucopyranoside)		16.8	3.13	0.9764	α-D-Glc-(1↔1)-α-D-Glc
Stachyose (Lupeose, 6^{Gal}-α-D-Galacto-raffinose)		60.5	0.014	0.9935	α-D-Gal-(1→6)-α-D-Gal-(1→6)-α-D-Glc-(1↔2)-β-D-Fru
Isomaltose (6-O-α-D-Glucopyranosyl-D-glucose)		7.50	3.11	0.9738	α-D-Glc-(1→6)-α-D-Glc

Glc: glucose: Gal: galactose: Fru: fructose

6.2 Discussion: Substrate Specificity of *Lipomyces starkeyi* α-Glucosidase

According to the definition of Chiba *et al.*, (1973b), yeast α-glucosidases preferably hydrolyse aryl glucosides compared to maltose and sucrose -and on this criterion -from the kinetic data summarised in Table 6.1, the α-glucosidase from *Lipomyces starkeyi* NCYC 1436, is a typical yeast α-glucosidase. The enzyme displays a broad substrate specificity, capable of acting on substrates with α-1,2, α-1,3, α-1,4 and α-1,6 sugar linkages. The enzyme showed a marked preference for α-1,4 linkages, but by-passed β-1,4 glucosidic linkages in substrates such as β-lactose.

Although α-glucosidases are believed to be highly specific for α-D-glucose residues, their specificity for the aglycone portion of the substrate is not as distinct, so aryl and alkyl-α-glucosides, maltose and maltose derivatives can all act as substrates - but to varying degrees. Gottschalk (1950), suggested that it was the closeness of the lower side of the pyranose ring (glycone) to the active site of the enzyme which makes α-glucosidases so sensitive to any configuration change at carbon atoms 1,2,3 and 4 of the glycone component of their substrates. Any change in configuration of the ring carbon atoms will either remove a hydroxyl group from its correct spatial position or produce steric hindrance by replacing a hydrogen atom with a larger hydroxyl group. Either process will interfere with the formation of the enzyme-substrate complex.

The preferred substrates for the α-glucosidase purified here, from *L. starkeyi*, NCYC 1436, as indicated by low *K*m (mM/ %) and high *V*max (μmol/min/mg) values, were: soluble starch; (0.07%, 5.5μmol/min/mg); PNPG (0.3mM, 14.1μmol/min/mg); and nigerose (0.8mM; 14.3μmol/min/mg).

The *L. starkeyi* α-glucosidase is unusual in its ability to hydrolyse soluble starch, a property reported in few yeast α-glucosidases. The α-glucosidase purified by Kelly *et al.*, 1985, from *L. starkeyi* CBS 1809, was also active on soluble starch. This could possible be attributed to the type of starch used, as starch derived from different sources has different physical and chemical properties. High molecular weight starches can have a higher proportion of steric hindrances and therefore hydrolysis can be slow, as the active centre is less accessible. Sanroman *et al.*, (1996) studied the activity of two glucoamylase enzymes using different starches. The higher molecular weight

starch offered more steric hindrance, which led to more points to attack, and hence a difference in the kinetic behaviour was observed.

In fact, a few of the substrates investigated in this study showed anomalous Michaelis-Menten behaviour. Amylopectin, maltohexaose, trehalose and dextran displayed non-linear curves at high substrate concentrations. This was attributed to the increased viscosity, which affected the rate of reaction of the *L. starkeyi* α-glucosidase. Therefore, *K*m and *V*max values could only be determined for these substrates using data points obtained at the lower concentration values, where the curve was linear.

Based on the kinetic data obtained from the linear part of the curve, hydrolysis of amylopectin by the *L. starkeyi* α-glucosidase was very slow, possibly suggesting that the enzyme was inefficient in cleaving glucose polymers rich in α-1, 6 branch points. Amylose was also chosen for evaluation but problems arose in solubilizing the substrate to form a homogeneous solution without visible particulates, so the solute concentrations could not be calculated, and the data was therefore not included.

Pullulan, a substrate that has a high proportion of α-1,6 bonds (1 in 3), and isomaltose - a disaccharide with α-1,6 bonds - showed some similarity to the amylopectin results; both substrates were hydrolyzed with difficulty compared to the preferred substrates of soluble starch and PNPG.

In the case of the structural series of maltose to maltoheptaose, it was difficult to establish an obvious trend as the enzyme did not seem to follow a simple kinetic model i.e. *V*max did not increase or decrease with the increase in chain length but remained about the same for all the maltodextrins. However, low *K*m values were obtained with maltose (2mM).

The low activity of the *Lipomyces starkeyi* α-glucosidase against the maltodextrins suggests that none of these substrates tested were primary targets for the enzyme. However, it is possible that as the substrates tested were not of analytical grade quality, any residual glucose present in the preparation, could have led to inaccurate kinetic determinations for the α-glucosidase when using the coupled enzyme assay. When examining α-glucosidase activity with each substrate, the hexokinase enzyme and buffers were incubated with the substrate for 15minutes prior to measuring the rate of reaction (see section 2.7). In some instances this led to a high background absorbance

of the blank tube and therefore estimating activity after the addition of α-glucosidase could not always be accurately measured.

Some of the other substrates investigated here, such as raffinose (80mM, 0.014μmol/min/mg) and stachyose (60.5mM, 0.014μmol/min/mg), contain α-1,2 as well as α-1,6 bonds, and were not readily cleaved by the α-glucosidase. Sucrose was hydrolyzed, but more slowly than maltose, (5mM, 1.61μmol/min/mg), which further demonstrated that the enzyme preferred α-1,4 bonds to α-1,2 bonds. Despite the problems of increased viscosity at high substrate concentrations, the *V*max (3.13μmol/min/mg) and *K*m (16.8mM) values were determined for trehalose (the yeast storage sugar). This data suggests that the α-glucosidase did not readily cleave α-1,1 bonds.

Dextran, which consists of approximately 95% α-1,6 linkages and 5% α-1,3 linkages (which also accounts for the branching in dextran; Sigma, technical bulletin), was hydrolysed slowly by the *L. starkeyi* enzyme (15%, 2.86μmol/min/mg). The kinetic data was obtained from the linear part of the plot, as increased viscosity was also a problem with high concentrations of dextran.

Several groups have used dextran as the primary carbon source for the growth of certain bacteria and fungi, and have then found α-glucosidase-type activity in the culture medium. An extracellular α-glucosidase with dextran-hydrolysing activity was purified from *Thermomyces lanuginosus*, a thermophilic fungus (Jensen and Olsen, 1996). Another group, Mizuno and Tachiki (1998), also found two species of extracellular α-glucosidase-type of activity when the bacterium, *Bacillus circulans* KA-304, was grown on dextran.

However, *L. starkeyi* α-glucosidase showed some preference for nigerose, a disaccharide containing α-1,3 bonds. With a low *K*m of 0.8mM and a *V*max of 14.3μmol/min/mg, the affinity for this substrate is consistent with much of the data published on substrate specificity, for a number of yeast α-glucosidases (see Table 6.2).

Overall, the α-glucosidase secreted from the soil yeast, *Lipomyces starkeyi* showed an affinity for a range of substrates investigated in this work. It is possible that the most

natural substrate for the α-glucosidase has not been tested in this study and therefore an extensive screening of compounds typically found in soils would be appropriate. However, the kinetic properties are similar to other reported yeast α-glucosidases, as shown in Table 6.2.

Table 6.2: Substrate Specificities of some Yeast α-Glucosidases

Organism	Substrate specificity*	Reference
***Lipomyces starkeyi* NCYC 1436**	**soluble starch; PNPG, nigerose**	
Torulaspora pretoriensis YK-1	PNPG, isomaltose, methyl α-D glucopyranoside, sucrose	Oda *et al.*, 1993
Lipomyces tetrasporus (carbohydrase)	nigerose, soluble starch, dextran	Gallagher, *et al.*, 1991
Lipomyces starkeyi CBS 1809	maltose; isomaltose, PNPG, maltotriose, isomaltotriose, amylopectin, starch	Kelly *et al.*, 1985
S. cerevisiae: Type 1 Type 2 Type 3	 PNPG PNPG PNPG	Matsusaka *et al.*, 1977.
S. oviformis maltase isomaltase	maltose, isomaltose	Lai and Axelrod, 1975
S. logos	maltose, PNPG	Chiba *et al.*, 1973
S. cerevisiae 1412-4 D: Type I. Maltase Type II. α-Methyl glucosidase (isomaltase)	 maltose, sucrose isomaltose, sucrose	Khan and Eaton, 1967.
S. italicus Y1225	sucrose, turanose, maltose	Halvorson and Ellias, 1958

*Substrates are listed in order of preference

The kinetic behaviour of the *Lipomyces starkeyi* NCYC 1436 α-glucosidase is distinct from the α-glucosidase purified by Halvorson and Ellias (1958) from *Saccharomyces italicus* Y122S. Using a maltose growth medium, they were able to purify and characterise an intracellular α-glucosidase, which actively cleaved sugars joined by α-1,2 (sucrose), α-1,3 (turanose) and α-1,4 (maltose). The enzyme did not utilize trehalose and substrates containing α-1,6 bonds such as isomaltose gave atypical results, which did not follow the Michaelis-Menten kinetic model.

Khan and Eaton (1967), reported that the α-glucosidase from *Saccharomyces cerevisiae* could be fractionated into two, a maltase and an isomaltase, when using a YEP (yeast extract, peptone) growth medium containing either 2% (w/v) maltose or 2% (w/v) α-methyl glucoside as the carbon source. The maltase hydrolysed both maltose and sucrose while the isomaltase hydrolysed isomaltose, sucrose and methyl-α-D glucoside.

Chiba *et al.*, (1973b), explored the substrate specificity of the *Saccharomyces logos* intracellular α-glucosidase using maltose, phenyl-α-maltoside, phenyl-α-glucoside, methyl-α-glucoside, nigerose, kojibiose, isomaltose, kojibiose and turanose. They concluded that the enzyme differed from α-glucosidases purified from other *Saccharomyces* species in that it exhibited stronger hydrolytic activity towards malto-oligosaccharides than towards other oligosaccharides.

The α-glucosidase exhibited high activity towards maltose and to a lesser extent phenyl α-D-glucoside and displayed no activity towards sucrose. This enzyme closely resembled α-glucosidases purified from moulds - a typical maltase.

Lai and Axelrod (1975) isolated a maltase and isomaltase, but from a different species, *Saccharomyces oviformis*. Isomaltase only hydrolysed isomaltose, while the maltase not only hydrolysed maltose but, unusually, also hydrolysed isomaltose. Both enzymes hydrolysed PNPG.

Matsusaka *et al.*, (1977), examined brewers yeast and found three α-glucosidase activities, all of which had different substrate specificities. All three hydrolyzed phenyl α-D-glucosides preferentially to maltose, isomaltose and methyl α-D glucopyranoside. These results on substrate specificity were comparable to the results obtained from Lai and Axelrod (1974, 1975) using *S. oviformis*, and also with Khan and Eaton (1967), with *S. cerevisiae*.

In fact, the only other reported α-glucosidase purified from a strain of *Lipomyces starkeyi* followed a similar pattern to the α-glucosidase purified by Khan and Eaton, (1967) and Lai and Axelrod (1975). Although only one glucosidase-type activity was purified from *Lipomyces starkeyi* CBS 1809, Kelly *et al.*, (1985) showed that the α-

glucosidase had the highest activity on both maltose and isomaltose. The enzyme displayed relative rates of activity on maltotriose, isomaltotriose and PNPG but was defined as a typical maltase, as it was inactive against sucrose. Unusually, the α-glucosidase showed high affinity for amylopectin and soluble starch. This differed from the α-glucosidase purified from *Lipomyces starkeyi* NCYC 1436 in this study, which displayed little activity towards amylopectin.
Kelly *et al.* isolated a third, sucrose-hydrolyzing, activity but further investigation wasn't carried out.

The novel carbohydrase purified from *Lipomyces tetrasporus* by Gallagher *et al.*, (1991) which displays an exolytic mode of action and releases α-D-glucose, also showed a broad substrate specificity, similar to the α-glucosidase purified from *L. starkeyi* with high activity on nigerose and soluble starch. However, this enzyme was unusual in its ability to hydrolyse dextran. The enzyme was not characterised further.

Oda *et al.*, (1993), carried out studies to explore the substrate specificity of the intracellular α-glucosidase isolated from *Torulaspora pretoriensis* YK-1. PNPG was the preferred substrate and enzyme activity decreased with the increased polymerisation of glucose from maltose up to maltopentaose. Soluble starch was not utilised at all. The purified enzyme hydrolysed isomaltose, methyl α-D-glucoside and sucrose more rapidly than α-1,4 maltooligosaccharides.

Early kinetic data previously published on α-glucosidases has been used to classify the enzyme into one of three categories, dependent on substrate specificity (Phillips, 1959); Type 1: α-glucosidases which preferentially hydrolyse PNPG and sucrose; Type 2: α-glucosidases which possess a strong affinity for maltose, and finally Type 3: α-glucosidases, which are capable of hydrolysing soluble starch (section 1.6). However, work published most recently by Chiba (1997), which examined the homology of the amino acid sequence of the regions containing the catalytic site of 20 different α-glucosidases, characterized the enzyme into two families according to their primary structure.
Family I broadly (although not exclusively), includes α-glucosidases from yeast, bacteria and insects, while Family II includes α-glucosidases from mammals,

filamentous fungi and plants. Family 1 contains α-glucosidases from *Saccharomyces carlsbergensis* and *Bacillus cereus,* both of which showed a high degree of similarity in four conserved regions (1, 2, 3, and 4), which also included the active site. Interestingly, these four regions are also conserved in the protein sequences of many α-glucan (starch) hydrolyzing α-amylases: (although there in no similarity in the overall protein sequences with α-amylases). Family II lacks regions 1, 3, and 4 but does have a highly conserved region 5. Chiba suggested that these two families independently evolved by convergent evolution from different ancestral proteins. As more α-glucosidases are sequenced and characterised, the robustness of the Chiba classification will be tested, and then possibly refined.

Chapter 7: Concluding Discussion

Starch-degrading enzymes are amongst the most important enzymes in present-day biotechnology, and amylolytic enzymes derived from yeast sources, which possess a useful range of properties, have the potential to widen the current applications of amylolytic enzymes from the food, brewing and distilling industries into diverse areas such as the clinical and medicinal industries.

α-Glucosidases have long been recognised as a commercially significant class of enzymes in the starch industry, working alongside α-amylases and glucoamylases for the complete degradation of starch. The advantages of enzymes capable of utilizing starch efficiently have been outlined in section 1.5.3.

The commercial success or economic importance of a microbially-derived enzyme used in bulk, relies on a number of factors such as yield, purity, thermostability, with the selection of a suitable strain being the most significant factor of all (Pandey, 2000). Although many organisms can produce amylolytic enzymes, it still remains a challenging task to obtain a strain of organism capable of producing a complement of enzymes of commercially acceptable yield. A yeast strain capable of achieving this has a competitive advantage. The results of this study have concluded that *Lipomyces starkeyi* NCYC 1436 is capable of hydrolysing soluble starch by production of an α-glucosidase and multiple α-amylases, and has the potential to compete in the competitive biotechnology arena.

Cultures of *Lipomyces starkeyi* NCYC 1436 when grown on a medium enriched with 2% starch were able to secrete α-glucosidase and α-amylase enzymes extracellularly to hydrolyse soluble starch. Maximum α-glucosidase production was obtained after a 6-day incubation, with optimum α-amylase activity seen after 14 days. Despite the long incubation of the organism, which was necessary to obtain the maximum yield, liquid cultures remained free of contamination and, importantly, protease activity was not detected at this stage.

The enzyme yields obtained for the α-glucosidase suggested that the environmental factors required for the optimum growth of the yeast were different from those needed

for the optimum production of the enzymes, and that certain parameters could be optimized to increase the enzyme yield e.g. the degree of aeration or nutrient supplementation. (With the availability of published literature on optimising the production of α-amylases in other organisms, it may be possible to develop new strategies to increase the yield of α-glucosidase from *L. starkeyi*). For example, Milner *et al.* (1997) used a two-stage inoculation when examining α-amylase production, which they found significantly, improved the yield when compared to a single inoculation.

In the work described here, extracellular α-glucosidase was extracted from the cell-free supernatant using ammonium sulphate precipitation and then purified by hydrophobic interactive chromatography (HIC) followed by ion exchange chromatography (IEC). An advantage to this simple purification scheme was the short number of steps required to achieve an electrophoretically homogeneous enzyme preparation. HIC using a Phenyl Sepharose CL-4B column was an appropriate choice following an ammonium sulphate precipitation step, as the sample was in high salt and minimal pre-treatment of the sample was required prior to HIC. Although only partially successful in separating α-glucosidase from α-amylase, HIC was effective in removing a large proportion of other contaminating proteins present in the sample (Table 4.9), and therefore purified the α-glucosidase activity.

Choosing a suitable ion exchange column capable of reproducibly separating the two enzyme activities without losing activity to irreversible binding sites was an analytical challenge. However, Q-Sepharose was found to be efficient in separating α-glucosidase activity from α-amylase, when active fractions collected from the Phenyl Sepharose CL-4B column were run individually by ion exchange. Individual HIC fractions purified by ion exchange showed up to a 24.6-fold increase in purity for α-glucosidase and up to a 7.6 increase in purity for α-amylase. In the case of the most active α-glucosidase fraction isolated after running the 0.01M HIC fraction on ion exchange, the purification factor rose to 83.4.

The elution profile of the enzyme on HIC columns and the corresponding multiple band pattern seen when these HIC fractions were run by SDS-PAGE, could indicate

that more than one α-glucosidase species was present in the supernatant. However, after further purification of these fractions via ion exchange chromatography, only a single species of α-glucosidase was evident. It is therefore possible that isozymes of α-glucosidase were present in the growth medium but were differentially labile to factors such as purification temperature, high salt environment or contaminating proteases. Only 59% of the α-glucosidase was recovered after HIC, whereas 72% of α-amylase was recovered. This may indicate that a small percentage of α-glucosidase activity, possibly α-glucosidase isozymes may have been lost on purification.

The presence of α-glucosidase isozymes has been reported in certain bacterial and yeast strains (Table 6.2). Mizuno and Tachiki (1998), detected two α-glucosidases: α-glucosidase I and α-glucosidase II, when they grew *Bacillus circulans* KA-304, on dextran as the sole carbon source. Both enzymes were able to hydrolyse p-Nitrophenyl-α-D-glucoside but α-glucosidase I showed exo-type activity with an affinity towards α-1,6 glucosidic linkages. α-Glucosidase II showed no activity on dextran and was not characterized further.

Monitoring the purification process by polyacrylamide gel electrophoresis after each step supplied information as to the level of purity of the enzyme and allowed for the qualitative assessment of the purification process.

The molecular weight of the α-glucosidase was determined under denaturing and non-denaturing conditions after ion exchange chromatography. A single major band was detected by SDS-PAGE (Figure 5.4), with an estimated molecular weight of 93,000 ± 5,000 Daltons. Under native conditions, the molecular weight of the same species (from exactly the same IEC fraction) was estimated at 162,000 Daltons, and specific enzyme activity staining of the native gel confirmed that the single band was an α-glucosidase (Figure 5.10).

The large discrepancy between the two molecular weight estimates could reflect anomalous electrophoretic behaviour of the (monomeric) enzyme, or could conceivably suggest that the native enzyme is a dimer.

Molecular weight analyses of α-glucosidases isolated from different yeast and bacterial sources give widely different results, and can show surprising complexities of enzyme structure. For example, Oda *et al.*, (1993), purified an intracellular α-glucosidase from *Torulaspora pretoriensis* YK-1 and confirmed from the gel data, that the enzyme was indeed a monomer of 60-69KDa. However, Kinsella *et al.*, (1991), when cloning and characterizing an α-glucosidase gene from the yeast *Candida tsukubaensis,* expressed it in the yeast *Saccharomyces cerevisiae* and found a diffuse protein band - estimated to be 70-80KDa. Further analysis showed that the α-glucosidase was actually composed of two polypeptides which were clearly resolved by SDS-PAGE following deglycosylation with endoglycosidase H, and that the both polypeptides were required for functional α-glucosidase activity.

Nakao *et al.,* (1994) purified a thermostable α-glucosidase with transglucosylation activity from an *E. coli* recombinant transformed with the α-glucosidase gene from a *Bacillus* species SAM1606. The enzyme was purified by a combination of ammonium sulphate fractionation, anionic exchange, hydroxyapatite chromatography and gel filtration. Gel filtration revealed three major active fractions, corresponding to molecular weights of 68,000, 138,000 and >200,000, but SDS-PAGE of all three fractions gave a single band of 64,000. When all three fractions were subjected to amino acid analysis and Edman degradation, the amino acid analyses gave identical amino acid compositions with identical amino-terminal sequences. They hypothesised that because the results were consistent with the calculated molecular weight from the amino acid sequence of the α-glucosidase, that this indicated that the enzyme could exist as mono and multimeric forms of a protomer protein with a molecular weight of 64,000. Studies carried out to further characterize the enzyme in terms of substrate specificity, thermostability and transglucosylation activity for the synthesis of oligosaccharides, revealed that there were no significant differences in these properties between the monomeric or multimeric forms of the enzyme. They could not discover whether the multimeric form was the native one or an artefact due to over-expression by gene cloning.

Degnan (1994), reported on an α-glucosidase from *Bacteroides ovatus*, a bacterium found in the gut. This organism was capable of assimilating starch and glucose equally well and the ability of the bacterium to hydrolyse a variety of substrates was attributed to a single dimeric enzyme - approximately 126KDa in size, which was capable of

hydrolysing gelatinised starch and PNPG. This work followed the research published by McCarthy *et al.*, (1988), who had previously found that another species of *Bacteroides, B. vulgatus,* secreted a single monomeric protein with both α-glucosidase and α-amylase activities.

Much of the variation in the molecular weight estimates reported for some yeast α-glucosidases may be due glycosylation and related to this, whether the enzyme is found intracellularly or extracellularly. If, as in the case of the α-glucosidase purified from *Lipomyces starkeyi* NCYC 1436, the enzyme is secreted into the cell-free supernatant, it is likely to be glycosylated, and the degree of glycosylation can influence the molecular weight determinations when analysed by polyacrylamide gel electrophoresis, and can affect pI estimates. Indeed, phenol sulphuric acid analysis of the purified *L. starkeyi* enzyme gave a carbohydrate content of approximately 32% (w/w).

Glycosylation is a natural modification of the covalent structure of proteins. Most secretory proteins become glycosylated as soon as growing polypeptide chains enter the endoplasmic reticulum. The influence of the carbohydrate moiety on the stability of glycoproteins has been investigated by a number of groups with secretion, protection against proteolysis, stabilization of tertiary structure and enhancement of solubility, all being proposed as possible functions for the carbohydrate moiety of the glycoprotein (Rudd *et al.*, 1994).

Recently, Wang *et al.*, (1996) examined the role of carbohydrate moieties in glycoproteins using CD (circular dichroism) and DSC (differential scanning calorimetry). They found that deglycosylating proteins decreased the proteins thermal stability. Studies on the reversibility of heat denaturation revealed that the carbohydrate moiety is important *in vivo*, when the protein is folding. A newly synthesized polypeptide may be transiently concentrated in the ER, and glycosylation may increase solubility and prevent non-specific aggregation, until the polypeptide chains are correctly folded and transported to the secretory pathway. Once folded correctly, glycosylation may prevent attack by proteolytic/denaturing agents and therefore help to stabilize the protein. Wang *et al.* suggested that stability was due to

hydrogen bonds, which are formed between the carbohydrate moiety and the polypeptide backbone.

In fact, the degree of glycosylation could have been a factor in accurately determining the pI of the α-glucosidase. The isoelectric focusing data obtained for the purified *Lipomyces starkeyi* α-glucosidase, was comparable to other yeast α-glucosidases but with three bands detected within the pI range of 4.6-5.0.

Further characterisation studies on the purified *L. starkeyi* α-glucosidase revealed that maximum enzyme activity was achieved at a temperature of 55°C and a pH of 4.5.

From the kinetic data obtained on the purified enzyme, the α-glucosidase from *Lipomyces starkeyi* NCYC 1436 is a typical yeast α-glucosidase, preferring aryl glucosides compared to maltose and sucrose (Chiba *et al.*, 1973b). The enzyme showed a marked preference for α-1,4 linkages, with the preferred substrates - as indicated by low *K*m (mM/ %) and high *V*max (μmol/min/mg) values: soluble starch; (0.07%, 5.5μmol/min/mg); PNPG (0.3mM, 14.1μmol/min/mg), and nigerose (0.8mM; 14.3μmol/min/mg) respectively. Hydrolysis of amylopectin, isomaltose and pullulan was very slow and therefore suggested that the enzyme was inefficient in cleaving glucose polymers rich in α-1, 6 bonds.

Overall, the kinetic behaviour of the *L. starkeyi* α-glucosidase reported in this study suggests that the enzyme is effective in hydrolysing the soluble starch in the growth medium - by preferentially attacking α-1,4 bonds, and effectively utilising starch breakdown products such as maltohexaose (1.70mM, 7.69μmol/min/mg) and maltose (2mM, 7.74μmol/min/mg).

It has long been considered that α-amylases and glucoamylases are solely responsible for initiating the degradation of starch, and that the primary function of α-glucosidases is to hydrolyse a major product of the α-amylase reaction, maltose. However, no glucoamylase activity was detected in the cell-free supernatants of the *L. starkeyi* starch cultures, though conformation analysis (α or β) of the glucose released during starch hydrolysis could definitely exclude glucoamylase involvement.

This study, therefore suggests that the α-glucosidase purified from *L. starkeyi* - by virtue of being secreted into the extracellular space before α-amylase (when soluble starch is the sole carbon source) - has an important and distinct role in the degradation of starch. Regarding this role, there are three possibilities.

The first possibility is that *L. starkeyi*'s α-glucosidase is induced by the presence of disaccharide sugars in the original medium, and acts primarily on them. These disaccharides would be derived either as a result of the preparation process, where starch is solubilized by direct heating, or when the soluble starch preparation is of a relatively impure grade and contains a significant number of contaminating sugars. A high concentration of maltose in the medium could delay the synthesis and secretion of α-amylase until all the disaccharide sugar had been converted to glucose and assimilated, and this could explain why maximum α-amylase activity was achieved as late as 14 days.

Moulin and Galzy (1979) previously suggested that regulation of the biosynthesis of α-amylase by *Lipomyces starkeyi* depended mainly on the induction by the substrate, and that the presence of maltose in the medium would repress its synthesis. Subsequent accumulation of glucose in the medium could result in the catabolic repression of α-glucosidase, and so explain the levelling off of α-glucosidase activity seen after 12 days growth. A similar phenomenon was reported by Degnan and Macfarlane (1994), who examined the synthesis and activity of an α-glucosidase produced by *Bifidobacterium pseudolongum*, a bacterium found in the human large intestine. They found that cell-associated α-glucosidase was constitutively produced during growth on starch, but no α-amylase was detected. They concluded that the bacterium had adopted a strategy aimed at utilizing pre-existing starch breakdown products, and that the α-glucosidase was not involved in the initial depolymerization stage.

The second possibility is that the purified α-glucosidase – which showed an affinity for α-1, 6, and α-1,3 as well as α-1,4 bonds - or another type of enzyme indeed acted on the starch. The ability to de-branch soluble starch would be advantage as α-amylase is incapable of hydrolysing these bonds and bypasses them.

It we propose that *Lipomyces starkeyi* α-glucosidase isozymes are secreted into the growth medium, (some of which are subsequently degraded during the purification

process), then it possible that one of these could be responsible for hydrolysing α-1,6 bonds in starch during the growth of the yeast. However, further work characterising these other isozymes would need to be done to provide support for this hypothesis.

The third possibility is that the α-glucosidase from *L. starkeyi* has a particular role in initiating the starch degradation process and this hypothesis is promoted by some recent work on plant glucosidases. Sun and Hensen (1990), examined the degradation of native starch granules by barley α-glucosidases. They found that α-glucosidases isolated from germinating barley seeds were capable of initiating the degradation of native barley starch in the absence of α-amylase. Effective starch hydrolysis was achieved by the combined action of α-glucosidase and α-amylase, the action of α-glucosidase being known as "amylase activation". They attributed this "activation" to the fact that α-glucosidase from barley could hydrolyse several different types of α-glucosidic bonds such α-1,2 and α-1,3 in addition to the α-1,4 bond - hydrolysed by the majority of α-glucosidases.

They substantiated their hypothesis by examining raw starch granules after specific enzyme action using scanning electron microscopy (SEM). This revealed that α-amylases on their own, degraded starch at specific sites, in particular at the equatorial grooves of the lenticular granules. After hydrolysis using α-glucosidase, SEM revealed that there was access to areas on the granule previously unavailable to α-amylase activity when acting alone. Raw starch hydrolysis was 8-11 times faster with the α-amylase and the α-glucosidase acting synergistically than with the α-amylase acting alone.

Another study, by Sissons and MacGregor (1994), on the hydrolysis of barley starch granules by α-glucosidase from malt, confirmed that the α-glucosidase could hydrolyse intact granules - and they suggested that the α-glucosidase was capable of hydrolysing starch at branch points. However, this later study revealed that hydrolysis by α-glucosidase alone occurred at a much slower rate than by the α-amylase alone, which was considered to be primary enzyme involved in starch degradation.

Overall, the extracellular α-glucosidase secreted by *Lipomyces starkeyi* NCYC 1436, appears to have a significant role in hydrolysing soluble starch, distinct from the role of the secreted α-amylase.

α-Amylase activity was secreted and purified from *L. starkeyi* NCYC 1436. Maximum activity was seen after fourteen days and extracted from the cell-free supernatant by ammonium sulphate precipitation and then purified by HIC and IEC. SDS-PAGE and native gel electrophoresis revealed 3 bands of 125,000, 68,000 and 48,500, and specific activity staining confirmed that 3 distinct α-amylase activities had been purified.

Future work

As previously mentioned, the purified *L. starkeyi* α-glucosidase could be used in the enzymatic synthesis of oligosaccharides. It has been widely acknowledged that oligosaccharides have an important role in certain biological processes i.e. current medicinal applications as anti-tumour agents, cardiac glycosides or antibiotics (Palcic, 2000), and also applications as bio-control agents, particularly against fungal pathogens of plants (Scigellova and Crout 1999). The discovery of novel enzymes from microbial sources has led to rapid advances in the enzymatic synthesis of oligosaccharides for continued research.

Great strides have been made to synthesize oligosaccharides using a biocatalytic approach employing glycosidases and glycosyltransferases, as chemical synthesis of these novel compounds is expensive and laborious with several well-defined steps required to achieve the desired stereochemistry.

Glycosidases are attractive as they are widely available, robust and tolerant of organic solvents. Coupled with the fact that they utilize inexpensive glycosyl donors such as p-nitrophenyl glycosides, they can prove to be very useful. Glycosidases are stereospecific, only hydrolyzing and synthesizing either α- or β- linkages, but they are not regiospecifc and so mixtures of products can be produced. This can be overcome by having high concentrations of organic solvents.

Enzyme-catalysed glycosidic bond formation takes place in a single step avoiding complicated protection-deprotection mechanisms and with complete control of the configuration of the anomeric carbon. Synthesis is quicker and more efficient than using the traditional chemical route.

The α-glucosidase purified from *Lipomyces starkeyi* has a number of properties that make it potentially useful for oligosaccharides synthesis. Firstly, the enzyme has a broad substrate-specificity, therefore, depending on which sugars the α-glucosidase is incubated with - a range of oligosaccharides can be made i.e. if the enzyme is incubated with glucose, maltose will be the major disaccharide produced (Bucke, 1996).

Importantly, the *L. starkeyi* α-glucosidase preparation described here is highly purified, and doesn't contain β-glucosidases, which makes it a viable option in

oligosaccharide synthesis, as most commercial enzyme preparations are impure containing a variety of contaminants. In fact the *L. starkeyi* enzyme could be used for the preparation of complex branched hetero-oligosaccharides which occur N-linked to glycoproteins. Preparation of these oligosaccharides is particularly challenging, as they must be synthesised by enzymes, which employ sugar nucleotides as substrates. These enzymes are highly specific and their use in synthesis can be costly, due to their expensive substrates (Bucke, 1996).

Other future work, which could be considered, would be to develop a large-scale yeast fermentation process, to increase the yield of the amylolytic enzymes. This would be fundamental in continuing the characterisation of the *L. starkeyi* α-glucosidase. Greater yields would allow for the amino acid sequencing of the purified enzyme, as well as a variety of more detailed studies.

Recombinant technology (an expression system) is a recognized technique employed to combat low enzyme yields. Cloning of the α-glucosidase gene from *L. starkeyi* and its expression in the yeast *Saccharomyces cerevisiae* could be achievable. *Saccharomyces cerevisiae* lacks amylolytic enzymes but it is extremely versatile, with excellent fermentation capabilities such as fast growth, GRAS (generally regarded as safe) status, ethanol tolerance and resistance to infection. It is the ideal expression host for the production of recombinant products (Petersen, *et al.*, 1998).

This approach has been used by a number of groups, such as Steyn and Pretorius, (1995) who purified an α-amylase from *Lipomyces kononenkoae* and expressed its *LKA1* gene in *Saccharomyces cerevisiae.* The *Lipomyces kononenkoae* α-amylase gene was previously cloned as a cDNA fragment and expressed in *Saccharomyces cerevisiae* under the control of the *PGKI* promotor.

Similar work was carried out by Kinsella *et al.*, (1991), using an α-glucosidase gene from *Candida tsukubaensis,* which was also expressed in *Saccharomyces cerevisiae.*

Most recently, Ma *et al.* (2000) integrated an isoamylase gene from *Pseudomonas amyloderamosa* and the glucoamylase gene from *Aspergillus awamori* into *Saccharomyces cerevisiae* and were subsequently able to convert more than 95% of starch in the growth media to short chain sugars.

Appendix 1

The ionic strength of the ammonium sulphate buffer in 0.01M sodium phosphate was calculated using the following equation:-

Ionic strength = $\frac{1}{2}$ [$C_1 \times Z_1^2 + C_1 \times Z_2^2$]

Where C = molar concentration of ions

Z = ionic charges

0.01M $Na_2HPO_4^{2-}$

$\frac{1}{2}$ [(2 x 0.01) x (1^2) + 0.01 x (2^2)] = 0.03

0.01M $NaH_2PO_4^{1-}$

$\frac{1}{2}$ [(1 x 0.01) x (1^2) + 0.01 x (1^2)] = 0.01

The ionic strength of the 0.01M sodium phosphate buffer, pH 6.8 is 0.04.

1M $(NH_4)_2SO_4$

$\frac{1}{2}$ [(2 x 1) x (1^2) + 1.0 x (2^2)] = 3.5

0.01M $(NH_4)_2SO_4$

$\frac{1}{2}$ [(2 x0.01) x (1^2) + 0.01 x (2^2)] = 0.06

The ionic strength of 1M ammonium sulphate buffer in 0.01M sodium phosphate buffer was calculated as 3.54. This is 35-fold higher than the ionic strength of the 0.01M ammonium sulphate in 0.01M sodium phosphate buffer, which was calculated as 0.1.

Appendix 2

Calculation of Molecular Weight by SDS-PAGE

The molecular weight of α-glucosidase was determined by comparing the electrophoretic mobility with known protein standard markers. An approximately linear relationship was obtained when the logarithms of the molecular weights of standard proteins were plotted against their respective electrophoretic mobilities (Rf). The relative mobility (Rf) of the standards and unknowns were calculated by dividing the migration distance from the top of the separating gel to the centre of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

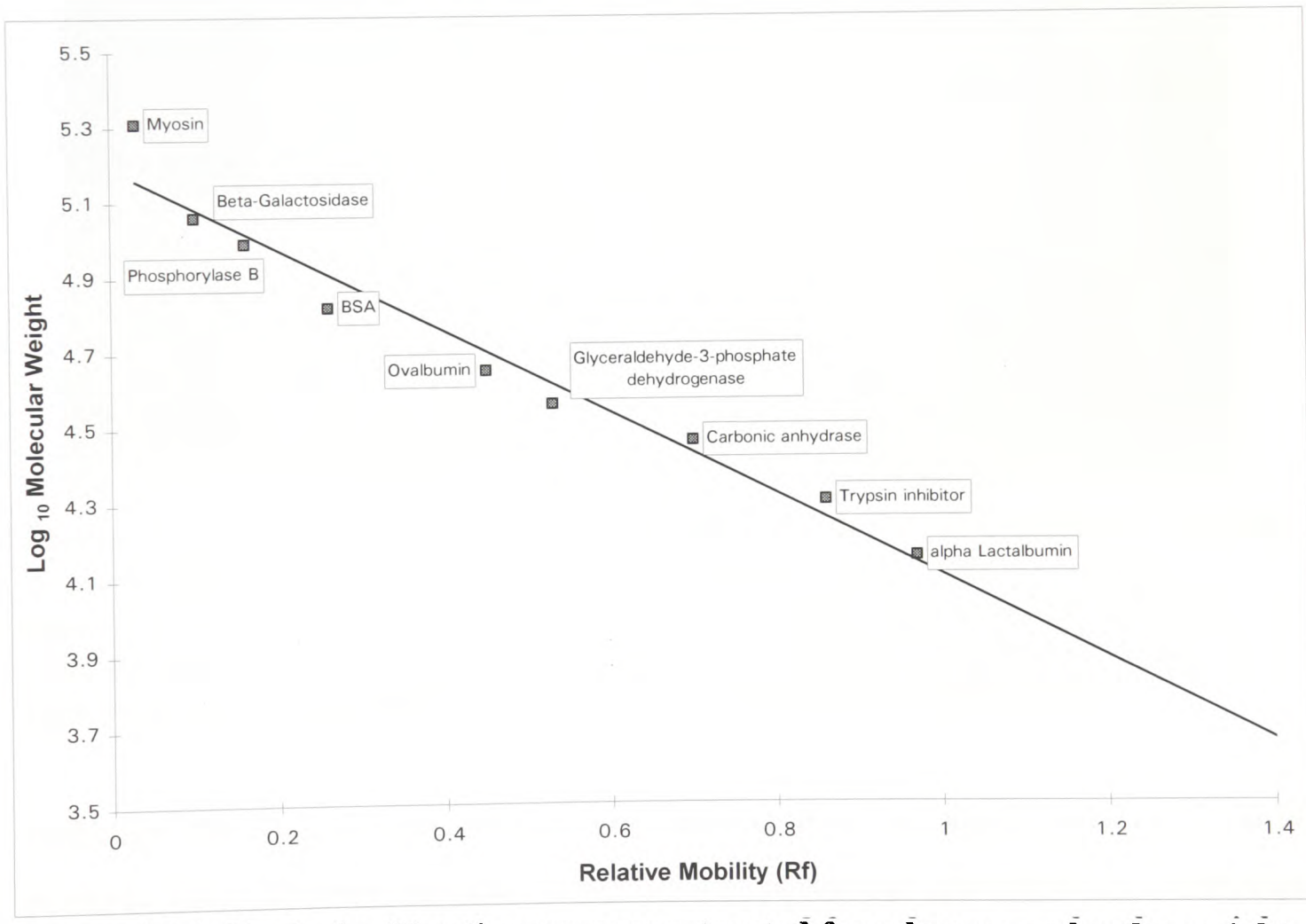


Figure A2.1: Typical calibration curve constructed from known molecular weight marker proteins (Sigma).

Appendix 3

PAGE of α-glucosidase derived from Baker's yeast (Sigma)

The purity of the post IEC purified preparation of α-glucosidase from *Lipomyces starkeyi* was compared to the commercially available α-glucosidase enzyme purchased from Sigma.

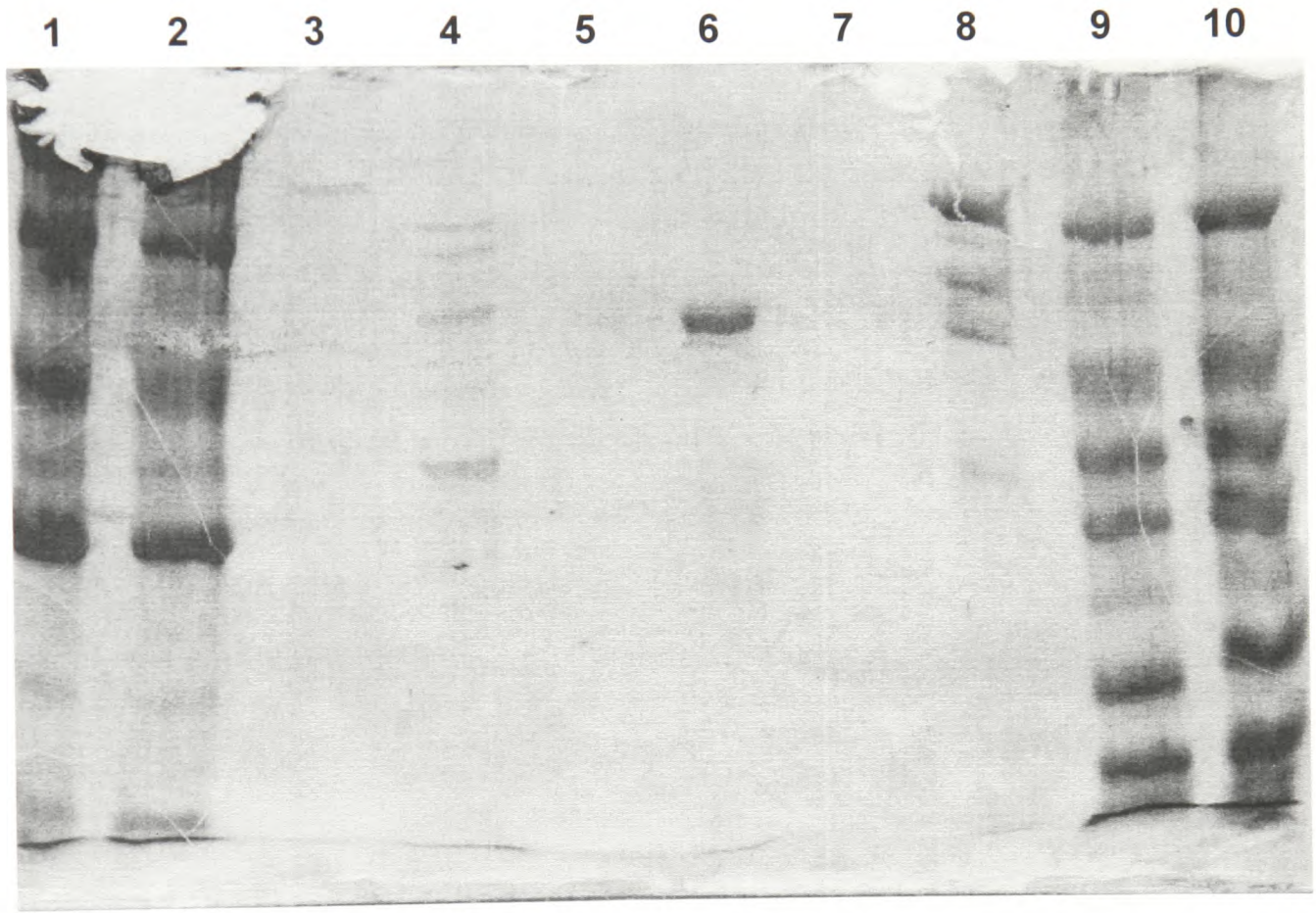


Figure A3.1: A 7.5% SDS-polyacrylamide gel. *Lane 3: post-IEC Lipomyces starkeyi α-glucosidase; Lane 4: Sigma α-glucosidase (Baker's yeast); lane 6: Sigma α-amylase (derived from Aspergillus niger); lane 8: Sigma α-glucoamylase (derived from Aspergillus niger). Enzymes were loaded at 0.1mg/ml concentration. Lane 1,2: high molecular weight markers; lanes 9,10: low molecular weight markers*

Although the Sigma α-glucosidase was derived from a fungal source and could possibly have different physical properties i.e. percentage glycosylation, molecular weight, from the α-glucosidase secreted from *Lipomyces starkeyi* NCYC 1436, it was still possible to assess how efficient the downstream purification process of the *L.*

starkeyi α-glucosidase was, by comparing the number of bands detected by SDS-PAGE in each preparation.

From the SDS gel shown in Figure A3.1 the purified α-glucosidase preparation from Sigma contains several bands (lane 4). This compares to a single band detected after ion exchange chromatography for the *Lipomyces starkeyi* α-glucosidase, (Lane 3) When the material was run under non-denaturing conditions, more than 10 bands were detected after staining with Coomassie Blue (Figure A3.2)

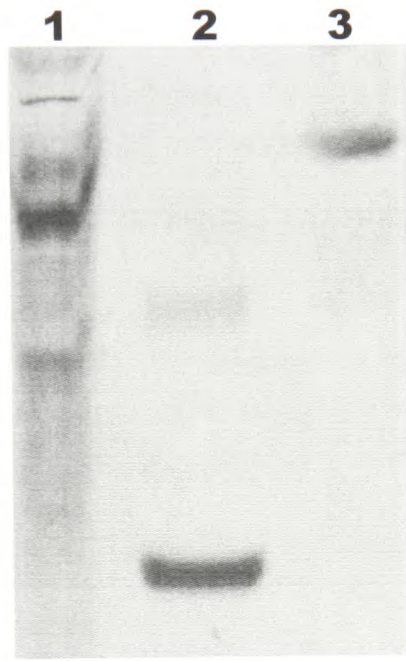


Figure A3.2: 7.5% non-denaturing polyacrylamide gel. *Lane 1: Sigma α-glucosidase (Bakers yeast); lane 2: BSA protein marker; lane 3: post-IEC Lipomyces starkeyi α-glucosidase.*

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