CHARACTERISATION OF THERMOSTABLE DEXTRANASES FROM MICRO-ORGANISMS FOR COMMERCIAL APPLICATION

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Contents

ABSTRACT

Currently, when dextranase is used in the sugar factory, modifications are required to normal factory operating conditions in order to achieve cost effective dextran 'removal'. The major change involves the decrease in primary heater temperature from 75°C to about 60°C. A number of processing problems result from this temperature change. Therefore, it was proposed to develop a thermostable dextranase which was active and stable at 75°C. The use of a high temperature dextranase would also overcome the problems associated with operating the primary heater at the lower temperature.

Existing culture collections, thermal environments and sites within raw sugar factories were used as the sources of microbial isolates screened for thermostable dextranase producers. Based on the amount of enzyme produced at elevated temperatures, five bacterial strains (SRI 2125, SRI 2128, AB11A, RT364 and DP17) were selected as the most promising sources of thermostable dextranases. These isolates were grown to pure strain using standard microbial techniques. Thereafter, broth culture of these strains was undertaken to produce sufficient crude extracellular dextranase for liquid assay. However, compared to fungal isolates, the amount of dextranase produced by the bacterial isolates was low.

Several assays to measure micro-quantities of dextranase activity were developed or modified to assess the thermal stability of the enzymes. Temperature and pH profiles of the crude extracts were determined using the PAHBAH assay to obtain a rapid and sensitive measure of optimal conditions required for enzymic activity. The subsequent development of the micro-haze test allowed the thermal stability and activity to be reliably assessed under simulated factory conditions.

Both new and existing enzymic assays were utilised to determine the activity and thermal stability of the most cost-effective commercial dextranase currently available *i.e.* the *C. gracile* dextranase. This enzyme was found to exhibit optimum activity at 55-60°C (pH 5.0) under all assay conditions. However, thermal stability at temperatures above 65°C was very low. A comparative assessment of the commercial potential of new thermostable dextranases was possible using the *C. gracile* dextranase as the 'bench mark'.

In addition, detailed studies on the *C. gracile* dextranase were performed to determine the physico-chemical characteristics of the enzyme(s) in the commercial preparation. The dextranase was separated into five distinct components by electrophoresis of the native enzyme. However, each of these components were found to possess a similar (if not identical) molecular size. Partial separation of these components was achieved using chromatofocusing. Each fraction obtained exhibited endo-dextranase activity.

Crude preparations of the five bacterial dextranases were found to exhibit optimal activity between 63 and 75°C (depending on the assay conditions and the method employed for activity measurement). The specific activities of these crude dextranase preparations were very low (0.021 to 0.68 μmole min^{-1} mg^{-1}) compared to the crude extracts obtained from fungal sources such as *C. gracile*. The presence of endo-dextranase activity was confirmed by determining the activity against cane dextran under simulated factory conditions.

The dextranase produced by the RT364 isolate was selected for purification investigations as it was the most active in the crude form. Several standard techniques for protein purification were utilised and the purity of the resulting preparations established by electrophoretic analysis. The active dextranase in the fraction of highest purity and activity was shown to represent in excess of 50 per cent of the protein in the preparation. Using this estimate, a final specific activity for the purified thermostable dextranase from RT364 was calculated to be about 20 μmole min^{-1} mg^{-1}. At this level, the specific activity of the RT364 enzyme is approximately 150 times less than that of the commercial *C.gracile* dextranase measured its optimum at temperature of 55°C. On this basis, the commercial potential of the thermostable dextranase from the RT364 isolate appears to be limited.

To date, the specific activities of purified forms of the remaining four thermostable dextranases have not been determined. It is possible that one of these isolates may be the source of a thermostable dextranase with greater commercial potential. However, further investigations with these enzymes to establish their commercial viability would require high developmental costs in relation to the very small market (the Australian sugar industry) currently available for these enzymes.

FOREWORD

The program of study described in this thesis formed an important part of a joint project between the Sugar Research Institute, the University of Queensland, and Griffith University to develop a high-temperature dextranase for use in the sugar industry.

The initial phases of the project required the development of screening methods for dextranase producing microorganisms and screening of microbial isolates from many and varied sources, in particular thermal environs. The microbiological aspects of the research were carried out by microbiologists and associated staff at their respective research institutions.

This thesis describes the biochemical characterisation of thermostable dextranases produced by the microbial isolates identified in the earlier microbial study. Initial assessment of the temperature optima, thermal stability and pH optima of the dextranases present in crude fermentation extracts of both aerobic and anaerobic thermophiles was carried out under defined conditions. Subsequently, methods were developed to allow micro-scale assessment of the crude dextranases under simulated factory conditions. A detailed physico-chemical characterisation of the most promising of the thermostable enzymes was undertaken in conjunction with a comparative analysis of the best dextranase available commercially. Preliminary investigations were also carried out to develop a purification scheme for a promising enzyme. These studies are ongoing in our group.

Future work will be aimed towards selection of dextranase-producing microorganisms which exhibit the potential for further development to the commercial stage. The selected enzyme(s) will be purified and characterised prior to cloning and expression in suitable microbial hosts. These proposed investigations would be carried out by researchers at the Centre for Molecular Biology and Biotechnology (UQ).

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LIST OF ABBREVIATIONS

AS	-	ammonium sulphate
ATCC	-	The American Type Culture Collection
CM	-	carboxymethyl
DEAE	-	diethylaminoethyl
EDTA	-	ethylenediaminetetra acetic acid (disodium salt)
HPLC	-	high performance liquid chromatography
ME	-	2-mercaptoethanol
$\bar{M}_n$	-	number average molecular weight
MW	-	molecular weight
NCIB	-	The National Collection of Industrial and Marine Bacteria
NRRL	-	The United States Department of Agriculture Culture Collection
PAGE	-	polyacrylamide gel electrophoresis
PAHBAH	-	*p*-hydroxybenzoic acid hydrazide
ppm on brix	-	parts per million on brix
RI	-	refractive index
SDS	-	sodium dodecyl sulphate
Tris	-	tris (hydroxymethyl) aminoethane

DEFINITIONS

In the course of this thesis certain terms have been used which are peculiar to the technology of the sugar industry, *e.g.* brix as a measure of solution concentration. Short definitions of some of these terms follow.

Brix : An appropriate measure of dissolved solids content derived from measurement of the density or the refractive index of the sample expressed as the percentage by weight of sucrose in a solution which has the same specific gravity (brix or °bx) or refractive index (Refractometer brix) as the sample.

Mud Filter : Mixed cane is clarified to remove suspended or insoluble impurities prior to evaporation to syrup. The 'mud solids' obtained are removed from process using a rotary vacuum filter (the mud filter).

ACKNOWLEDGMENTS

The work described in this thesis was carried out at the Sugar Research Institute, Mackay, Queensland during the period February, 1991 to July, 1992, with exception of some investigations on the *C. gracile* dextranase (Chapter 3). This work was performed prior to this time as part of the appraisal of commercial enzymes for the sugar industry.

The author gratefully acknowledges the following individuals, institutions and corporations.

Dr. P.A. Inkerman, whose vast experience with the application of enzymes to the sugar milling process led to the instigation of such an interesting and rewarding project, allowing the author the opportunity of working on the biochemical aspects for the purpose of this degree, and continuing to act as a supervisor for this thesis after leaving Sugar Research Institute.

Dr. G. Pegg for his advice and encouragement throughout the project.

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Dr. S. Hamilton and Dr. C. Wynter) and the Griffith University (Dr. B. Patel), who contributed significantly to the project "Development of a High Temperature Dextranase", particularly in the areas of isolation and screening of microorganisms and preparation of crude microbial extracts containing extracellular dextranases.

Mrs. A. Bates for her calm and uncomplaining endurance while typing this manuscript.

My husband, John, whose understanding and support helped to make this research degree possible.

PUBLICATIONS

A list of publications to date, arising from this study.

(i) Lucas, S.R., Wynter, C., Brown, C.F.*, Dawson, M., Inkerman, P.A, Patel, B., de Jersey, J., Hamilton, S. (1990). "Screening procedures for high-temperature dextranase from thermophilic bacteria". Poster Session, Aust. Biotech. Conf.

(ii) Wynter, C.V.A., Chang, M., Hamilton, S., de Jersey, J., Galea, C. and Inkerman, P.A. (1992). "Purification and properties of a thermostable dextranase". Proc. Aust. Soc. Biochem. and Molecular Biol., 24.

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* Author's maiden name was Brown.

CHAPTER 1

General Introduction

The high molecular weight polysaccharide dextran is a product of the deterioration of sugar cane, which occurs during delays between harvesting and crushing of the cane (Hidi *et al.*, 1974). The enzyme dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11) has been used successfully since the 1970's in Australian sugar factories to alleviate problems caused by the presence of dextran during the production of raw sugar (Fulcher and Inkerman, 1974b; 1976). However, the commercial fungal dextranases used for this purpose are unstable at the normal operating temperature of the primary juice heater (75°C). Reducing the temperature of the heated juice to the temperature optima of the commercial enzymes (57-60°C) results in a number of deleterious effects on processing (Inkerman, 1980). For this reason, the development of a commercial dextranase exhibiting a temperature optimum closer to the conditions used during normal processing of sugar cane *i.e.* approximately 75°C was proposed. This thesis describes work carried out to isolate, characterise and purify enzymes which possess high thermostability and which are derived from thermophilic microorganisms.

1.1 DEXTRAN

Dextran is the collective name for a large class of extracellular bacterial polysaccharides composed exclusively of the monomeric unit, α-D-glucanopyranose, and linked mainly by 1→6 glycosidic bonds (Alsop, 1983). Dextrans of varying structures have been obtained from a number of microbial fermentations and cell-

free enzyme extracts. Jeanes and co-workers (1954) isolated the dextrans produced in sucrose culture media by almost one hundred strains of bacteria. These dextrans contained from 50 to 97 per cent α,1→6 glycosidic bonds. The non-α,1→6 linkages were comprised of α,1→3 (as high as 35 per cent), α,1→4 (as high as 36 per cent) and possibly α,1→2 bonds. The molecular structure of a typical dextran molecule is shown in Figure 1.1.

The bacteria which produce dextran belong to the family *Lactobacillaceae*, but more specifically, to the genera *Lactobacillus, Leuconostoc,* and *Streptococcus* (Sidebotham, 1974). These bacteria produce an enzyme, dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase, EC 2.4.1.5), which catalyses the formation of dextran by utilising sucrose as the D-glucosyl-donor substrate. Dextran synthesis proceeds via the transfer of the glucose moiety from sucrose to the growing polymer chain (Robyt, 1979). Due to differences in the degree of branching and molecular weight, the chemical and physical properties of dextrans exhibit considerable variation.

1.1.1 Early History of Dextrans

As early as 1813, reports described the mysterious thickening or solidification of juice obtained from sugar cane and sugar beet (Jeanes, 1977). About 50 years later, Louis Pasteur explained that these "viscous fermentations" were due to microbial action. Subsequently, van Tieghem (1878) isolated and named the causative bacteria, *Leuconostoc mesenteroides* (Alsop, 1983). Finally, Scheibler (1880) established that the viscous product was a glucan which he named dextran

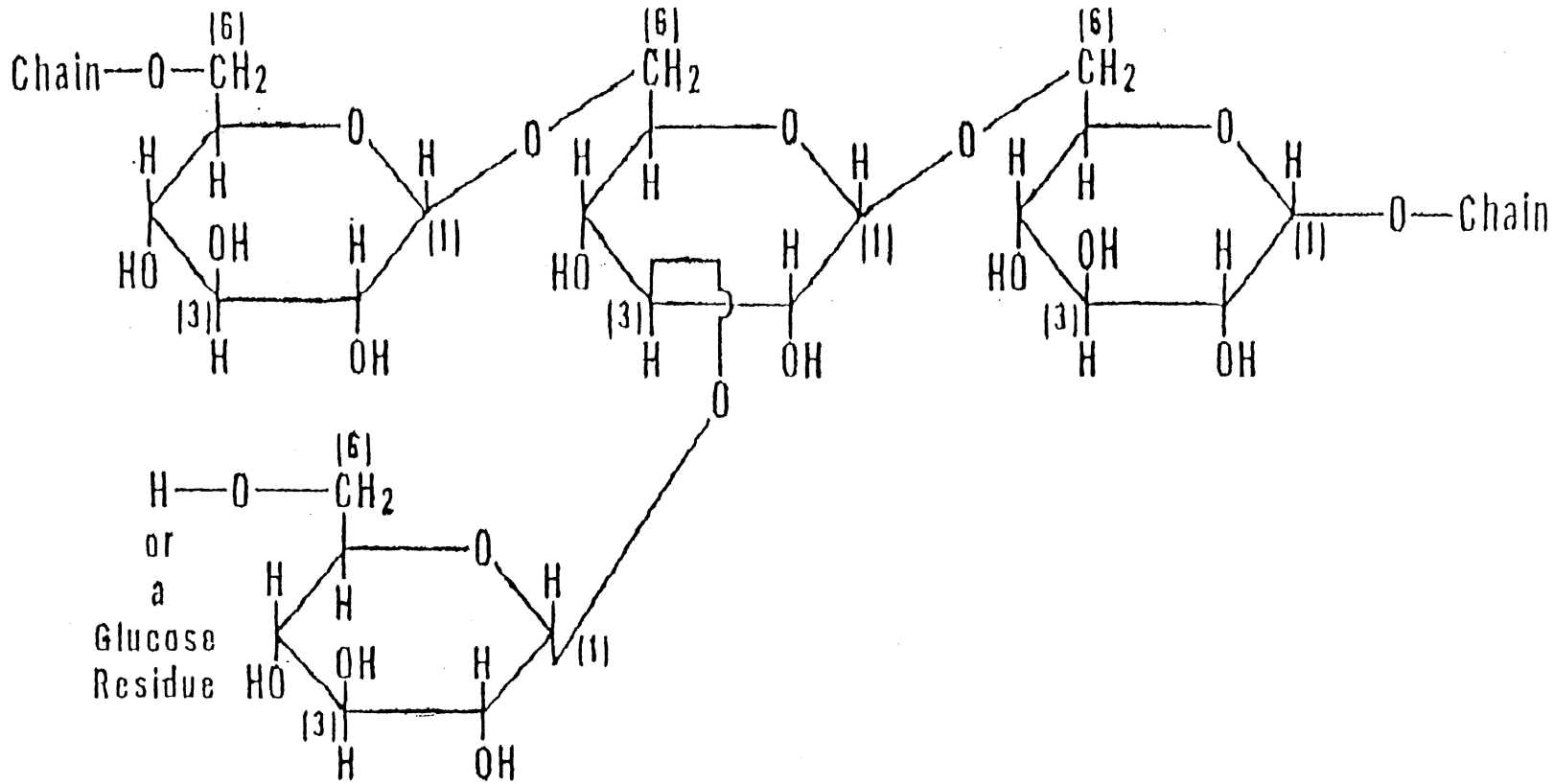


Figure 1.1 Molecular structure of a typical dextran molecule.

(Jeanes, 1977). Thus, dextrans were the first extracellular microbial polysaccharides to be the subject of systematic scientific investigation.

1.1.2 Commercial Dextrans

Dextrans from several bacterial strains were also the first extracellular microbial polysaccharides to be produced and used industrially. In 1948, Gronwall and Ingelman patented the use of clinical dextran as a plasma substitute. These small molecular weight (MW 75 000 ± 25 000) dextrans are prepared by acid hydrolysis of the native dextran produced by *Leuconostoc mesenteroides* strain NRRL B-512 (Alsop, 1983). The dextran produced by this bacterium is one of the simplest known, as the backbone consists of 95 per cent glucopyranosidic α,1$\rightarrow$6 linkages with 5 per cent α,1$\rightarrow$3 branching (Jeanes, 1977). Industrial production of dextran initiated further studies on dextran derivatives such as ethers, esters, acetates and amyl carbonates (Baker, 1959). Today, dextran and dextran derivatives are produced commercially from different strains of *Leuconostoc mesenteroides* and *L. dextranicum* mainly for use in pharmaceuticals and fine chemical production.

1.1.3 Cane Dextrans

Healthy green cane is resistant to infection by the dextran-producing bacteria (Foster *et al.,* 1980). Pre-harvest burning of cane destroys the plant's ability to resist infection. With the subsequent introduction into Australia of chopper-harvesting in the 1960's, processing problems were experienced with some cane supplies. Extensive studies by various research organisations associated with the sugar

industry demonstrated that cane deterioration was the cause of these problems (Egan, 1971). In addition, dextran was found to be the only polysaccharide produced in sufficient quantities to cause the observed processing difficulties (Keniry *et al.*, 1967; Foster, 1969; Hidi *et al.*, 1974).

The major factors influencing cane deterioration, and thereby dextran levels, are the climatic conditions during harvesting and delays both in cutting and in crushing the burnt cane (Fulcher and Inkerman, 1974a). Other factors contributing to the variability in dextran levels are the billet size, degree of mutilation of the billet and cane variety (Ivin and Bevan, 1973).

The dominant dextran-producing micro-organism present in deteriorated cane entering a sugar factory has been identified as *Leuconostoc mesenteroides* (Egan, 1971). An extensive survey has confirmed this finding within the Australian sugar industry (McNeil and Inkerman, 1977). In addition, *Lactobacillus* species were also identified as being involved in dextran production (McNeil and Inkerman, 1977; Foster *et al.*, 1980).

Independent studies (Covacevich and Richards, 1974; Blake and Inkerman, unpublished) demonstrated that cane dextrans isolated from different sources possess a similar structure *viz.* 95 per cent α-1$\rightarrow$6 and 5 per cent branching (probably α,1$\rightarrow$3 linkages). These structures are also similar to the dextran produced by strain NRRL B-512. However, detailed enzymic studies (Fulcher and Inkerman, 1976; Inkerman, 1980; Brown and Inkerman, 1982), have demonstrated that cane dextrans are hydrolysed at a slower rate compared to NRRL B-512

dextrans. This result was interpreted in terms of a different pattern of branching in these polymers.

Dextrans isolated and purified from cane juices possess a molecular weight of about 5 x 10^6 daltons, as measured by light scattering (Inkerman, 1980). A detailed examination of the data indicated the dextrans were extremely polydisperse *i.e.* the preparations consist of a wide range of molecular sizes.

1.2 DEXTRAN PROBLEMS IN RAW SUGAR PRODUCTION

The effects of cane dextran on raw sugar manufacture and raw sugar quality have been well documented (Foster, 1969; Imrie and Tilbury, 1972; Wells and James, 1976). Processing problems associated with dextran are observed when deteriorated canes are supplied to the sugar factory. Some factory operations may be affected for up to three days following an initial influx of badly deteriorated cane (Inkerman, unpublished).

The major by-products of cane deterioration are reducing sugars, organic acids, microbial biomass and dextran (Egan, 1971). Dextran accounts for only a small portion (about 5 per cent) of the total sucrose loss due to cane deterioration (RSQTC, 1987). However, dextran is primarily responsible for the major processing problems of a physical nature which reduce sucrose recovery during raw sugar manufacture. This loss of sucrose to the molasses occurs at various stages of raw sugar production and is a serious economic problem for raw sugar manufacturers. The principal physical effects of dextran on raw sugar processing are associated with viscosity, crystallisation, clarification and polarimetry (Fulcher and Inkerman, 1976).

1.2.1 Viscosity

The primary problem caused by the presence of dextran is an increase in the viscosity of the concentrated factory products such as syrups and massecuites which are already very viscous. Exhaustion trials on a series of molasses with a range of dextran levels found that the dextran effect was significant in determining the viscosity (and hence exhaustibility) of the molasses. The loss in exhaustion performance was estimated to be about 1.2-1.4 units of true purity for each 1000 ppm of dextran (on solids) present (Miller and Wright, 1977). An increased viscosity of the high brix process streams also directly affects boiling rates and purging (Greenfield and Geronimos, 1978).

1.2.2 Crystallisation

During crystallisation, some of the dextran is preferentially adsorbed onto one of the faces of the sucrose crystal (Day, 1971). Consequently, the rate of crystal growth is reduced and the shape of the crystal changed (Day, 1971). The resultant 'needle grain' or elongated crystals cause problems in separation of the mother liquor from the crystal during centrifugation (Miller and Wright, 1977). A significant decrease in growth rate and increase in elongation of these crystals are evident at dextran levels of 450 ppm on brix (Day, 1971).

Figure 1.2 illustrates the differences between normal raw sugar crystal shape and elongated crystals due to the presence of dextran. Inhibition of crystallisation slows production rates and at worst, this problem can stop the factory (Wells and James, 1976). In addition, the amount of sucrose able to be recovered from the

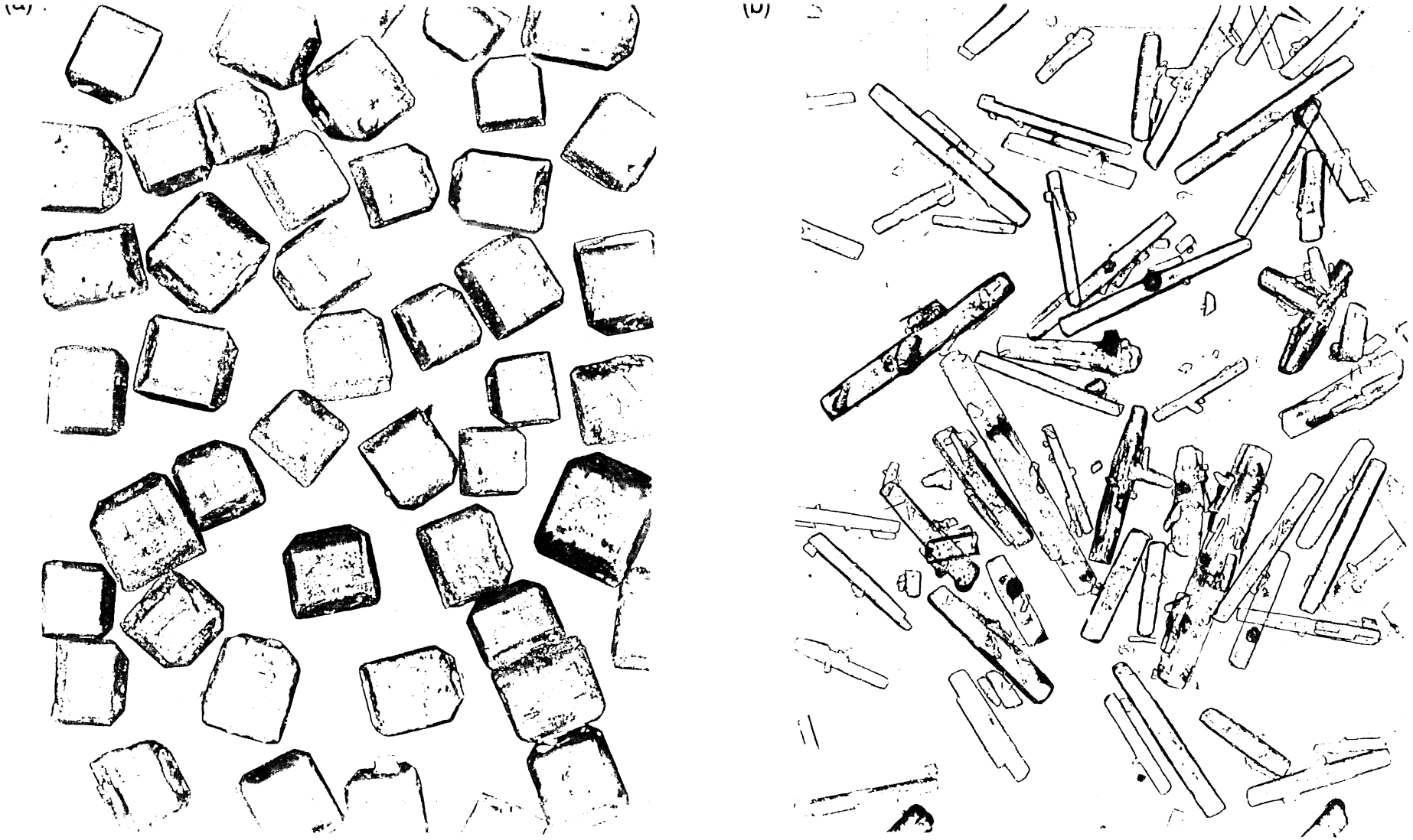


Figure 1.2. Sugar crystals obtained from burnt, chopper-harvested cane.
(a) No delay between harvesting and crushing; (b) 3 days delay between harvesting and crushing (x20).
Photos courtesy of Mr. K.F. Miller, Sugar Research Institute.

syrup during crystallisation is decreased (Miller and Wright, 1977). The additional sucrose is lost into the molasses.

1.2.3 Clarification

The efficiency of the clarification process is reduced in juices containing high levels of dextran. Poor clarification and increased juice turbidity are indicators of the presence of dextran in deteriorated cane entering the factory. Davis (1959) suggested that dextran acts as a protective colloid and inhibits flocculants and clarifiers. Poorly clarified juice results in down-stream processing problems caused by the carry over of insoluble material in normally clear juice.

1.2.4 Polarisation

NRRL B-512 type dextrans are dextrorotatory with a specific rotation ($[\alpha]_D^{20}$) of +200° (Sutherland and Paton, 1968) which is approximately three times the value of an equivalent weight of sucrose ($[\alpha]_D^{20}$ = + 66.5°). This property is important because of its effect on commercial transactions between growers, raw sugar producers and refiners. Under present arrangements, polarisation measurements are used to determine the amount of sucrose present in sugar cane juice and raw sugar. Therefore, the presence of dextran would result in an enhanced payment to both the grower and the raw sugar producer. Determination of polarisation typically involves clarification with basic lead acetate. This step has been found to remove more than two thirds of the high molecular weight dextran (110 000 daltons or greater; CSR, 1985). However, the removal of low molecular weight dextrans (40 000 daltons or less) has not been shown to be significant by this method (Dickey, 1984).

In summary, processing sugar canes containing high levels of dextran is costly for the miller due to associated factory problems and sugar losses. Dextran containing raw sugars create subsequent problems for the refiner. In most cases the amount of dextran entering the factory is kept to a minimum by efficient harvesting practices and cane transport systems. However, unforeseen delays due to weather or factory breakdowns do occur and, at such times, reduction of the incoming dextran levels by enzymic hydrolysis is a cost effective alternative (Inkerman, 1980). The cost benefits to the overall raw sugar processing operation would be further enhanced if the conditions for optimal enzyme performance matched those existing during normal process in Australian raw sugar factories.

1.3 DEXTRANASE

Dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11) hydrolyses the α-1,6 glycosidic bond of dextran, to produce low molecular weight oligosaccharides such as glucose, isomaltose, isomaltotriose and linear or branched higher molecular weight isomaltooligosaccharides. The enzyme is produced extracellularly by a number of fungi and bacteria upon induction by dextran (Janson and Porath, 1966). Since the initial report on dextranase activity from culture fluid broth from *Cellvibro fulva* (Ingleman, 1948) many papers have appeared describing endo- and exo-dextranases from microbial sources.

Microorganisms have been found which produce both exo- and endo-dextranases to ensure the rapid hydrolysis of dextran. The exo-dextranases attack the non-reducing terminal linkages of the substrate producing glucose as the only low molecular weight product (Walker, 1978). In contrast, the endo-dextranases

fragment dextrans by hydrolysing 1,6-α-glycosidic linkages within the polymer and between branch points (Walker, 1978). A sharp decrease in viscosity is produced early in the reaction, when the release of reducing groups is relatively low. To ensure the viscosity of process streams is rapidly reduced it is the endo-dextranase activity which is required for commercial application in the sugar industry.

Most microorganisms will produce dextranase only when grown in a medium containing dextran. Other known inducers of the enzyme are its oligosaccharide products. However, growth on isomaltose results in low yields of dextranase because of catabolite repression of enzyme synthesis (Walker, 1978). Oxidised dextran, insoluble dextran, or ester derivatives of isomaltose provide higher enzyme yields than soluble dextran, because the product is slowly and continuously released (Walker, 1978). A limited number of microorganisms have been found to produce the enzyme in small amounts in the absence of dextran (Hattori and Ishibashi, 1981).

1.3.1 General Characteristics of Dextranases

Most 1,6-α-glucanases (dextranases) are acidic proteins, with isoelectric points in the pH range 4.1-4.6. An exception is the dextranase from *Chaetomium gracile* which has a pI = 5.7-6.2 (Hattori *et al.,* 1981). Amino acid compositions show a high content of acidic amino acids, a relatively high content of aromatic amino acids and a low content of thiol groups (Walker, 1978). Optimal activity of dextranase is generally exhibited between the pH values of 4.5 to 6.5. Mould dextranases are characterised by a high temperature for optimal activity (50°C), with more recent fungal isolates (such as *Chaetomium gracile*) exhibiting a temperature

optimum of 55-60°C. In contrast, dextranases from bacteria *e.g. Streptococcus spp.* are rapidly inactivated at temperatures above 40°C.

Dextranases exhibit full activity without the aid of any cofactors *i.e.* they do not show metal ion dependence, and chelating agents such as EDTA do not inhibit enzyme activity. Inactivation by heavy metals has been demonstrated, particularly mercuric and cupric salts (Hattori *et al.*, 1981).

Dextranases are much smaller molecules than dextrans. Therefore, the enzyme must diffuse to a suitable site on the substrate surface for the reaction to proceed. This situation contrasts with many other enzymic reactions in which the small substrate fits into a cleft in the enzyme *e.g.* the sucrose-invertase reaction.

1.3.2 Commercial Applications for Dextranases

Dextranases have received attention because of their potential commercial applications. As described above, these enzymes have a very large-scale application in the 'removal' of dextran from process streams in sugar cane factories (Inkerman, 1980). In addition, the enzyme has been shown to be useful as an anticarie agent and is currently utilised in Japan as an additive to toothpaste (Dr. S. Minato, private communication). At present, this application is not possible for countries which rely on the Food and Drug Authority (FDA) of the United States of America to give approval of products prior to their introduction to the market.

The significance of dextran as a factor in dental caries arises from its role in the adherence and colonisation of the *Streptoccus spp.* to the tooth surface. Both the soluble and insoluble dextrans are involved in the mechanism by which the microorganisms become established in plaque. For this reason, dental research has focused on hydrolysis of the water-soluble and insoluble glucans, (Pulkownik and Walker, 1977), produced by oral streptoccal species such as *Streptococcus mutans, S. sanguis, S. sobrinus, S. cricetus* and *S. rattus.* The presence of these bacteria in dental plaque has been associated positively with the induction of dental caries (Barrett *et al.*, 1987).

Dextrans produced by oral bacteria are highly branched and in this respect differ significantly from the dextrans associated with deteriorated sugar canes. For example, native dextran produced by *S. sanguis* contains predominantly 1,6-α-glucosidic linkages (68-70 %) and a high proportion of 1,3 branch linkages (15-17 %) together with a further proportion of 1,3 linkages (14-21 %) not involved in branching (Walker, 1978). Despite these structural differences, it has been shown that both types of dextrans are readily hydrolysed by the enzyme, dextranase (Guggenheim and Burckhardt, 1974). In addition, the ability of the streptococcal cells to adhere to surfaces is significantly reduced by pretreatment with dextranase produced by a *Streptoccus sp.* (Barrett *et al.*, 1987). Treatment with commercial dextranases has been shown to destroy the dextran-induced aggregating activity of the oral bacteria (Walker, 1978).

1.3.3 Sources of Dextranases

By the 1970's, the search for dextranase producing microorganisms concentrated on fungi from the genus Penicillium. Chaiet *et al.* (1970) isolated and purified a dextranase from *Penicillium funiculosum* which exhibited a pH optimum 5 to 7 and rapidly lost activity at temperatures above 55°C. Pfizer Chemicals produced a commercial dextranase (Glucanase D-I) from a selected strain of *Penicillium sp.* A competitive enzymic product from a strain of *P.funiculosum* (Talozyme D) was produced commercially by Tate and Lyle (England). Both dextranase preparations have been utilised to some extent in the sugar industry since 1970 (Imrie and Tilbury, 1972; Tilbury and French, 1974; Fulcher and Inkerman, 1974b). Another mould dextranase (DN 25 L) produced by a different species of *Penicillium, Penicillium lilacinum,* has also been exploited commercially by Novo Industri (Denmark) since the early 1970's (Fulcher and Inkerman, 1974b; 1976).

In the search for a dextranase suitable for the treatment of dental plaque Guggenheim and Burkhardt (1974) isolated a dextranase from *S. mutans* which exhibited maximum activity at 37-40°C and pH 5.0. The conditions for optimal activity of the enzyme corresponded very closely to those experienced at the source of its isolation *i.e.* the human mouth. Thereafter, Pulkownik and Walker (1977) showed that most strains of *S. mutans* produce extracellular dextranases, in addition to the plaque forming polysaccharide, dextran. Other endo-dextranases have been purified and characterised from the genus *Cytophaga* (Janson and Porath, 1966) and from a fusobacterium isolated from dental plaque (da Costa *et al.*, 1974). Recently,

Igarashi and Yamamoto (1988) have purified and characterised multiple forms of both intra- and extra- cellular dextranases from *Bacteroids oralis* Ig4a.

Hattori and Ishibashi (1981) screened dextranase-producing microorganisms for enzymes suitable for use as anticarie agents. The screening temperature selected was 40°C, the isolation of a dextranase that was also useful in the sugar industry (at 55 - 60°C) was an unexpected bonus. Initial screening resulted in the isolation of 30 different microbial strains from 15 different genus. Further screening based solely on the amounts of enzyme produced (induced by dextran) led to the selection of only eight strains, two of which belonged to the genera *Chaetomium*. Most of the isolates produced dextranases which had a pH optima of ~5, however, varying pH stabilities were obtained. The *Chaetomium spp.* produced dextranases which were typical endo-dextranases and were substantially higher in activity than enzymes from other isolates. A commercial dextranase from *C. gracile* has been available since the early 1980's as a product of the Sankyo Company (Japan).

Overall, there appears to be a wide range of microorganisms which produce dextranases. However, despite the diversity in the microbial sources of dextranases isolated to date, none have been found which exhibit a temperature optimum above 55-60°C in defined media or sugar cane juice.

1.3.4 Commercial Dextranases

Currently, a number of dextranases from fungal sources are available commercially for analytical and industrial applications. An analytical bacterial

dextranase from a strain of *Bacillus coagulans* (Taylor *et al.*, 1990) was produced by Calbiochem, but it is no longer available.

Major industrial sources of dextranase are species of *Penicillium funiculosum* (Tate and Lyle); *Penicillium lilacinum* (Novo; DN L25 and DN L50); *Chaetomium gracile* (Sankyo, DL-2; Solvay (Miles), Dextranex L 4000 and Amano (Pfizer), Dextranase L). The Pfizer glucanase D-I has been superseded by a new Pfizer product from a *Chaetomium* species. These enzyme preparations have approval (under British regulations) for use in early stages of food processing (FACC, 1980). However, as it is possible that these fungi might also produce various antibiotics and toxic metabolites, these enzyme preparations do not have FDA approval for application to food processing in the U.S.A.. To address this problem an application has been lodged with the FDA to obtain approval for the use of the industrial enzymes in food production in the U.S.A (Dr. S. Minato, private communication).

The problems associated with FDA approval led Koenig and Day (1988) to isolate and purify a dextranase from an *ascosporogenous* yeast, *Lipomyces starkeyi*. This yeast is not known to produce antibiotics or toxic metabolites and has been used in food related applications. These factors increase the possibility of FDA approval being granted for a dextranase produced by this microorganism. The *Lipomyces* dextranase exhibited a temperature optimum of 50-60°C when assayed for dextran hydrolysis in a refined sucrose/dextran mixture. However, the temperature optimum for this dextranase measured in cane juice (Koenig and Day, 1988) was only 30-40°C (about 15°C lower than the values for the *Penicillium* and *Chaetomium* dextranases). Therefore, the enzyme from *Lipomyces* does not appear to possess the necessary thermal stability for application in the sugar industry.

These authors proposed that the presence of impurities in the cane juice may contribute to the observed shift in temperature optimum. To date, no supporting evidence for this statement has been published.

1.4 INDUSTRIAL USE OF DEXTRANASES IN THE SUGAR FACTORY

1.4.1. The Australian Experience

The Australian raw sugar industry has always endeavoured to minimise and control dextran at its source, by implementing harvest and transport controls which reduce cane deterioration. However, lengthy delays due to unforeseen weather conditions or factory breakdowns result in post-harvest cane deterioration and dextran entering the factory. Early attempts to 'remove' dextran from process streams in Australian factories were not successful (Hidi and Staker, 1975). However, an efficient enzymic procedure was developed during the mid 1970's in which dextranase was used in Australian raw sugar factories to hydrolyse dextran (Fulcher and Inkerman, 1974b; 1976; Inkerman,1980).

The procedure entailed incubating mixed juice with the dextranase at 60°C for 30-40 minutes at the natural pH of the mixed juice (Fulcher and Inkerman, 1974b). Considerable benefits in factory 'performance' and sugar quality were gained from the process (Inkerman, 1980). Though the turbidities of clarified juice are significantly improved, the filterabilities of raw sugars produced from deteriorated cane juice are lower in value (5 - 10 units) than the raw sugars produced from fresh cane juice of the same initial purity. Other areas of improvements in processing were the exhaustibility of molasses, crystallisation rates, purging times, the viscosity

of high-brix factory streams and the reduced fouling of heating surfaces (Inkerman and James, 1976).

Hydrolysis of the dextran to a molecular weight of less than 10^4 (designated 'complete removal') produces a marked reduction in the percentage of elongated sugar crystals. In comparison, 'partial removal' at high incoming dextran levels can result in severe problems with crystal elongation and fine grain, although other parameters such as viscosities of high-brix factory products are improved (Inkerman, 1980).

The normal method of enzyme addition is at an initial constant rate in excess of the predicted requirements. This addition rate is based mainly on the residence time available in the mixed juice tank and adjustment is made at a later stage once the incoming and residual dextran levels have been determined by analysis (Inkerman and Riddell, 1977). A more economical method which utilises the formation of acid by the dextran-producing bacteria as an instantaneous monitor of incoming dextran levels was proposed by Inkerman *et al.* (1983). An automatic control system uses variations in the juice pH to enable the correct dosage of dextranase to be added instantaneously, thereby overcoming some of the problems associated with usual methods of enzyme addition. Though some shortcomings still exist, especially in the pH range associated with fresh cane, the application of the system under factory conditions results in a more efficient and cheaper process for the enzymic hydrolysis of dextran in deteriorated cane juice (Inkerman, 1984).

Hence, the use of dextranase has been a viable process in Australian sugar factories during limited periods of processing deteriorated cane. The process is

represented diagrammatically in Figure 1.3. However, the temperature optima of the currently available commercial dextranases restricts the upper limit of the incubation temperatures for hydrolysis of dextran in the factory to 57-60°C, some 15°C below normal operating temperature. Therefore, some prior warning of the possibility of deteriorated cane entering the factory is necessary in order that the appropriate temperature adjustment can be made and to allow the enzyme addition system to be set up. Failure to do so results in dextran entering into process. The lack of treatment of deteriorated cane juice appears to be the major cause of high levels of dextran found in Australian raw sugars.

When dextranase is used in the sugar factory, some modifications are necessary to normal factory operating conditions in order to achieve cost effective dextran 'removal'. As mentioned above, the major change involves the decrease in primary heater temperature from 75°C to about 60°C. Unfortunately, at the same time, a number of processing problems result from this temperature change. These are a disruption to the overall routine operation of the factory; a continual loss of sucrose at the lower temperature due to microbial action; a time delay (as the temperature is reduced from 75°C to 60°C) before the dextranase can be added allowing untreated dextran into process with its subsequent deleterious effects both on sugar quality and processing efficiency; an additional cost is incurred for extra commercial α-amylase required in the evaporators to control the high starch levels resulting from the decreased efficiency of starch hydrolysis by the naturally occurring α-amylases in the incubators at 60°C compared to 75°C; and finally the efficiency of clarification is also affected by the lower incubation temperature which again results in lower sugar quality.

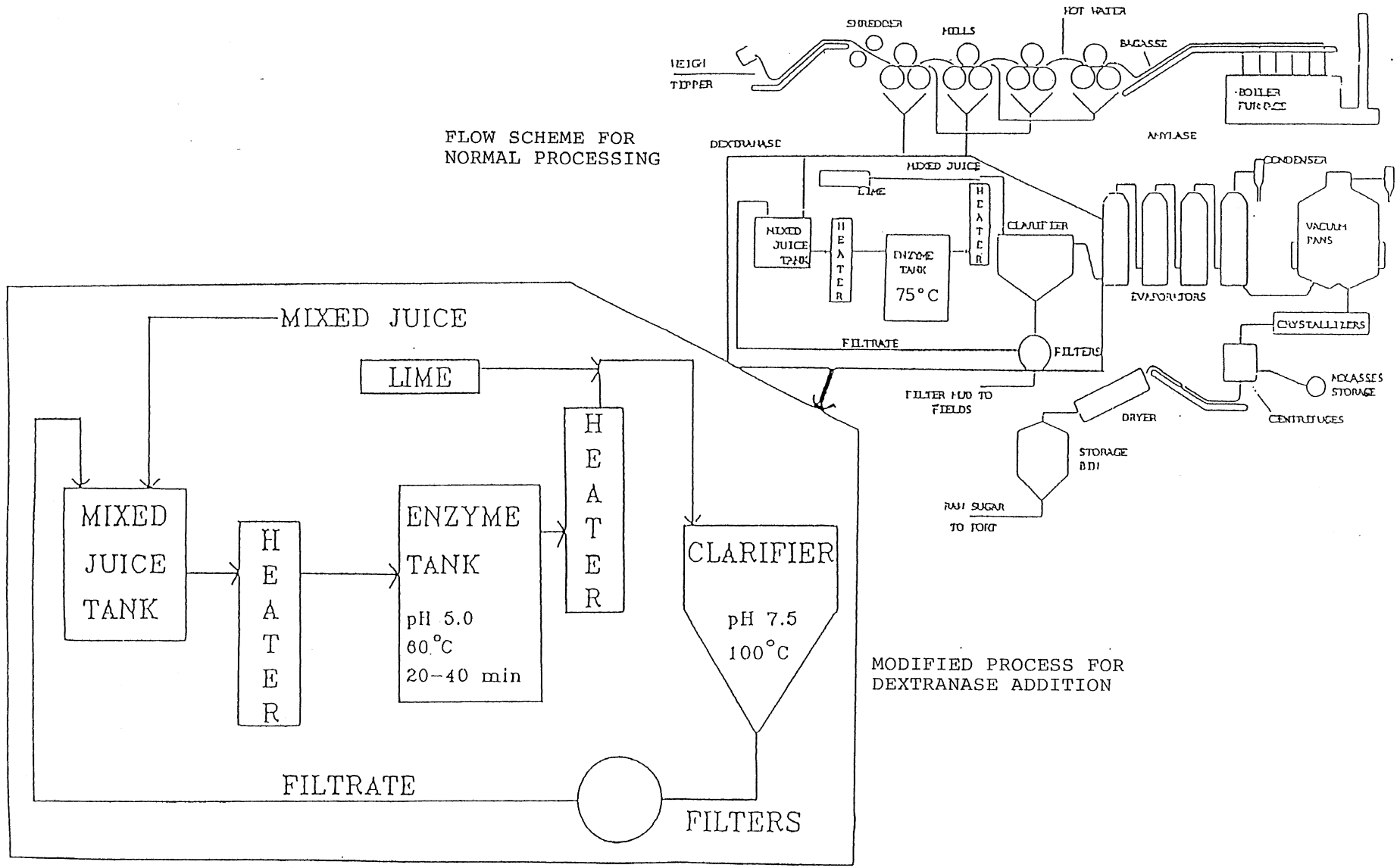


Figure 1.3. Diagrammatic representation of the dextranase process in an Australian sugar factory.

Furthermore, tight control of primary juice heater temperature is essential in order to minimize denaturation of the enzyme as the dextranase is rapidly denatured above 60°C.

Alternative dextranase addition sites in the factory have been investigated. Hidi and Staker (1975) compared enzymic removal of dextran from juice, syrup and molasses under process conditions. Their results indicated that about the same total dose of enzyme can be used to treat any process stream under the best practical mill conditions. This assumes that a compromise is obtained between the ideal and real values of the parameters (brix, temperature and pH) for optimum enzyme activity in the different process streams.

In contrast, Fulcher and Inkerman (1974b) found that the best site in the factory process to add dextranase was to the mixed juice before it was subject to clarification. After this process, the effectiveness of the enzyme would be compromised by such properties of the medium as pH (>7.0), sucrose concentration (>40 brix) and temperature (> 60°C). Further studies (Inkerman and Fulcher, 1976) showed that the addition of dextranase to mixed juice was more favourable than syrup (or other process stream) by a factor of in excess of 100. Similar values have been reported recently by Galea and Inkerman (1991). These authors found that low residual dextran levels were obtained in syrups (70 brix, pH 7.5) only after prolonged incubation (4 hr) at very high enzyme concentrations. In comparison, a similar residual dextran level can be achieved at pH 5.0 in deteriorated cane juice (18 brix) after an incubation time of only 30 minutes using an enzyme concentration about 100 times less (Figure 1.4). The 'enzymic rate' estimated from this data is

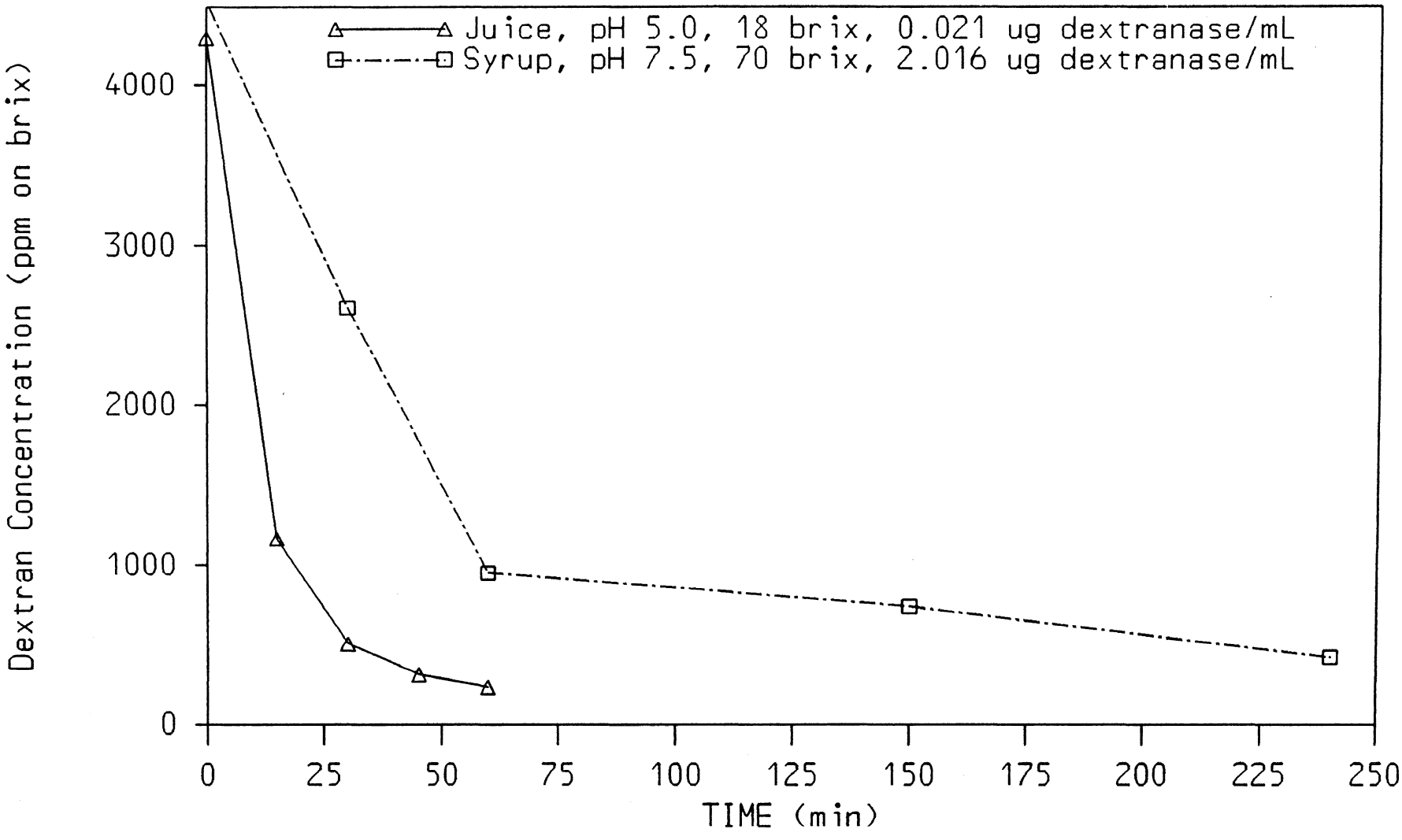


Figure 1.4. Progress curves for dextranase-catalysed hydrolysis of cane dextran in deteriorated juice and syrup at 60°C and 65°C, respectively (from Galea and Inkerman, 1991).

approximately 200 times the value obtained in syrup (calculated from the respective enzyme concentrations, the respective incubation times and the different volumes of juice and syrup to be treated).

The chemo-physical properties of commercially available dextranases governed the selection of the mixed juice as the point for dextranase addition, and the temperature alteration to normal process required for efficient use of the enzyme. Although, the temperatures at which these dextranases are currently operating are comparable with most other industrially thermostable enzymes (with exception of the α-amylase derived from *Bacillus lichenoformis*) they are not the normal process conditions in a raw sugar factory in Australia. Therefore, interest has developed in isolating a thermostable dextranase which is active and stable at 75°C. Such an enzyme could be added into process under normal operating conditions without any disruptions to subsequent unit operations.

1.4.2 The Overseas Experience

Research workers from a number of countries have studied the possibility of dextranase addition into process to overcome the problems caused by dextran. Early attempts in the West Indies were unsuccessful (Tilbury, 1971).

As stated above, the juice stream has the most appropriate pH for enzyme addition and is the best site for temperature adjustment in Australian sugar factories. Use of this point for enzyme addition leads to practical problems for most overseas factories as these processes, as currently operated, do not achieve sufficient residence time in the mixed juice stream. This problem could be overcome by either

modification of the process to include large mixed juice tanks, as is the case in Australia, or selection of the next most cost-effective site for enzyme addition.

Workers from outside Australia have found that factory juice clarification removed variable amounts of dextran (75-85 per cent, Jolly and Prakash, 1987; 40-70 per cent, De Stephano, 1988) and that soil present in the mixed juice may contain enzymic inhibitor (Madhu *et al.*, 1984). Therefore, treatment of unclarified juice may be 'wasteful' and the inclusion of mixed juice tanks into process was considered unnecessary. The best alternative, enzyme addition to syrup, was the preferred option. The data on removal of dextran by factory clarification has not been substantiated by factory data collected under Australian conditions nor has any inhibitory effects of soil been documented.

The heat stability of dextranases (*Penicillium spp.*: Inkerman and Fulcher, 1976; *Chaetomium gracile*: Hattori and Minato, 1985) has been shown to increase markedly with sugar density. In a refined sucrose syrup (65 brix), the *Chaetomium* enzyme was most active at 75-80°C and stable at 80°C for at least 60 minutes (Hattori and Minato, 1985). In comparsion, in a refined sucrose juice (20 brix) the dextranase was most active at 50-60°C. However, the relative dose rates for the refined syrup was of the order of 40 times that required in the refined juice for removal of equivalent levels of dextran. These results were obtained using an ideal system.

De Stephano (1988) showed that the *C. gracile* dextranase rapidly lost activity at temperatures of 60°C or above (10-30 min) in syrups of 65 brix. He also carried out a comparison of the activity of the enzyme in process juice (16.2 brix,

50°C) and syrup (60 brix, 65°C) for a constant time (30 minutes). The results showed that a dextranase level of over 10 times the amount used in juice was required in syrup for dextran removal. However, at syrup brix of 60 rather than 65 the viscosity of the solution would be significantly lower, and hence more favourable for enzymic reactions. Based on his results, De Stephano considered dextranase addition to syrup to be a viable alternative to juice because clarification had produced a 'cleaner' solution. Also, the volumes to be treated were smaller and the necessary incubation times were available at that stage of the process.

Large scale factory trials on the effectiveness of dextranase were carried out in Jamaica and Trinadad (Menzes, 1988). In these factories because of the lack of residence time in the mixed juice tank and 'the more favourable pH of syrup' (contrasting with Australian conditions where juice pH is more favourable for dextranase activity than syrup pH) the latter was selected as the enzyme addition point. The addition of *C. gracile* dextranase for the reduction of dextran levels in syrup and subsequent liquors was evaluated. All factors compared for the trial period were better than the control period *e.g.* tonnes of high grade sugar produced per day increased by over 28 per cent. Other savings included a reduction of molasses recycle in magma streams, increased exhaustibility of final molasses and a reduction in maintenance and fuel demand. However, while the benefits of dextranase addition were adequately demonstrated by this trial, a cost-benefit analysis was not determined.

Based on results obtained in Australia and overseas, it is apparent the selection of the addition point for dextranase depends on the factory process

conditions, the economics of enzyme addition and the characteristics of the enzyme selected for factory application.

To date, only in Australia has dextranase addition (to juice) been demonstrated to be a cost-effective process on a commercial scale. For example, figures from one Australian factory show that controlled dextranase addition results in a cost-benefit of a factor of 10 (Inkerman and Riddell, unpublished). This figure is derived from gains obtained from the production of extra sugar from increased molasses exhaustablilty and does not include gains obtained from other improvements in factory operations or raw sugar quality (L. Riddell, private communications).

1.5 THERMOSTABLE ENZYMES

Enzyme thermostability can be defined as the retention of activity after heating to temperatures of 60°C or above for a prolonged period (Ng and Kenealy, 1986). Currently, most thermostable enzymes used commercially have been derived from mesophiles. For example, *Bacillus lichenoformis*, which has an optimum growth temperature of 37°C, produces a highly thermostable α-amylase with a temperature optimum of approximately 95°C or higher in starch slurries (Krishnan and Chandra, 1983) and 75-85°C in the presence of low starch levels in mediums such as sugar cane juice or syrup (Saito, 1973). However, thermophilic bacteria are potentially the best sources of thermostable enzymes. This is due largely to the fact that thermophilic microorganisms must possess intrinsically thermostable cellular components. Thermophilic organisms, especially sporeformers, have been shown

to produce a large variety of extracellular thermostable enzymes, though in varying amounts (Brock, 1986).

There are many industrial applications for thermostable enzymes. Some of the key industrial thermostable enzymes and their uses are listed in Table 1.1. In addition to exhibiting the characteristics required, enzymes to be used on an industrial scale have to meet other criteria if they are to gain wide acceptance. These criteria are that the enzymes have to be produced economically and in large quantities, be easily recoverable (preferably extracellularly), and be produced by a microorganism with low cultivation cost.

TABLE 1.1 APPLICATION OF KEY INDUSTRIAL ENZYMES (from Ng and Kenealy, 1986)

Operating Enzyme	Temperature (°C)	Major application
Carbohydrases		
α-amylase (bacterial)	90-110	Starch hydrolysis, brewing baking, detergents
dextranase	55-60	Dextran removal
glucoamylase	50-60	Maltodextrin hydrolysis
α-amylase (fungal)	50-60	Maltose
pullulanase	50-60	High glucose syrups
xylose isomerase	45-55	High fructose syrups
pectinase	20-50	Clarification of juices/wines
cellulase	45-55	Cellulose hydrolysis
lactase	30-50	Lactose hydrolysis, food processing
Proteases		
acid proteases	30-50	Food processing
fungal proteases (neutral proteases)	40-60	Baking, brewing, food processing
alkaline proteases	40-60	Detergent
Lipases	30-70	Detergent, food processing

The upper temperature limit for most of these industrial enzymes is 50-60°C, with the exception of α-amylase from *Bacillus licheniformis* with an operating temperature of 90-110°C. The dextranases currently used in the cane sugar industry are thermostable industrial enzymes. However, for Australian raw sugar factories to operate under normal processing conditions a dextranase with an optimal temperature 15°C higher (*i.e.* 75°C) is required.

1.5.1 Sources of Thermostable Enzymes

Thermostable enzymes can be categorized into two groups according to their sources, those derived by natural selection and those created by physical or chemical modification of thermolabile enzymes.

1.5.1.1 Natural Sources

As stated above, the majority of thermostable enzymes currently used in industrial applications are produced by mesophiles. Normally, this type of thermostable enzyme has been obtained by extensive screening and subsequent strain improvement.

The thermophiles as a group have failed to gain a higher share of the market for several main reasons (Ng and Kenealy, 1986). Firstly, thermostable enzymes from thermophiles have less activity than their mesophilic counterparts under similar conditions at elevated temperatures. Secondly, thermophilic bacteria grow at a much slower rate than mesophiles, thus producing lower amounts of enzymes (Ng and Kenealy, 1986). The thermophilic bacteria are also more difficult to 'handle' as many

are strict anaerobes requiring specialised equipment to carry out culture and growth manipulations. Finally, the maximum temperature of operation is not the only criterion for selection of an "effective" industrial enzyme.

Despite these drawbacks, thermophiles may still be good sources of thermostable enzymes since the yield and the cost of cultivation can be lowered by strain manipulation *e.g.* gene cloning and expression into an organism such as *Escherichia coli* (Kristjansson, 1989). This source of enzymes will expand as more novel microorganisms from extreme environments are discovered and investigated.

1.5.1.2 Induced Thermostability

Thermostability can be induced by association with stabilizing non-protein compounds or physico-chemical modifications. Such enhancement includes interactions with substrates, solvents and salts as well as chemical modifications.

Enzymes are generally more stable when they are complexed with substrates or co-factors than in the free form. Thus, glutamate synthetase from *B. stearothermophilus*, which normally loses its activity at 65°C, is stable in the presence of NH_4^+, glutamate, Mg^{2+} and ATP. It is even stabilized by feed back inhibitors such as alanine and histidine (Wedler and Hoffman, 1974).

An example of substrate exploitation for enzyme stabilisation can be found in the starch industry (Godfrey and Reichelt, 1983). High substrate concentrations are employed throughout the starch hydrolysis process and with the added stabilizing

effect of calcium, α-amylase from *B. licheniformis* continues to function at temperatures above 100°C.

The forces that stabilize a protein are derived from the interaction of the protein with the solvent. Some organic solvents e.g. polyhydroxyl alcohols have been observed to enhance thermostability (Schmid, 1979). Glycerol, sucrose and ethylene glycol are generally used to stabilize enzymes during storage. Most salts have deleterious effects on enzyme stability, however, enzymes stored in 3 M ammonium sulphate are known to be very stable.

Chemical modification of the enzyme to enhance thermostability involves alteration of the protein surface by chemical reagents or covalent linkage to polymeric materials. The general effect of such modifications is to provide extra energy of stabilization which varies from enzyme to enzyme (Schmid, 1979).

1.6 THERMOPHILES

A thermophile is an organism capable of growth at high temperature. However, there is no universally accepted temperature limits, hence, no universally accepted definition of the term thermophile. Therefore, for the purpose of this thesis the definitions utilised by Sonnleitner (1983) will be employed. This author defines thermophily of microorganisms using the generally accepted categorization of thermophiles into several classes *i.e.* thermotolerent, thermophilic and extreme thermophilic species based on arbitrarily chosen cardinal temperatures as shown in Table 1.2.

TABLE 1.2. DEFINITION OF THERMOPHILY BY MEANS OF CARDINAL TEMPERATURES (from Sonnleitner, 1983).

Technical term	Cardinal temperatures (°C)		
	minimal	optimal	maximal
Thermotolerant	<30		
Faculative thermophilic		<45	>45
Moderate thermophilic			<70...75
Thermophilic	≥40 >30	≥55 >50 ≥45	≥65 >60 >55 >45 but <70
Extremely thermophilic or 'caldoactive'	≥40	≥65	≥70 (90?) >70...75 ≥65

The temperatures listed in Table 1.2 are for growth not survival. Many thermophilic bacteria are capable of forming spores and can tolerate temperatures much higher than the temperatures at which they can grow. For the purpose of enzyme production it is the temperature range over which a bacterium is able to maintain a population which is important (Brock, 1986). A wide variety of genera have thermophilic representatives. In recent years, a number of new and interesting thermophilic bacteria have been isolated and characterised. Many of these have proven to be of considerable interest to both the molecular biologist and biotechnologist. A number of recent publications (Hyun and Zeikus, 1985a; 1985b; Saha *et al.,* 1988; Mathupala *et al.,* 1990) detail the isolation of thermostable enzymes from thermophilic microorganisms.

1.6.1 Thermostable Enzymes from Thermophilic Microorganisms

Hyun and Zeikus (1985a) isolated an anaerobic thermophilic bacterium which produced a thermostable pullulanase and glucoamylase. The organism was identified as a strain of *Clostridium thermohydrosulphuricum* which grew readily at a temperature of 65°C. The pullulanase and glucoamylase exhibited optimal activities at 85°C and 75°C, respectively. Co-cultures of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum* growing on a starch medium lead to enhanced production of ethanol and a thermostable amylase (Hyun and Zeikus, 1985b).

A highly thermostable pullulanase from *Clostridium thermohydrosulphuricum* (mutant Z 21-109) was purified and studied (Saha *et al.*, 1988). Characterisation of a cell-bound cyclodextrinase with a temperature optimum of 60°C and a cell-bound α-glucosidase with a temperature optimum of 75°C from *C. thermohydrosulfricum* 39E have been reported (Saha and Zeikus, 1990;1991). Using starch as the substrate, Mathupala *et al.* (1990) isolated a highly thermostable pullulanase activity from this bacterium.

A bacterial glucoamylase has been purified and characterised from the anaerobic thermophilic bacterium *Clostridium thermosaccharolyticum* (Specka *et al.*, 1991). This enzyme attacks α-1,4 and α-1,6 glycosidic linkages and exhibits an activity optimum at 70°C and pH 5.0.

To date, a limited number of reports have been published on thermophilic fungi which produce thermostable enzymes. However, Christakopoulos *et al.* (1990)

have described the isolation of thermostable α- and β- galactosidases from the thermophilic fungi, *Aspergillus niger*.

1.6.2 Cloning and Gene Expression

Improved yield and reduced production costs for a number of thermostable enzymes from thermophiles have been reported after cloning and expression of the enzyme by mesophilic microorganisms. For example, a thermostable pullulanase from a new isolated strain of *B. stearothermophilus* was cloned and expressed in *Bacillus subtilis* (Kuriki *et al.*, 1988). This enzyme hydrolyses the α-(1→6)-D-glucosidic linkages of pullulan and exhibited a temperature optimum of 65°C. Sen and Oriel (1989) demonstrated that elevated levels of extracellular secretion of α-amylase were obtained through cloning of the α-amylase of *B. stearothermophilus* into *E. coli*. *B. sterothermophilus* is a poor producer of a thermostable alanine dehydrogenase. The cloning and expression of the enzyme from the *Bacillus sp.* (an anaerobic thermophile) in *Escherichia coli* was reported by Sakamoto *et al.* (1990). Enzyme productivity was enhanced 60-fold by gene cloning.

Of relevance to the project being described herein was the work by Okushima *et al.* (1991) on the molecular cloning of the Arthrobacter dextranase gene and its expression in *E. coli* and *Streptococcus sanguis*. Unfortunately, the enzyme possessed a temperature optimum of 45°C *i.e.* well below current commercial dextranases (60°C) and even further from the proposed temperature optimum for raw sugar factory dextranases (75°C). This dextranase exhibits characteristics which would appear to be more suitable for application in the prevention of dental caries than for use in the sugar industry.

1.7 AIMS OF THE PRESENT STUDY

The experimental work described in this thesis involved the assessment of activity and thermostability of dextranases in crude fermentation media of aerobic and anaerobic microorganisms. Physico-chemical characterisation of the selected enzyme(s) would be carried out and purification attempted. The commercial dextranase from *C. gracile* will also be characterised to enable an assessment of the commercial potential of the novel thermostable dextranases. Should suitable enzymes be obtained, a further program of study would be required. The aim of that study would be to take the dextranases which exhibit the characteristics required for commercial application and clone them into suitable microbial hosts (*e.g. E. coli* or *B. subtilus*).

CHAPTER 2

Development of Assays for Assessment of Dextranase Activity

2.1 INTRODUCTION

Dextranases (EC 3.2.1.11) isolated from microbial sources exhibit either endo- or exo- activity against the $\alpha,1\rightarrow6$ glycosidic bonds of the dextran molecule. The endo-enzyme randomly hydrolyses glycosidic linkages within the dextran molecule, resulting in a rapid reduction in molecular weight. In contrast, the exo-enzyme hydrolyses only the non-reducing end terminal glycosidic linkages producing glucose as the only low molecular weight product. The molecular weight of the residual dextran polymer is relatively slowly reduced by this 'step-wise' process.

Assays for dextranase activity have been described which are based on the determination of the liberated reducing sugars by iodometric titration (Tsuchiya *et al.*, 1952) or by the 3,5-dinitrosalicylic acid reagent (Miller, 1959; Janson and Porath, 1966) and other sensitive reducing sugar assays (Lever, 1972; 1973; Plant, 1987). Measurement of the increase in reducing power during the hydrolytic reaction gives a quantitative assessment of the exo-dextranase activity. These methods also detect endo-dextranase action, however, activity is generally under-estimated because the resultant polymers and oligomers exhibit a lower response to the reagents than glucose, the usual reducing sugar standard. Despite these limitations, the use of reducing sugar assays for determination of dextranase activity, (even endo-dextranase activity) has become common place because of simplicity and rapidity (Janson and Porath, 1966; Hidi and Staker, 1975). To assess dextranase

activity reliably under factory conditions, *i.e.* in the presence of high concentrations of sucrose and in solutions containing impurities which also exhibit reducing power, the reducing sugar methods are of limited use. However, the sensitivity offered by these methods suggests that they could be employed for preliminary screening procedures.

Significant improvement in factory performance has been noted when either complete (MW below 10 000 daltons) or partial 'removal' of dextran (Inkerman and James, 1976; Inkerman, 1980) from sugar cane juice was achieved. These observations confirm that it is the large size of the cane dextran (MW ~5×10^6; Inkerman, 1980) which has the most deleterious effects on the processing efficiency of the factory as well as on the product quality (Imrie and Tilbury, 1972). Hence, it is the endo-activity of a dextranase which is of greatest importance in the sugar factory as this activity leads to a rapid decrease in the molecular weight of the dextran.

Methods specific for assessment of endo-dextranase activity require the measurement of dextran levels and the rate of dextran hydrolysis. The reaction can be followed by measuring changes in some physical property of the solution, such as viscosity (Geronimos and Greenfield, 1978). However, viscometric techniques are very slow and require large volumes of solution for each determination. Hence, these procedures offer little in the way of rapidity, simplicity and sensitivity.

Only the alcohol-haze method (Nicholson and Horsley, 1959; Keniry *et al.*, 1969) has been used on a routine basis within the Australian sugar industry for measurement of dextran in factory products. This method has also been utilised to

directly assess the 'removal' of dextran from first expressed juice in factory trials (Fulcher and Inkerman, 1974b; Inkerman and James, 1976; Inkerman and Riddell, 1977). The concentration of dextran in sugar cane juice was obtained by spectrophotometric measurement of the haze produced and the dextranase activity determined by following the reduction in the dextran haze.

The alcohol-haze method is based on the measurement of the haze developed in 50 per cent ethanol following enzymic removal of starch, elimination of interfering salts by use of ion-exchange resin and the precipitation of proteinaceous compounds by trichloroacetic acid. An enzyme step may be incorporated into the methodology to give a practical assessment of dextranase activity under simulated factory conditions (Fulcher and Inkerman, 1974b; Brown and Inkerman, 1990).

Alternatively, a more rapid colorimetric test has been used over the past decade to measure the activity of commercial dextranases in the presence of sucrose. The test is designated the Blue Dextran assay (Koh and Khouw, 1970; Brown and Inkerman, 1989; 1992). The technique involves the exploitation of the high molecular weight polymer, Blue Dextran 2000 (a dye-conjugate dextran used in calibration of gel filtration columns), as the substrate. Dextranase catalyses the hydrolysis of the polymer to smaller molecular weight fragments. Residual polymer fragments are separated from the soluble dye by precipitation with two volumes of ethanol. The absorbance of the resulting blue coloured solution is a quantitative measure of the endo-dextranase activity.

In summary, the commercial potential of a dextranase for use in the sugar industry is dependent on the endo-activity exhibited under factory conditions.

Currently, there are two assays routinely used which give a specific measurement of endo-dextranase activity under factory or simulated factory conditions. However, the concentrations of extra-cellular dextranases produced by microorganisms have been reported to vary widely (Hattori *et al.,* 1981). Preliminary results suggested that the amount of enzyme produced by most of the isolates under study would not be sufficient for quantitation by either the alcohol-haze method or the Blue Dextran test described above *i.e.* the macro-scale procedures. Therefore, several micro-scale methods were developed for assessment of dextranase-producing microorganisms and enzyme characterisation under defined and/or factory conditions.

2.2 EXPERIMENTAL

2.2.1 Reagents

All chemicals were the best available analytical grade reagents and were supplied by Ajax Fine Chemicals (Auburn, Australia) unless otherwise noted. Denatured and absolute ethanol (CSR Limited, Sydney, Australia) was used as supplied or aqueous solutions were prepared by dilution (v/v) with distilled water. UltraPure ammonium sulphate (Enzyme grade) was obtained from Bethesda Research Laboratories (Gaitersburg, MD).

The standard sucrose (starch and dextran free) was obtained from CSR Limited (Sydney, Australia). Commercial dextrans (MW $2x10^6$) T2000 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The sucrose and dextrans were used without further purification.

High purity glucose and *p*-hydroxybenzoic acid hydrazide (PAHBAH) of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO).

Potato starch was purchased from BDH Chemicals Limited (Poole, England). Pullulan ($\bar{M}_n$ 8.6 X 10^4) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). Acid-washed celite was prepared by adding 50 mL of concentrated hydrochloric acid (analytical grade, SG 1.16) to 50 g of celite (hyflo supercel, Johns-Manville, Denver, Co) suspended in 1 L of distilled water. The suspension was stirred for five min, collected by vacuum filtration on a Whatman No. 42 filter paper and washed free of acid with distilled water. The precipitate was dried for 16 h at 104°C, cooled and stored in a sealed container.

Mixed ion-exchange resin was prepared by thoroughly mixing equal weights of dried Amberlite IR 45-OH anion exchanger and dried Amberlite IR 120-H cation exchanger (BDH Chemicals, Australia). The resins were normally supplied wet and were washed with at least twice their weight of distilled water. After draining to dryness, the resins were washed briefly (no longer than two min) with an equivalent weight of acetone. The solvent was immediately removed and the resins air-dried at room temperature.

Purified cane dextrans were isolated from the first-expressed juice extracted from deteriorated sugar cane billets by repeated precipitation with alcohol (50%, v/v). The precipitate was lyophilised and stored at approximately 20°C and 10-15 per cent moisture.

Dextranases (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11) isolated from *Penicillium lilacinum, Penicillium funiculosum* and *Chaetomium gracile* (in 50 % (v/v) glycerol), were supplied as liquid preparations of different partial purities by Novo Industri (Denmark), Tate and Lyle (England) and Miles-Kyowa (Japan), respectively. The commercial enzyme preparations were stored at 4°C. These preparations were used as supplied for activity assays. However, because of the high activity exhibited by these enzymes, dilution with buffer (pH 5.0, 0.05 M acetate or citrate) was carried out as described below.

The thermostable α-amylase, Termamyl 120L, was purchased from Novo Industri, Denmark. The liquid preparation is a product of a selected strain of *Bacillus licheniformis.*

2.2.2 Preparation of deteriorated Juices

Burnt cane billets were collected during late October from a Mackay factory and stored in a large bin at ambient temperature for ten days. After crushing the deteriorated cane in the Sugar Research Institute laboratory mill, the first expressed juice (pH 4.0, 25 brix) was clarified in the laboratory. The resultant "cloudy" juice was concentrated to 72 brix (pH 6.5) in the SRI experimental pan and stored at 5°C. Deteriorated juices were prepared as required by dilution with distilled water to 18 brix.

2.2.3 Preparation of Crude Extracellular Culture Fluid

Dextranase producing isolates which were shown to be pure cultures were grown in either batch or continuous culture to produce sufficient quantities of the dextranase for characterisation using liquid assays. At the end of the exponential growth phase of the culture (determined by measurement of OD at 600 nm) the cells were separated from the broth by centrifugation (5°C, 30 min, 15 000*g*). The supernatant was the culture extract and was assayed directly where possible. Alternatively, the protein present in the cell-free extract was concentrated (by up to 100 times) by precipitation with ammonium sulphate. A gently stirred extracellular culture extract was slowly brought to 80 % saturation by the addition of solid ammonium sulphate. Once the salt had dissolved, the solution was allowed to stand for 1 hour prior to centrifugation at 20 000*g* for 30 min at 5°C. The resultant precipitate was redissolved in a minimum volume of the buffer to achieve maximum concentration. This crude dextranase solution was dialysed exhaustively against the same buffer at 5°C. To ensure that an optically 'clean' liquid enzyme preparation was obtained for assays, the dialysate was centrifuged (5°C, 30 min, 20 000*g*).

2.2.4 Preparation of Dextran Solutions

Dextrans were dissolved by the addition of small quantities of CO_2-free distilled water to an accurately weighed amount of the lyophilised material with constant stirring to form a homogeneous slurry. The particles were allowed to become uniformly hydrated by standing with occasional stirring of the solution (A. Jeanes, private communication). Additional water was added gradually so as to avoid the presence of an excess while gel masses still remained. After transferring

to a suitable container, the sample was autoclaved for 20 min at 100 kPa (121°C). The sample was allowed to cool to room temperature and the solution made to volume. Dextran solutions were prepared daily. (Due to solubility problems, prolonged storage of solutions is not recommended even under refrigeration.)

2.2.5 Preparation of Buffers

Buffers were prepared using analytical grade reagents. Measurements of pH were made at 20°C with a Radiometer pH meter 26, equipped with a combination glass electrode.

2.2.6 Measurement of Dextranase Activity

(i) The Alcohol-Haze Method

Preparation of Standard Curve (Figure 2.1)

Aliquots of a standard dextran solution (T2000, 1.0 mg mL^{-1}) containing 0.1 to 7.0 mg dextran were pipetted into 25-mL volumetric flasks containing 0.5 mL of 10 % trichloroacetic acid (TCA) and 5.0 mL of sucrose solution (0.2 g mL^{-1}). The total volume of the aqueous solution was adjusted to 12.5 mL by adding the required volume of distilled water. Denatured alcohol (96 %) was then added (with swirling) from a burette to the 25-mL mark. The contents were gently mixed and the timing started. After 20 min the haze formed was read in 2 cm cuvettes against water dilutions of the aliquots at 720 nm.

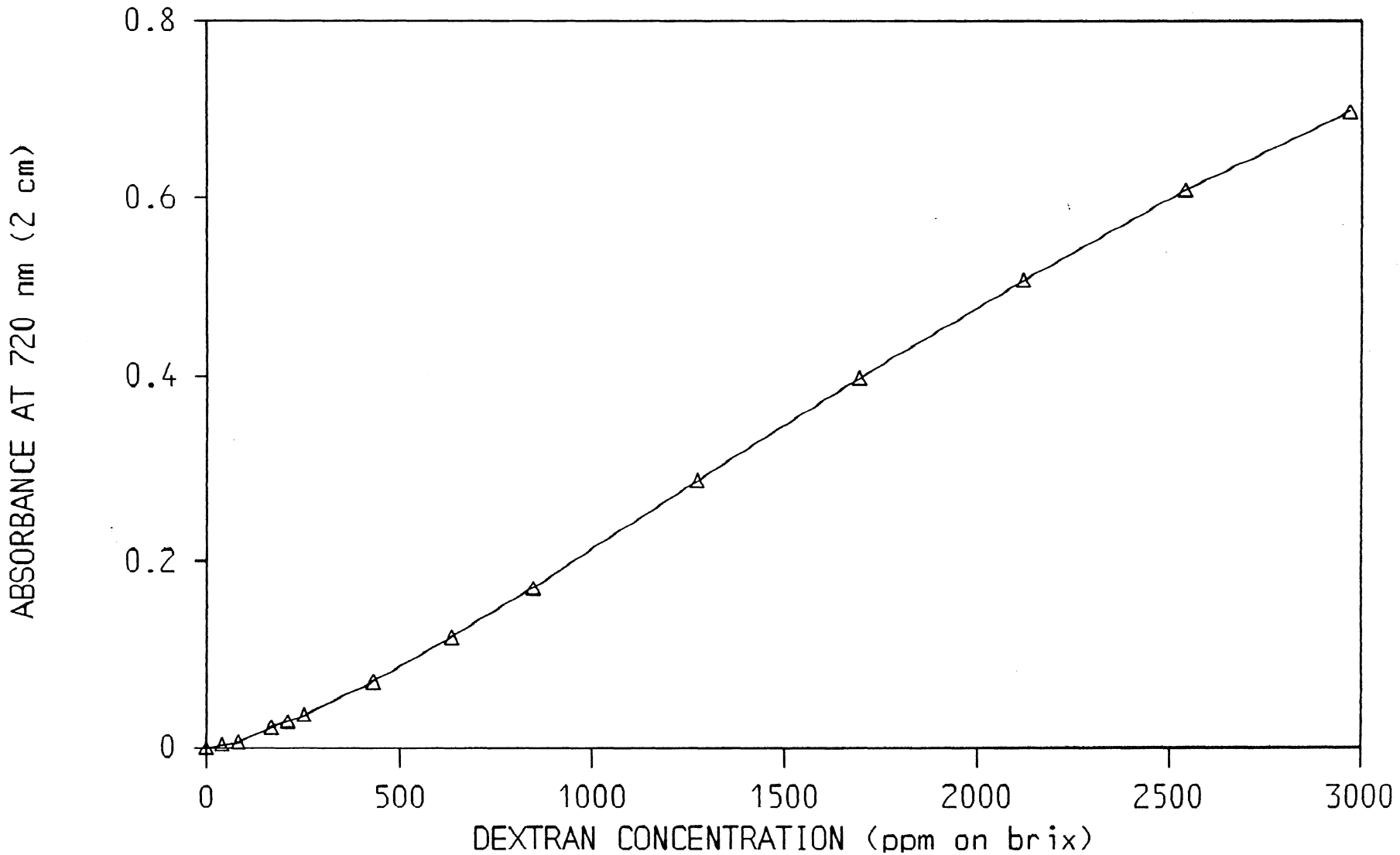


Figure 2.1 Standard curve for the alcohol haze test.

Dextranase Activity in Purified Dextran Solutions

An incubation mixture (2 L) containing approximately 10 000 ppm dextran on brix was prepared using dextran T2000 (or purified cane dextran), sucrose to a final concentration of 20.0 brix and 0.05 M buffer (pH 5.0, citrate). An aliquot (200 mL) of the incubation mixture was taken for each assay temperature. Two additional aliquots were used as controls incubated at the lowest and highest temperatures. After equilibration of the solution at the assay temperature, dextranase was added and hydrolysis allowed to proceed for 30 min. The reaction was stopped when a 50-mL aliquot of the mixture was transferred to a flask containing 10 % TCA (10 mL). A subsample (adjusted to give a final volume of 12.5 mL) was added to each of two 25-mL volumetric flasks. One of the flasks was made to volume with denatured alcohol while the blank was prepared by the addition of distilled water. The absorbance of the haze at 720nm was determined as described previously. Initial and residual dextran levels were determined and the dextranase activity was calculated as the amount of dextran hydrolysed (ppm on brix) per min per mg of enzyme.

Dextranase Activity in Raw Cane Juice.

Deteriorated cane juice (approximately 300 mL) was allowed to equilibrate in a thermostatically-controlled water bath at 90°C (± 5°C) for approximately 5 min. Thereafter, Termamyl 120L (0.3 mL) was added and the juice incubated for a further 15 min to remove starch. The solution was cooled to room temperature and a 250 mL aliquot of the juice (of known brix, ~18) was shaken for 10 min with mixed ion exchange resin (8.0 g). The resin was removed by filtration of the juice through a

150 μm gauze. The salt free solution was pre-equilibrated at the required temperature for 5-10 min. Dextranase was added to the incubation mixture and the reaction was stopped after 30 min when a 50-mL aliquot of the reaction mixture was transferred to a flask containing 10 % TCA (10 mL). Acid washed celite (5g) was added to the solution prior to vacuum filtration to remove any proteinaceous precipitate. An aliquot of this filtrate (adjusted to give a final volume of 12.5 mL) was added to each of two 25-mL volumetric flasks and the haze development procedure continued as outlined above. In calculation of dextran levels, allowance was made for dilution of the sample at the trichloroacetic acid addition step.

(ii) The Blue Dextran Assay

Dextranase activity was determined by measurement of the amount of dye released from Blue Dextran 2000 according to the method of Koh and Khouw (1970), modified as follows : 3 mL of substrate (Blue Dextran 0.4%, w/v) and 0.4 mL of citrate buffer (0.025 M), pH 5.0, were incubated for 5 min at the required temperature. A 100-μL aliquot of suitably diluted enzyme was added and the reaction mixture incubated for a further 15 min. The reaction was stopped by the addition of 7 mL of absolute ethanol and the solution then placed in an ice bath for 15 min. After centrifugation at 20 000*g* for 15 min at 20°C, the absorbance was read at 630 nm. The absorbance was corrected for any obtained for the distilled water blank. One unit of dextranase activity is defined as the amount of enzyme that catalyses the release of 1 mg of dye complex ($\varepsilon_{1\,cm}^{1\,mg\,mL^{-1}} = 0.965$) in 15 min under the prevailing assay conditions (Brown and Inkerman, 1992).

(iii) The PAHBAH Assay

The amount of colour produced during the reaction of PAHBAH with the reducing sugars produced during enzymic hydrolysis was used to determine dextranase activity. The micro-method of Lever (1973) was modified as follows: A mixture of 0.4 mL substrate (dextran T2000, 0.4%) and 0.1 mL buffer (citrate, 0.5 M, pH 5.0) was pre-incubated at the required temperature for 5 min. The appropriate amount of enzyme solution (20-200 μL) was added and the total volume adjusted to 0.7 mL. Aliquots (50 μL) of the reaction mixture were withdrawn and transferred to a reaction tube containing 1.0 mL of PAHBAH reagent (PAHBAH 0.1M, sodium sulphite 0.1 M, calcium chloride 0.02 M, trisodium citrate 0.05M, and sodium hydroxide 0.5M; Lever, 1973; Appendix 2.A). The reaction mixtures were heated for 10 min in a boiling water bath, and after cooling to room temperature, absorbances were measured at 420 nm. Substrate and enzyme blanks were also prepared using buffer to replace the enzyme and substrate, respectively, in the reaction mixture. Absorbance measurements were corrected for the appropriate blank absorbances. Reducing sugar concentrations were determined from a standard curve prepared using glucose (1-10 μg). One unit of dextranase activity is defined as the amount of enzyme producing 1 μmole of reducing sugar per min under the prevailing assay conditions. The detailed experimental procedure is given in full in Appendix 2.A.

(iv) The Micro-Blue Dextran Assay

The sensitivity of the Blue Dextran assay ((ii) above) was improved by reducing the overall volume of the assay and increasing the relative amount of the enzyme per assay. Alterations to the macro-Blue Dextran method were as follows:

0.4 mL of incubation mixture (Blue Dextran 0.4%, w/v; citrate buffer, 0.025 M, pH 5.0; ± sucrose 20 brix) was preincubated for 5 min at the assay temperature. A 100-µL aliquot of suitably diluted enzyme was added and the reaction mixture incubated for a further 30 min. The reaction was stopped by the addition of 1.2 mL of absolute ethanol and the solution cooled in an ice bath for a further 15 min. After centrifugation at 12 000*g* for 15 min (Sorval MICROSPIN 24S) at 20°C, the absorbance was read at 630 nm. The absorbance was corrected for any value obtained for a distilled water blank. One unit of dextranase activity was identical to that used for the macro-Blue Dextran assay.

(v) The Micro-Haze Method

Preparation of Standard Curve (Figure 2.2)

A standard curve was prepared using the relative concentrations of components as for the alcohol-haze method; however, the overall volumes were reduced by a factor of 12.5. A total volume of 1.0 mL was used for the TCA/sucrose/dextran/water component. An additional 1.0 mL of ethanol (or distilled water for the blanks) was added to a 2-mL vial, the contents were gently mixed and the timing started. After 20 min the haze formed was read in 1 cm cuvettes (1.5 mL) against the water dilution at 720 nm.

Dextranase Activity in Purified Dextran Solutions

An incubation mixture (100 mL) containing approximately 10 000 ppm dextran on brix was prepared using dextran T2000 (or purified cane dextran),

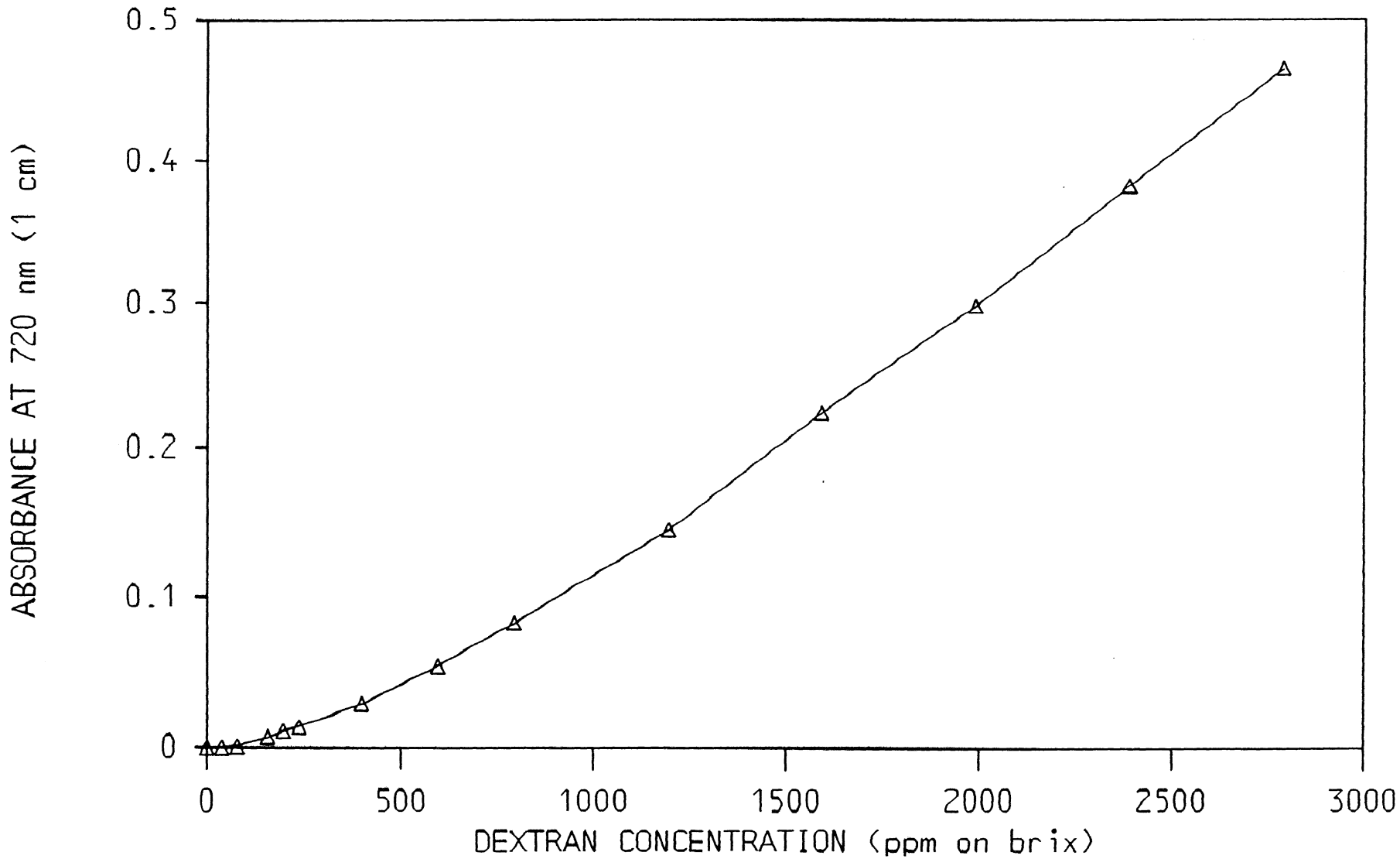


Figure 2.2 Standard curve for the micro-haze test.

sucrose to a final concentration of 20.0 brix and 0.05 M buffer (pH 5.0, citrate). Duplicate aliquots (1 mL) of the incubation mixture were transferred to a 2-mL vial (sealed to minimise evaporation) for each assay temperature. Two additional aliquots were taken to act as controls incubated at the lowest and highest temperatures. After equilibration of the solution for 5 min the dextranase solution (0.1 mL) was added to the test assays and an equivalent volume of distilled water added to the controls. Enzymic hydrolysis was stopped after 30 min by transferring the reaction mixture (1.1 mL) to a 1.5-mL Ependorf centrifuge tube containing 10 % TCA (0.2 mL).

The mixture was chilled on ice for 5 min then centrifuged at 12 000 *g* for 15 min at room temperature to remove any precipitate. Two subsamples (0.2 mL) were withdrawn from the solution and added to separate 2-mL vials containing 0.8 mL sucrose solution (20.0 brix). Denatured alcohol (1.0 mL) was added to one vial while distilled water (1.0 mL) was added to the blank. The method for reading absorbance followed exactly that employed in construction of the standard curve. Initial and residual dextran levels were determined and the dextranase activity was calculated as the amount of dextran hydrolysed (ppm on brix) per min per mg enzyme.

Dextranase Activity in Raw Cane Juice

Deteriorated cane juice (100 mL) was treated with α-amylase and deionised as described for the methodology of the alcohol-haze method. Thereafter, aliquots (1 mL) of the juice were pipetted into 2-mL assay tubes and the procedure followed

was as described for the purified dextran solutions using the micro-haze method. The detailed experimental procedure is given in full in Appendix 2.B.

2.3 RESULTS AND DISCUSSION

2.3.1 The Alcohol-Haze Test

Assessment of dextranase activity under factory conditions was readily determined using the alcohol-haze test. This method gave a quantitative measure of dextran hydrolysis in raw sugar cane juice. The temperature profile obtained by the alcohol-haze method for the *Penicillium lilacinum* dextranase (Novo 25 L) is given in Figure 2.3. The resulting curve was typically 'bell-shaped', exhibiting an optimum around 55°C with evidence of enzymic denaturation above this temperature. Enzyme activity fell rapidly as the incubation temperature approached 70°C. The assays were performed in deteriorated cane juice (pH 5.0) with an initial dextran concentration of about 10 000 ppm on brix. The use of the alcohol-haze method is of practical importance as results can be directly related to enzymic activity in the juice stream of a raw sugar factory.

This method has been utilised to follow the rate of dextran hydrolysis under factory conditions (Fulcher and Inkerman, 1974b; 1976). The rate curve obtained for the *Chaetomium gracile*-catalysed hydrolysis of cane dextran measured by the alcohol-haze test is presented in Figure 2.4.

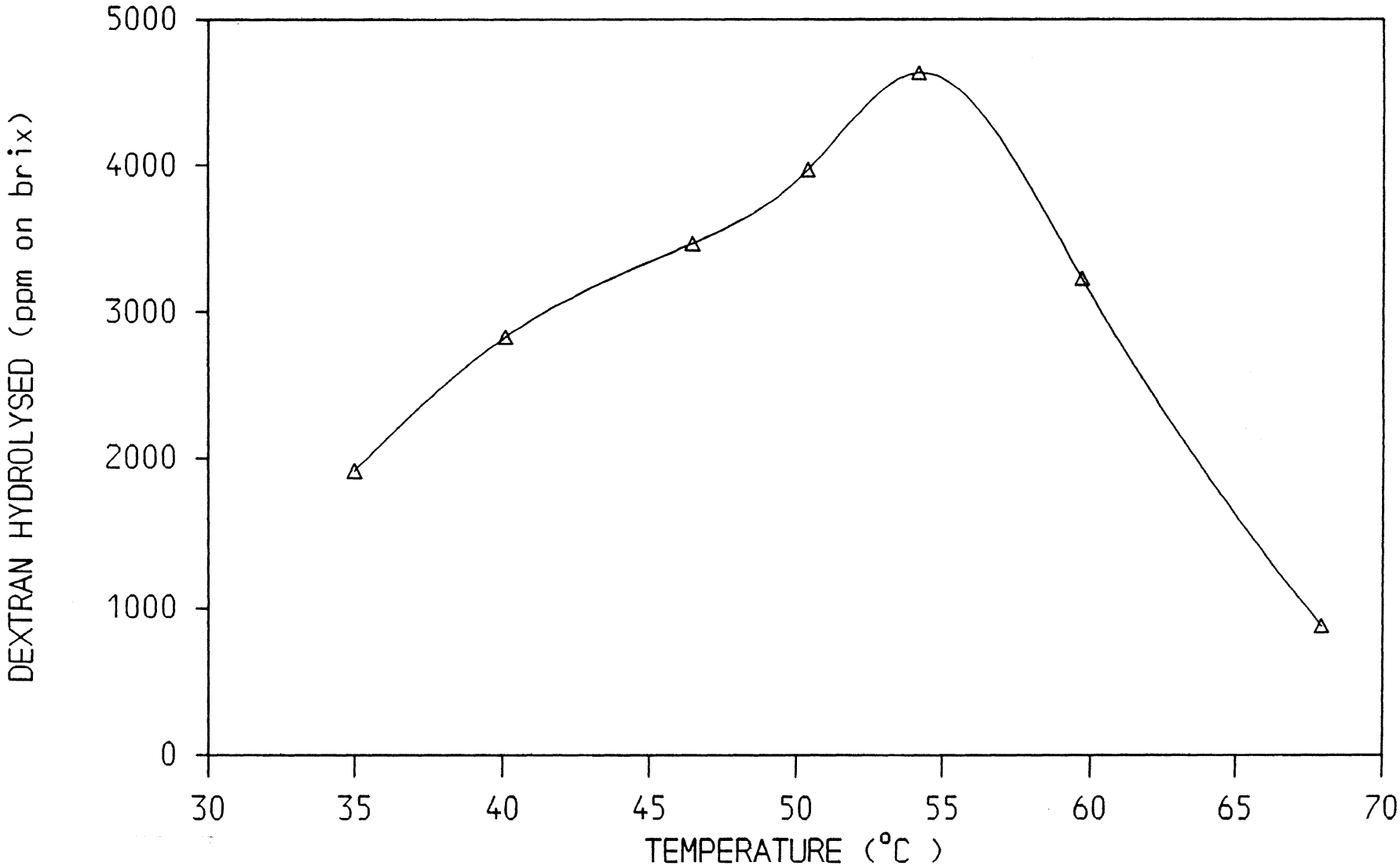


Figure 2.3. Temperature profile for *Penicilium lilacinum* (Novo 25L) in deteriorated cane juice, dextran 10 000 ppm on brix, pH 5.0, 30 min as measured by the alcohol-haze test.

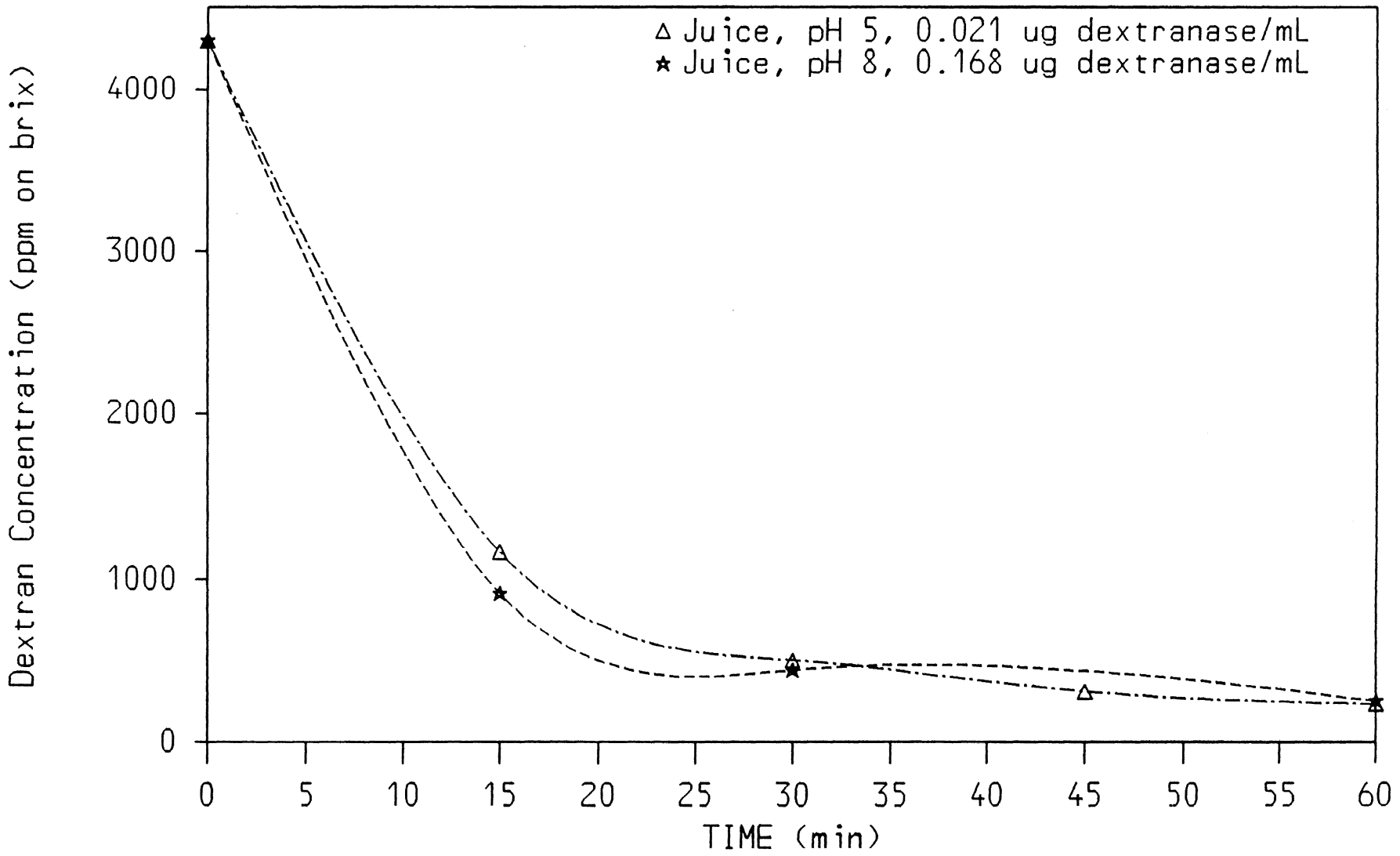


Figure 2.4 **Progress curve for *C. gracile* dextranase - catalysed hydrolysis of cane dextran in deteriorated juice at pH 5 and 8 at 60°C as measured by the alcohol-haze method.**

Assays with the dextranex L 4000 (Miles-Kyowa) commercial preparation from *C. gracile* were carried out using deteriorated cane juice as the substrate (initial dextran concentration 4 500 ppm on brix) and enzyme concentrations of 0.021 and 0.168 μg dextranase mL^{-1}. Assay conditions were 60°C, pH 5.0 and pH 8.0 and the reaction was followed for 60 minutes. These conditions are representative of those operating in the mixed juice streams during normal and 'specialised' processing in Australian sugar factories.

Other characteristics of the dextranases *e.g.* the pH optimum, have been determined using the alcohol-haze method. The volume of incubation mixture required for this test is fairly large (at least 50 mL of substrate for a single assay with larger volumes required for reaction rate experiments). Hence, the amount of dextranase required is also quite large. This feature of the method does not present a problem if dextranase is readily available as is the case when assessing commercial dextranase preparations. However, to assess the dextranase produced from new microbial isolates (generally expected to be expressed into the culture extract at very low concentrations) smaller scale methods were sought.

2.3.2 The Blue Dextran Assay

The suitability of new or 'improved' commercial dextranases for application in the raw sugar factory has been routinely assessed using the rapid Blue Dextran assay. This method was also found to be specific for measurement of endo-dextranase activity (Brown and Inkerman, 1992). Assessment of the relative activity of the enzyme preparations in the presence (or absence) of sucrose has been determined. A comparison of the activity of some commercial dextranases

measured by the Blue Dextran assay is given in Table 2.1. The incubation mixture included sucrose (20 brix) and the Blue Dextran substrate (pH 5.0).

TABLE 2.1. A COMPARISON OF THE ACTIVITY OF SOME CURRENTLY AVAILABLE COMMERCIAL DEXTRANASE PREPARATIONS[a]

Sample	A_{630} per g Enzyme[b]	Relative Activity (%)
Miles Dextranex (*C. gracile*)	1.756	100
Novo 25 L (*P. lilacinum*)	0.418	23.8
Novo 50 L (*P. lilacinum*)	0.810	46.1
Tate and Lyle (*P. funiculosum*)	1.212	69.0

[a]Conditions of assay were 60°C, pH 5.0 for 30 minutes. [b]Activity determined, by weight, on the commercial enzyme solution.

Under these assay conditions the *C. gracile* dextranase was the most active of the four commercial dextranases tested. As such it has been recommended for use within Australian sugar factories since the early 1980's.

Temperature and pH optima were determined with appropriate changes in the assay conditions. To investigate the effect of sucrose on the thermal stability of the *C. gracile* dextranase, temperature profiles with and without sucrose (20 brix) were determined (Figure 2.5). In the presence of sucrose an increase in temperature optimum of about 5°C was obtained. Similar effects on thermal stability

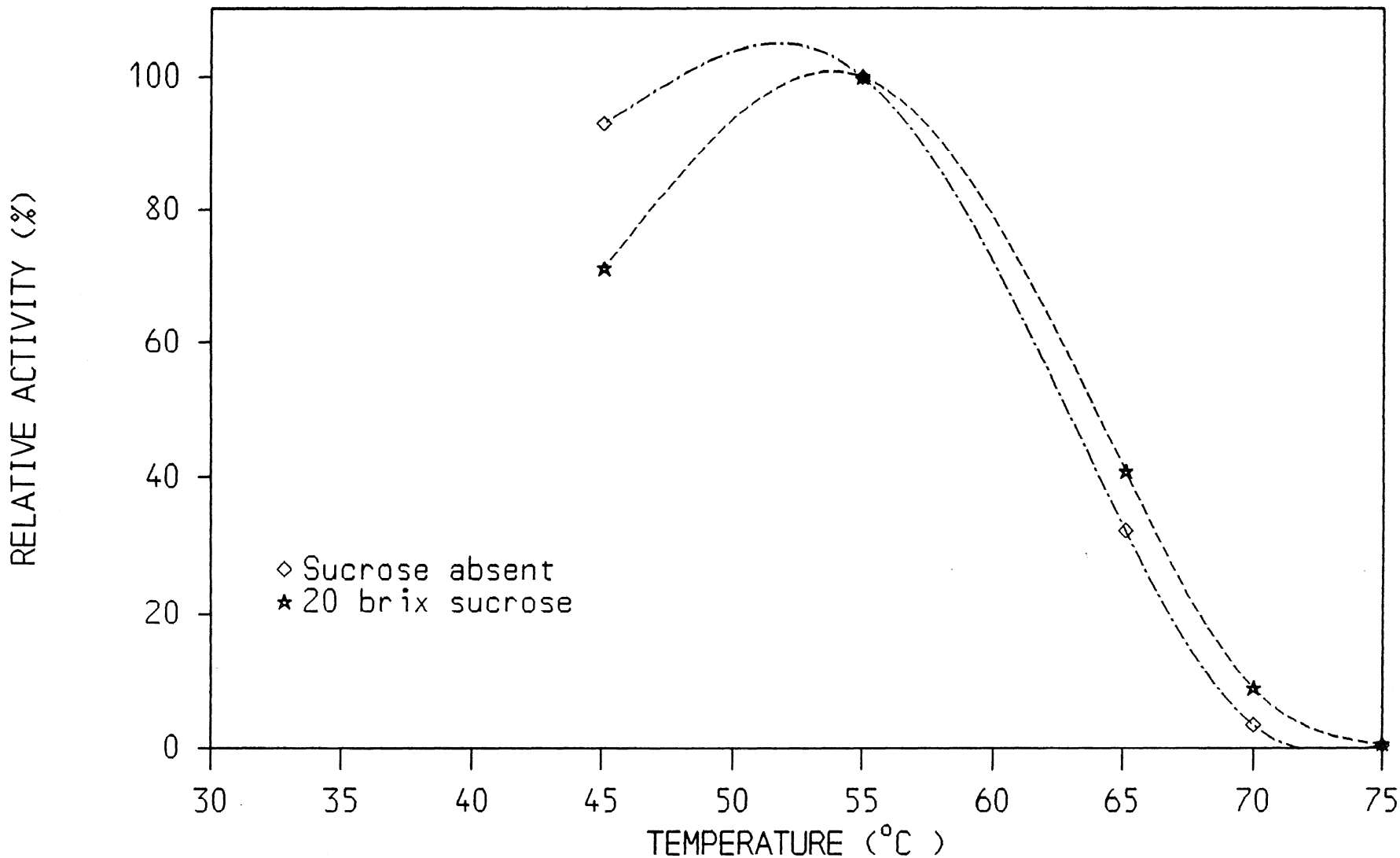


Figure 2.5. Temperature profile for *C.gracile* dextranase in the presence and absence of sucrose as measured by the Blue Dextran assay, pH 5.0, 30 min.

of enzymes by agents such as sucrose, has been reported previously (Graber and Comes, 1989).

The Blue Dextran method was found to be suitable for assessment of dextranases expressed by early fungal isolates (Table 2.2). However, as a technique for the assessment of dextranases produced by bacterial isolates it was shown to be too insensitive as no absorbance was detected after the 30 minute incubation period (A_{630} = 0.000) with the maximum volume (100 μL) of SRI 2116 culture extract.

TABLE 2.2 DEXTRANASE ACTIVITY OF THE CRUDE EXTRACT OBTAINED FROM THE FUNGAL ISOLATE ATCC 60154.

Temperature	Absorbance (1 cm, 630 nm)	Relative Activity
45.5	1.065	99.9
60.2	1.066	100
75.2	0.049	4.2

2.3.3 The PAHBAH Assay

The search for a sensitive assay to follow the enzymic hydrolysis of the polysaccharide, dextran, led to the selection of the reducing sugar method developed by Lever (1972; 1973). This spectrophotometric method measures the reaction between an aromatic acid hydrazide and reducing carbohydrates released by the dextranase under alkaline conditions. The sensitive procedure selected used *p*-hydroxybenzoic acid hydrazide (PAHBAH) to obtain optimum results for assay of

reducing sugars. This assay gives a quantitative measure of the exo-dextranase activity and only a semiquantitative measure of the endo-dextranase activity. However, it's sensitivity could not be matched by the existing methods for endo-activity (e.g. the alcohol haze test and the Blue Dextran assay).

Initial experiments with the *C. gracile* dextranase demonstrated that the amount of the commercial dextranase required per assay was of the order of 0.02 μg to give a readily detectable increase in reducing sugars (determined as glucose). This is approximately 60 and 25 times less commercial enzyme per assay than required by the alcohol-haze and Blue Dextran methods, respectively.

Prior to application of the PAHBAH procedure for the measurement of dextranase produced by microbial isolates, the effect of 'background' was investigated. The cell-free culture filtrate containing the extracellular enzyme might also contain additional components which exhibit reducing power. Therefore, investigations into the response of the enzyme blank at different time intervals were carried out. The reducing sugar concentrations on the direct culture filtrate and the concentrated crude extract were measured at 0 and 30 minutes (the standard reaction time). Although initial levels were high (absorbance 0.304 at 420 nm for the enzyme blank) for the direct extract, no measurable increase in the levels of background reducing sugar were detected over the time course of the experiment. Results obtained for the concentrated crude extract exhibited relatively low initial background levels (Table 2.3) and no significant increase in the values were measured during the incubation period.

TABLE 2.3 THE EFFECT OF INCUBATION TIME ON BACKGROUND REDUCING SUGAR LEVELS FOR A CONCENTRATED CRUDE EXTRACT FROM THE ISOLATE SRI 2116 MEASURED BY THE PAHBAH METHOD[a].

Sample	Time (min)	Absorbance (1 cm, 420 nm)
Dextran Substrate Blank	0	0.045
	30	0.043
SRI 2116 Enzyme Blank	0	0.049
	30	0.045
SRI 2116 Test	0	0.082
	30	1.781

[a]The enzyme was 50 µL of concentrated extract from the culture of the isolate SRI 2116; substrate 0.1 % dextran T2000; assay conditions were 55°C at pH 5.0.

Previous workers had encountered problems with precipitate formation during the colorimetric reaction (Dr. C. Wynter, private communication). The need for a centrifugation step prior to the measurement of absorbance was investigated using glucose standard solutions. A visual inspection found no evidence of precipitate formation and absorbances before and after centrifugation (Table 2.4) demonstrated no significant change had occurred. Therefore, for routine analysis no centrifugation step was included in the procedure.

TABLE 2.4. EFFECT OF CENTRIFUGATION ON THE COLORIMETRIC REACTION OF THE PAHBAH ASSAY[a].

Sample	Treatment	Absorbance (1 cm, 420 nm)	Difference
Reagent Blank	None	0.022	0.003
	Centrifugation	0.025	
2.0 μg Glucose	None	0.261	0.001
	Centrifugation	0.262	
5.0 μg Glucose	None	0.598	0.017
	Centrifugation	0.615	
7.0 μg Glucose	None	0.861	0.025
	Centrifugation	0.886	

[a]Centrifugation was at 12 000 *g*, 2 min using the Sorval Microspin.

The effect of enzyme concentration on the rate of the reaction was investigated at two substrate concentrations (0.1 % and 0.4 % dextran T2000). To ensure that the reaction rate was limited only by the substrate concentration, the assay temperature was reduced to 45°C and the incubation time to 15 min. The effect of the various concentrations of the *C. gracile* dextranase on the reaction rate at two substrate levels is presented in Figure 2.6. The non-linear curve obtained for an initial dextran concentration of 0.1% is indicative of a substrate limited reaction. In contrast, a linear curve was obtained when an initial dextran concentration of 0.4% was employed, indicating that the rate of the reaction was not substrate limited. Therefore, the initial substrate concentration for this reaction was increased from 0.1% to 0.4%.

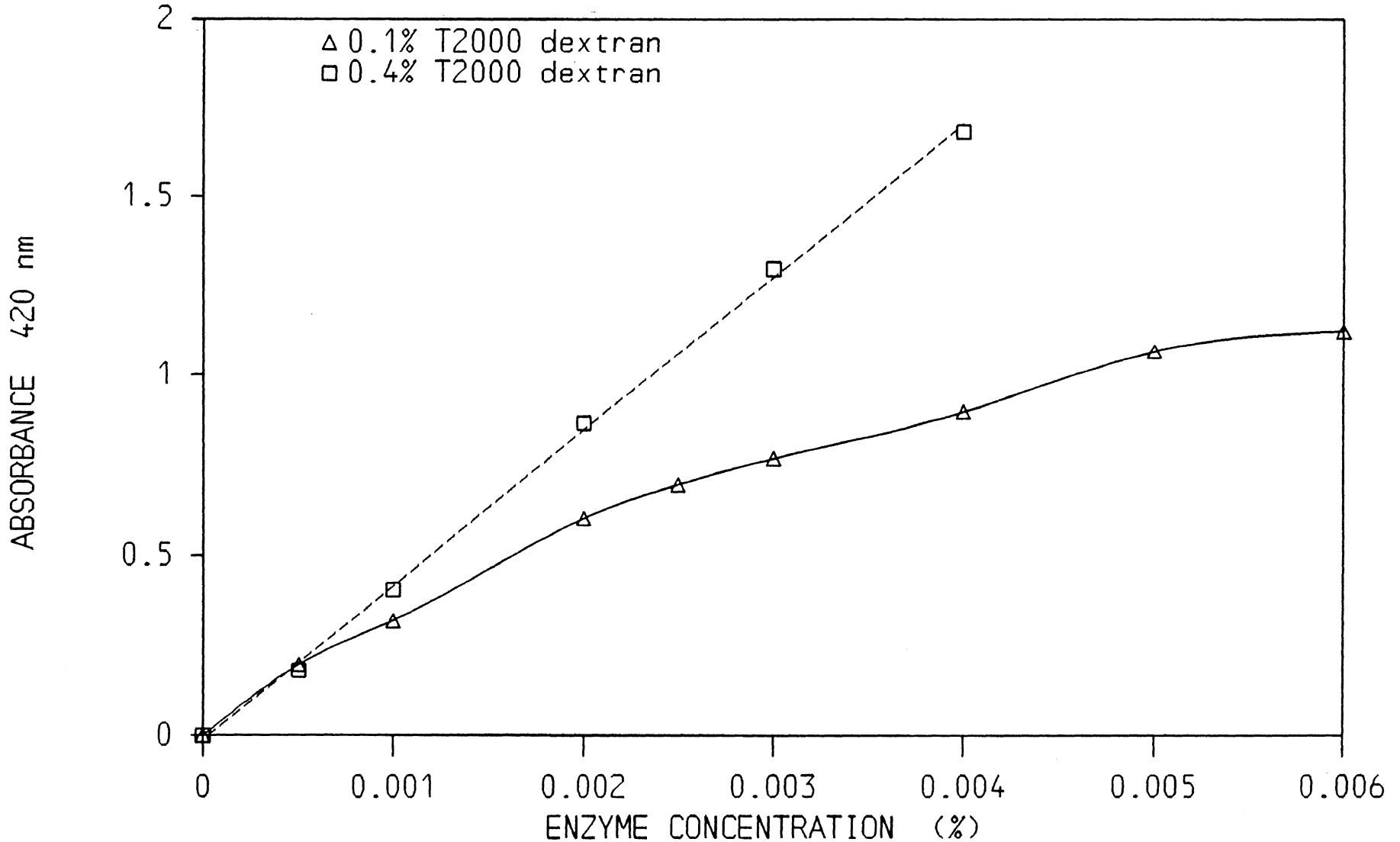


Figure 2.6. Effect of enzyme concentration on the degree of hydrolysis of dextran T2000 (pH 5.0, 15 min) as determined by the PAHBAH method.

Further data obtained for application of the PAHBAH method for measurement of the temperature profiles for extra-cellular dextranases obtained from fungal and bacterial isolates are presented in Chapter 5.

2.3.4. The Micro-Blue Dextran Assay

An increase in the 'sensitivity' of the Blue Dextran assay was obtained by introducing two changes to the methodology described previously. Namely, a reduction in the overall assay volume and a doubling in the volume of enzyme extract utilized per assay. These modifications actually led to the detection of lower dextranase concentrations rather than an increase in the inherent sensitivity of the method. The incorporation of the buffer and sucrose into the substrate stock solution allowed the decrease in the detection limits without creating problems such as substrate limitation. The actual concentration of the Blue Dextran substrate in the incubation mixture for the micro-Blue Dextran assay is 0.27 % compared to a concentration of 0.34 % in the standard procedure. Any future attempt to further increase the sensitivity of the micro-method by increasing the volume of enzyme extract (above the current volume of 200 μL) must be verified for substrate limitation under the new assay conditions.

The *C. gracile* dextranase was used to experimentally determine the apparent increase in method sensitivity. The amount of dextranase which could be detected using the micro-Blue Dextran assay was calculated to be about 10 times lower compared to the standard Blue Dextran assay.

The micro-Blue Dextran assay was found to be sensitive enough to measure the dextranase activity in concentrated extracts from the bacterial isolates but not in the direct culture filtrate. A typical result obtained for temperature profiles for concentrated extracts by the micro-Blue Dextran method is presented in Figure 2.7.

2.3.5 The Micro-Haze Method

The two micro-scale methods outlined above (the PAHBAH and micro-Blue Dextran assay) have led to an improvement in sensitivity for measurement of both exo- and endo- dextranase activity. However, a sensitive and practical method for the assessment of enzymic activity under factory conditions was not available. Therefore, it was proposed to refine the alcohol haze method to obtain maximum sensitivity on a micro-scale. The minimum volume for micro-scale spectrophotometric measurement of the haze was determined to be 1.0 mL (0.5 mL aqueous mixture and 0.5 mL denatured alcohol) if micro-cuvettes could be used successfully.

(i) Dextran T2000 as substrate

Initially, a series of standard solutions was prepared to give a final volume of 1.0 mL. The resulting curve (Figure 2.2) was typical of that obtained for the alcohol-haze test. The range of dextran concentrations covered by this curve (and all other alcohol haze standard curves assuming a sucrose concentration of 20 brix) was 0-3000 dextran (ppm on brix). A temperature profile for the *C. gracile* dextranase was determined using dextran T2000 as the substrate and an initial

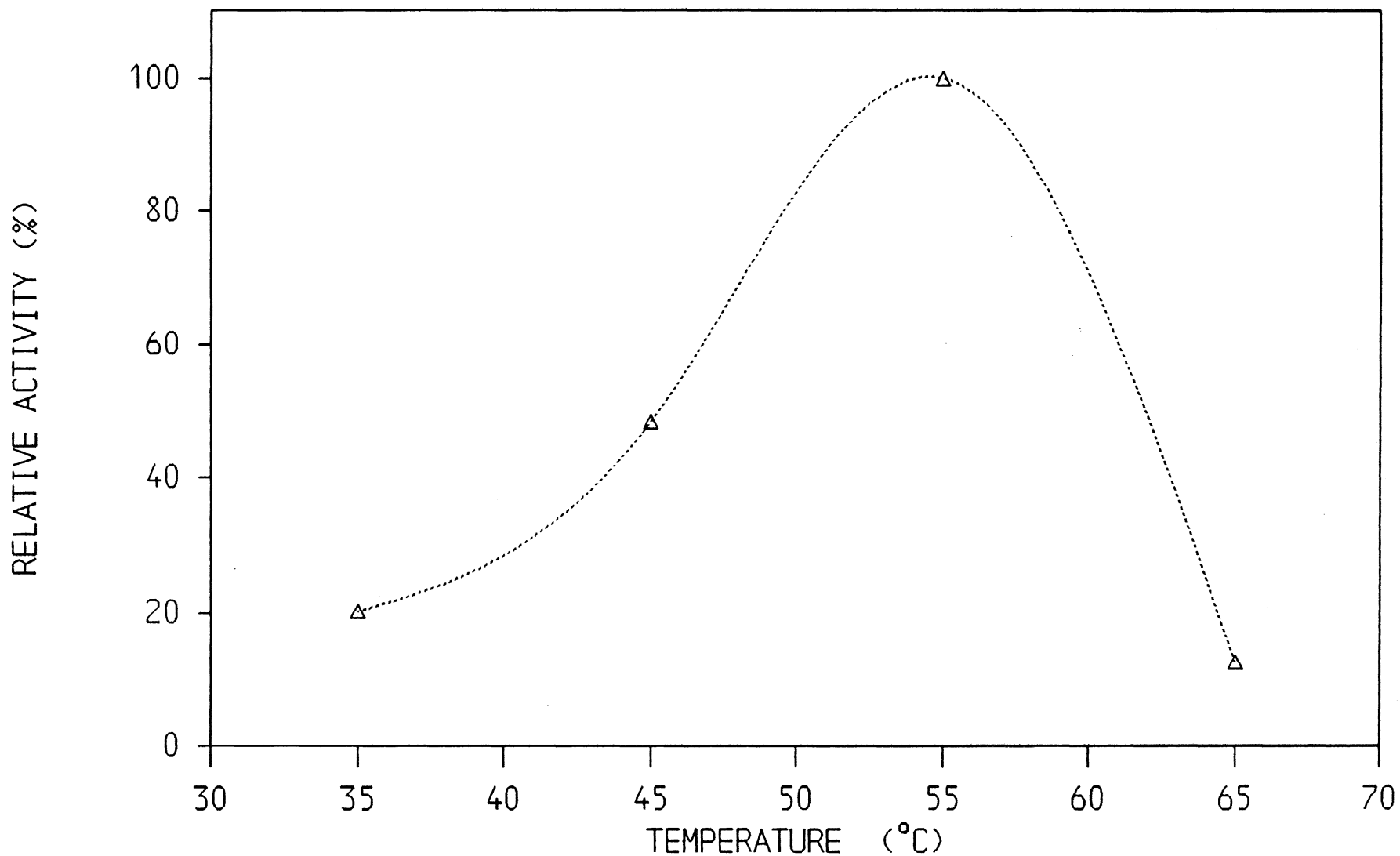


Figure 2.7. Temperature profile for a bacterial isolate (SRI 2116) as determined by the micro-Blue Dextran assay (pH 5.0, 30 min).

dextran concentration of about 3000 ppm on brix. This substrate level was selected to avoid a dilution step prior to haze formation which would reduce any gains in sensitivity. However, the curve obtained for the temperature profile suggested that the reaction rates, particularly at temperatures near the optimum, were substrate limited (see Figure 2.8; *C. gracile* dextranase, 3.86×10^{-9} g mL^{-1}). Subsequently, the dextran concentration in the incubation mixture was increased to about 12 000 ppm on brix. To compensate for the higher dextran levels present in the incubation mixture, only a subsample of this mixture could be taken for haze development. The volume of the subsample was selected to be 0.2 mL and the overall volume of the assay mixture was increased to 2.0 mL to allow some excess solution for spectrophotometric measurement. A sucrose solution (20 brix, 0.8 mL) was used to adjust the volume of the aqueous solution to 1.0 mL prior to addition of denatured ethanol (1.0 mL). The temperature profile obtained for the *C. gracile* dextranase (7.64×10^{-9} g mL^{-1}) under the new assay conditions is shown in Figure 2.9. The resulting curve was typical of a temperature profile obtained for this enzyme by the various methods outlined above when substrate limitations had been overcome.

The micro-haze method described above (with dextran T2000 as the substrate) was used to determine the temperature profiles and thermal stability of dextranase activity obtained from liquid cultures of both fungal and bacterial isolates.

(ii) Cane Dextran as substrate

Assessment of dextranase activity under factory conditions was obtained by changing the substrate for the micro-haze method from dextran T2000 to deteriorated sugar cane juice. Pretreatment of the juice (*i.e.* removal of starch, salts

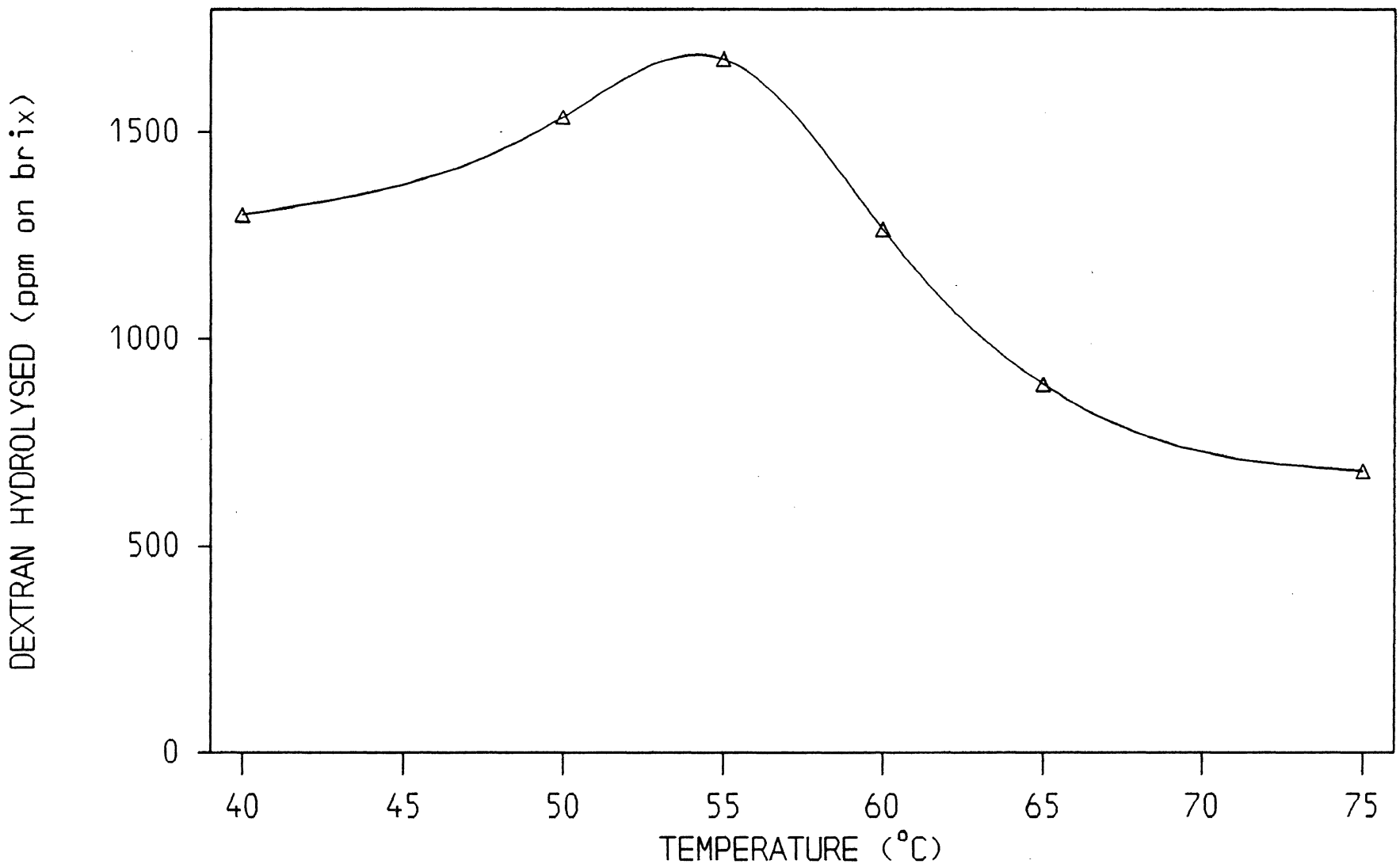


Figure 2.8 Temperature profile for *C.gracile* dextranase, determined using an initial substrate (T2000 dextran) concentration of ~3000 ppm on brix, measured by the micro-haze method.

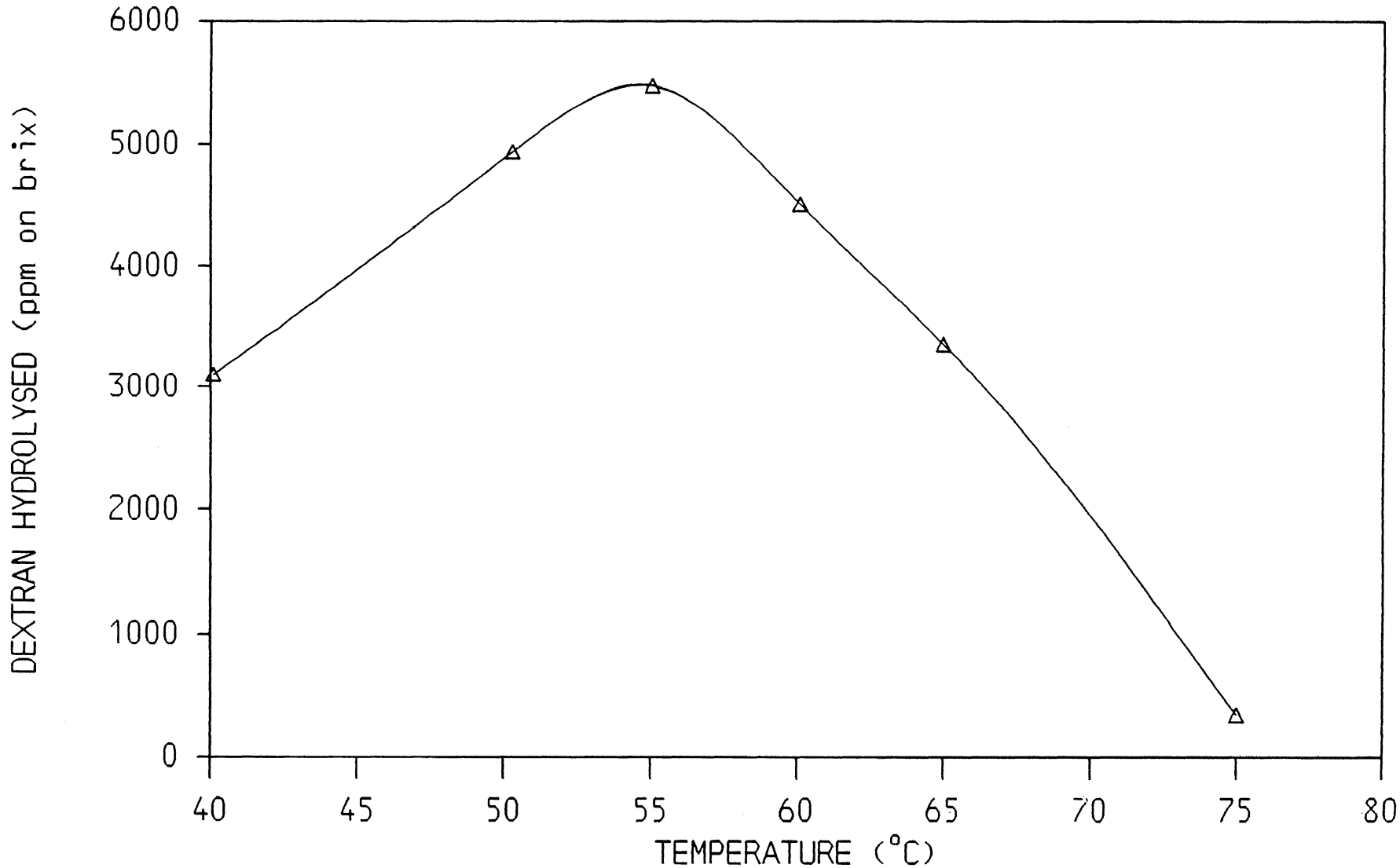


Figure 2.9 Temperature profile for *C. gracile* dextranase, determined using an initial substrate (T2000 dextran) concentration of ~10 000 ppm on brix, as measured by the micro-haze method.

and proteinaceous compounds) was carried out as previously described for the alcohol-haze test. Deteriorated cane juice (cane dextran levels about 10 000- 12 000 ppm on brix) was prepared as the incubation mixture for the determination of temperature profiles for the four most promising thermostable dextranases (see Chapter 5). Results obtained by this method are related directly to enzyme performance in the raw sugar factory.

Thus, the micro-haze test has been shown to be a sensitive and low volume assay for the assessment of endo-dextranase activity under factory conditions. A technique capable of determining this characteristic for the enzymes under study is invaluable to achieve assessment of the commercial potential of the thermostable dextranases early in the developmental process.

2.3.6 A Comparison of the Methods for Determination of Dextranase Activity

A number of parameters from all the methods used for the measurement of dextranase activity are listed in Tables 2.5 and 2.6. The values were obtained from experimental results obtained for the *C. gracile* dextranase at 55°C and pH 5.0, using a incubation time of 30 min. The usefulness of the methods for assessment of very low dextranase activity was determined from a comparison of the amount of dextranase required per assay and their relative sensitivities (Table 2.5).

Based solely on the amount of enzyme required per assay the PAHBAH assay and the micro-haze test detect dextranase concentrations of much lower

TABLE 2.5 A COMPARISON OF THE SENSITIVITY OF THE METHODS FOR THE ASSESSMENT OF DEXTRANASE ACTIVITY[a].

Parameter	PAHBAH Method	Blue Dextran	Micro-Blue Dextran Assay	Alcohol-Haze Test	Micro-Haze Test
Substrate Concentration	0.4%	0.4%	0.4%	0.405%	0.55%
Absorbance	1.007 (420 nm)	0.266 (630 nm)	0.560 (630 nm)	0.300 (720 nm)	0.200 (720 nm)
Dextranase Solution Concentration (as supplied)	0.001%	0.10%	0.04%	0.10%	0.002%
Aliquot of Dextranase Solution per Assay (µL)	200	100	200	100	100
Assay Volume	700 µL	3.5 µL	600 µL	50 mL (60 mL)	1100 µL (1300 µL)
Subsample	50 µL	None	None	12.5 mL	200 µL
Final Volume	1500 µL	10.5 mL	1800 µL	25.0 mL	2000 µL
Dextranase Concentration in Incubation Mixture ($g \times 10^{-9}$ mL^{-1})[b]	12.0	120	560	8.4	7.6
Amount (g) of Dextranase per Assay	8.4×10^{-9}	4.2×10^{-7}	3.4×10^{-7}	4.2×10^{-7}	8.4×10^{-9}
Relative Sensitivity[c]	1.2×10^{8}	6.3×10^{5}	1.6×10^{6}	7.1×10^{5}	2.4×10^{7}

[a]The substrate for all assays was dextran T2000; for the haze tests the substrate concentration is equivalent to 8 000 and 10 000 ppm on brix assuming a sucrose concentration of 20 brix; [b]Dextranase concentration is adjusted for a protein concentration of 4.2 mg mL^{-1} for the *C. gracile* dextranase preparation of Dextranex L 4000. [c]Relative sensitivity is calculated as absorbance ÷ amount of dextranase per assay.

levels (at least 50 fold) than other methods studied. A low detection limit was important for measurement of the micro-quantities of dextranase produced by the bacterial isolates under investigation. Hence, these two methods were used to rapidly assess of the thermal characteristics of the crude enzymes.

A comparison of the relative sensitivities (calculated from the absorbance, as well as, the amount of dextranase used per assay) the PAHBAH assay is five times more sensitive than the micro-haze test. The micro-Blue Dextran assay (the other micro method used for dextranase measurement) was 75 times less sensitive than the PAHBAH assay. In comparison, the two macromethods (the Blue Dextran Assay and the alcohol-haze test) were, respectively, 190 and 170 times less sensitive than the PAHBAH assay. This comparison highlights the need for the development of the micro-scale methods. The dextranase produced by all microbial isolates could now be rapidly assessed in the cell-free culture extracts.

APPENDIX 2A

Laboratory Notes for The PAHBAH ASSAY

Reagents:

Dextran Solution (0.4 %)

Dextran T2000 (Pharmacia) 0.4 g is dissolved gradually in distilled water and prepared using the method given in the experimental section.

Citrate buffer (0.5 M), pH 5.0

Weigh out 147.0 g tri-sodium citrate and dissolve in approximately 900 mL distilled water. Adjust the pH to 5.0 with concentrated HCl and make to 1000 mL with distilled water. This solution will keep for 2-3 weeks if stored at 4°C.

Citrate Buffer (0.05 M), pH 5.0

Dilute 100 mL of the 0.5 M citrate buffer to 1 L (adjust pH to 5.0 if necessary). This solution is prepared fresh daily.

PAHBAH Reagent

PAHBAH Solution A:- 0.5 M tri-sodium citrate

- Weigh out 73.5 g tri-sodium citrate, transfer to 500 mL volumetric flask and make to mark with distilled water.

PAHBAH Solution B:- 1.0 M sodium sulphite

Weigh out 63.0 g of sodium sulphite, transfer to 500 mL volumetric flask and make to the mark with distilled water.

PAHBAH Solution C:- 0.2 M calcium chloride

Weigh out 14.70 g $CaCl_2.2H_2O$ (MW 147.02 g), transfer to 500 mL volumetric flask and make to the mark with distilled water.

PAHBAH Solution D:- 5.0 M sodium hydroxide

Weigh out 100 g NaOH, dissolve in 1 L plastic beaker with distilled water, cool to room temperature in water bath. Transfer to 500 mL volumetric flask and make to the mark with distilled water.

PAHBAH REAGENT:

Into a 50 mL volumetric flask pipette -

5 mL solution A (0.5 M tri-sodium citrate)

5 mL distilled water, mix well

5 mL solution B (1.0 M sodium sulphite), mix well

5 mL distilled water, mix well

5 mL solution C (0.2 M calcium chloride), mix well

5 mL distilled water, mix well

5 mL solution D (5.0 M sodium hydroxide), mix well

Add 0.761 g PAHBAH, wash into flask with distilled water and make volume to the mark.

Test Procedure

1. **Incubation Mix:- Total Volume 0.7 mL.**

   Using micro pipettes prepare the following incubation mixes, omitting the enzyme component until after step 2 has been carried out.

   a) Substrate Blank : 400 µL dextran solution
   100 µL buffer solution
   200 µL distilled water

   b) Enzyme Blank : 400 µL distilled water
   100 µL buffer solution
   200 µL enzyme solution or { x µL enzyme solution
   { y µL distilled water
   { where x + y = 200}

   Enzyme blank solutions are prepared in single for each of the enzyme solutions being tested under the assay conditions.

   c) Test Solution : 400 µL dextran solution
   100 µL buffer solution
   200 µL enzyme solution or { x µL enzyme solution
   { y µL distilled water
   { where x + y = 200}

Test solutions are prepared in duplicate for each of the enzyme solutions being tested under the current assay conditions.

2. Incubate the tubes for 5 min in a water bath equilibrated at 55°C (or the required temperatures as used for determination of temperature optima).

3. At the end of the 5 min (in step 2 above), pipette 200 µL (or the required amount) of the test solution into the sample tubes. Mix thoroughly and return to the water bath for a further incubation period of exactly 30 min (or the required time *e.g.* 15 min for pH optimum and velocity (V) vs [E] experiments).

4. Stop the enzymic reaction by adding 50 µL of the incubation mix into the second set of tubes containing:-

   PAHBAH reagent (1.0 mL)

   distilled water (450 µL)

   Mix the solutions well after addition of the 50 µL aliquot.

   Prepare a Reagent blank to contain :-

   PAHBAH reagent (1.0 mL)

   distilled water (500 µL)

   Prepare a set of Glucose standards (1 - 10 µg) to contain :-

   PAHBAH reagent (1.0 mL)

   distilled water (450 µL)

   glucose solution (50 µL)

5. Place the second set of tubes (colour reagent mixes) in a boiling water bath for 10 min.

6. Transfer the tubes to a water bath at about 20°C and cool to room temperature for 5 min.

7. Read the absorbance of the solutions at 420 nm in a 1-mL cell against distilled water on a Cary 118C or similar spectrophotometer.

8. If the absorbance is higher than 1.5, the analysis must be repeated using a lower enzyme concentration.

9. Average the absorbance readings obtained for the test solutions.

10. Subtract the reagent blank value from all other absorbances. Further subtract the substrate blank and enzyme blanks from the test solutions. The final absorbance value can then be equated in terms of glucose released (μg) per minute at the prescribed temperature. From this value calculate the enzyme activity in μmole glucose produced per min per mL enzyme.

APPENDIX 2B

Laboratory Notes for The Micro-Haze Method

PREPARATION OF STANDARD CURVE

Prepare standard curve as per alcohol-haze method ratios but volumes are reduced *i.e.* 1.0 mL TCA/sucrose/water/dextran and 1.0 mL ethanol therefore, total volume would be 2.0 mL. Detailed compositions of the standard solutions are presented in Table 2B.1.

Solutions:

TCA (10 %)	:	0.04 mL
Sucrose(50 %)	:	0.40 mL
Dextran solution	:	0 - 0.56 mL
Distilled Water	:	0.56 - 0 mL
Total Volume	:	1.0 mL
Ethanol	:	1.0 mL
Total Volume	:	2.0 mL

Read and record the absorbance at 720 nm at 20 min in a 1 cm cuvette. Prepare standard curve.

TABLE 2B.1 STANDARD DEXTRAN SOLUTIONS

Flask Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15 Blank
Volume of 100 g/L TCA (mL)	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Volume of 500 g/L Sucrose (mL)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Standard Dextran Solution (mL)	0	0.008	0.016	0.032	0.04	0.048	0.080	0.12	0.16	0.24	0.32	0.4	0.48	0.56	0
Distilled Water (mL)	0.56	0.552	0.544	0.528	0.520	0.512	0.48	0.44	0.40	0.32	0.24	0.16	0.08	0	0.78
Dextran Concentration (mg/L)	0	3.62	7.24	14.48	18.1	21.7	36.2	54.3	72.4	108.6	144.8	181	217	253.3	0
Dextran Concentration (ppm on brix)	0	39.8	79.6	159.3	199.1	238.9	398	597.3	796.4	1194.6	1593	1991	2389	2786	0
A_{720} (Corr)	0.000	0.000	0.001	0.007	0.011	0.014	0.029	0.054	0.083	0.146	0.224	0.298	0.383	0.465	0.000

Test Procedure for Temperature Profile

1. A number of water baths were set up at temperatures of 40, 50, 55, 60, 65, 70 and 75°C (higher if required).

2. 1 mL of the incubation mixture (dextran/buffer/sucrose) transferred to each 2-mL vials (to avoid evaporation at the higher temperatures the vials are of the type with screw caps). Each assay was carried out in duplicate. A zero-time assay or control was also prepared.

3. Pre-equilibrate solutions at the respective temperatures for 5 min.

4. Add 0.1 mL enzyme solution to test vial and 0.1 mL distilled water to the control, mix well and seal. Incubate the reaction mixture for 30 min.

5. Stop the enzymic reaction by transferring the solution to a 1.5 mL Ependorf tube to which 0.2 mL 10 % TCA had been added. Mix well and seal. Chill in ice bath for 5 min. Centrifuge the samples (Sorval Microspin 24S, 15 min, 12 000 rpm, room temperature) to remove any precipitate.

6. The test solution was prepared by transferring a 0.2 mL aliquot of the reaction mixture to a 2-mL tube containing 0.8 mL sucrose solution (20 brix). Denatured alcohol (1.0 mL) was slowly added and the solution was mixed by gently inverting three times. For preparation of blank solutions the alcohol was replaced by distilled water. The absorbance at 720 nm in 1 cm micro cuvette was read at 20 min.

The micro-haze method can be modified to use an alternative dextran substrate in the incubation mixture *e.g.* cane dextran. The cane dextran may be either the highly purified cane dextrans (isolated from the first expressed juice of deteriorated cane) or deteriorated juice containing cane dextran.

CHAPTER 3

Characterisation of the *Chaetomium gracile* Dextranase

3.1 INTRODUCTION

The dextranase (EC 3.2.1.11) produced by the mesophilic fungus, *Chaetomium gracile* was first isolated and studied by Hattori and co-workers (Hattori and Ishibashi, 1981; Hattori *et al.,* 1981). A strain of *C. gracile* was selected from over 550 fungal and 115 streptomyces isolates as the most potent dextranase producer. The enzyme was found to be stable over a wide range of pH (4-12) and at relatively high temperatures (40-60°C).

Subsequently, the enzyme was purified and characterised (Hattori *et al.*, 1981). Two forms of dextranase were isolated by the purification procedure employed. Each form was shown to be electrophoretically pure. Using their purification protocol, Hattori *et al.* (1981) obtained an increase in the specific activity from 70 units for the crude extract to 1920-1960 units for the purified forms of the dextranase *i.e.* about a 30-fold increase in purity.

A commercial preparation of the *C. gracile* enzyme has been available for industrial application since the early 1980's. In 1983, Inkerman *et al.* reported investigations which established this commercial dextranase was more efficient, particularly at low dextran concentrations, and more cost-effective than other

commercial enzymes available at that time. Continued assessment of commercial dextranases available on the Australian market has shown that the *C. gracile* enzyme remains the most cost-effective dextranase for utilisation in a raw sugar factory (Brown and Inkerman, 1992). A comparative assessment of five commercial preparations of dextranase available in the United States of America (De Stefano, 1988) confirmed the *C. gracile* dextranase to be more active against dextran than the other enzymes tested.

Despite the significant changes which are required to normal processing operations in the raw sugar factory to incorporate dextranase addition, the procedure for use of the enzyme industrially (Fulcher and Inkerman, 1974b) remains unchanged after almost two decades.

Detailed studies on the *C. gracile* dextranase were performed as part of this study to determine the physico-chemical characteristics of the enzyme(s) in the commercial preparation. This allowed the properties of promising thermostable dextranases to be compared directly to those of the *C. gracile* dextranase *i.e.* parameters would be measured under the same conditions in the same laboratory by the same analyst. The most important characteristics for commercial application being thermostability and cost-effective enzymic activity under factory conditions.

3.2. EXPERIMENTAL

3.2.1 Reagents

All chemicals were the best available analytical grade reagents as supplied by Ajax Fine Chemicals (Auburn, Australia) unless otherwise noted. Denatured and

absolute ethanol (CSR Limited, Sydney, Australia) was used as supplied or as aqueous solutions diluted with distilled water. UltraPure ammonium sulphate (enzyme grade) was obtained from Bethesda Research Laboratories (Gaitersburg, MD).

High purity glucose, maltose, isomaltose, isomaltotriose and stachyose were purchased from Sigma Chemical Co. (St. Louis, MO), as was p-hydroxybenzoic acid hydrazide (analytical grade).

Potato starch and carboxymethylcellulose (high viscosity) were purchased from BDH Chemicals Limited (Poole, England). Arabinogalactan and cellulose were purified from sugar cane by Drs. J.D. Blake and K.E. McNeil, respectively, formerly of the Sugar Research Institute. Pullulan ($\bar{M}_n$ 8.6 x 10^4) was obtained from Hayashibara Biochemical Laboratories (Okayam, Japan).

The standard sucrose (starch and dextrans free) was obtained from CSR Limited (Sydney, Australia). Commercial dextrans (MW 2 x 10^6) T2000 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The sucrose and dextrans were used without further purification. Polybuffer (Type 74 and 96) and polybuffer exchanger (Type 94) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Sephadex G-200 and the gel filtration media, Sepharose CL-6B, were also obtained from this company.

The dry resins CM-cellulose (Selectacel CM-S Type 20) and DEAE-cellulose (Selectacel Type 20) were products of the Brown Paper Company, Berlin, New Hampshire.

Neuraminidase (influenza virus) was purchased from Calbiochem (Australia Limited).

Purified cane dextrans and deteriorated sugar cane juice were prepared as described in Chapter 2.

The thermostable α-amylase, Termamyl 120L, was purchased from Novo Industri, Denmark. The liquid preparation is a product of a selected strain of *Bacillus licheniformis.*

Dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11) isolated from *Chaetomium gracile* was supplied as a partially purified liquid preparation (in 50 % (v/v) glycerol) by Miles-Kyowa (Japan). The commercial enzyme preparation was stored at 4°C. A small 'purified' sample of the *C. gracile* dextranase was a gift from Miles-Kyowa, Japan. This sample was used without further purification.

3.2.2 Preparation of the Commercial *C. gracile* Dextranase for Experimentation

(a) Measurement of Enzymic Activity

The commercial dextranase preparation was used as supplied in 50 % (v/v) glycerol for activity assays. However, because of the high activity exhibited by this enzyme, dilution with buffer (pH 5.0, acetate or citrate) was carried out prior to assay.

(b) Preparation of a Dialysed Concentrate

A stock solution of the enzyme was prepared by the method of Brown and Inkerman (1992) from the commercial dextranase by exhaustive dialysis at 5°C against appropriate buffer. A gently stirred dialysed enzyme solution was slowly brought to 80 per cent saturation by the addition of solid ammonium sulphate. Once the salt had dissolved, the solution was allowed to stand for 1 hour and then centrifuged at 20 000 *g* for 30 min at 5°C. The resultant precipitate was redissolved in a minimum volume of the buffer and dialysed exhaustively against the same buffer. The dialysate was centrifuged at 5°C for 30 min to obtain an optically clear solution (Brown and Inkerman, 1992). This concentrated glycerol-free preparation was used for column chromatography, electrophoresis and the enzyme-HPLC method.

3.2.3 Measurement of Dextranase Activity

The dextranase activity was determined by one or more of the methods outlined in Chapter 2 *i.e.* the Blue Dextran assay, the PAHBAH Assay, the micro-Blue Dextran assay or the micro-haze method (using either purified dextran or deteriorated cane juice as the substrate).

3.2.4 Determination of Protein Concentration

(a) U.V. Absorption

The concentration of protein was determined by measuring the absorbance of an optically clear solution at 280 nm. This method was used for monitoring

protein in the eluent from column chromatography. A solution of the *C. gracile* dextranase containing 1 mg mL^{-1} has an absorbance of 1.9 at 280 nm (Hattori *et al.*, 1981) *i.e.* $A^{1\%}_{1cm}$ is 19.1.

(b) Biorad Standard Assay

Protein levels in the dextranase solutions were determined by the Biorad standard assay (Bradford, 1976).

3.2.5 Detection of Covalently Bound Carbohydrate

The detection of carbohydrate was by the phenol-sulphuric acid assay on exhaustively dialysed preparations of the enzyme. Colour development was carried out with minor modifications to the original method of Dubois *et al.* (1956). A 1-mL aliquot of each sample was pipetted into a clean, dry, lint-free test tube (24 mm x 150 mm) using A-grade pipettes. Each analysis was carried out in triplicate. Aqueous phenol (1 mL, 5% (w/v)) was added to each tube using a calibrated automatic dispenser. Thereafter, the solutions were thoroughly mixed using a vortex mixer (about 10 sec). A jet of concentrated sulphuric acid (5 mL) was added rapidly from a pre-calibrated automatic dispenser. The acid was directed onto the surface of the solution (rather than the side of the test tube) as this procedure results in instantaneous mixing of the reagents. After additional mixing (about 20 sec on a vortex mixer), the solutions were allowed to cool to room temperature (at least 30 min). The absorbance of the solutions was measured at 488 nm on a Cary 118C recording spectrometer against a distilled water blank.

3.2.6 Detection of Other Catalytic Activity

The dextranase from *C. gracile* was checked for other catalytic activity by estimation of the reducing sugars (Dygert *et al.,* 1965) released from the following carbohydrates: sucrose, potato starch, pullulan, carboxymethyl-cellulose, sugar cane cellulose and sugar cane arabinogalactan.

3.2.7 Measurement of the Hydrolytic Products by the Enzyme-HPLC Method

A detailed study of the products obtained upon hydrolysis of dextran, potato starch, isomaltose and stachyose was also carried out. These products were determined according to the enzyme-HPLC procedure described by Brown and Inkerman (1992).

(a) Enzymic Digestion and Sample Preparation. Solutions of the substrate (1.0%, pH 5.0, 0.025 M acetate) were incubated at 45°C for 4 hours with a total of 20 000 units (determined by the Blue Dextran assay) of dextranase added in three equal aliquots at zero time, 20 min and 40 min. The reaction mixture was then boiled for 5 min, cooled and deionised by shaking with 15 g of mixed bed ion-exchange resin for 30 min. The resin was removed by filtration through a 150 μ sieve and the hydrolysate evaporated to dryness. The hydrolysate was redissolved in 1 mL distilled water, filtered through an 0.45 μm membrane (Minisart, Satorius) and stored in 1 mL glass vials at -20°C until required for HPLC analysis.

(b) HPLC Analysis. Separation of the products of dextran hydrolysis was carried out using reverse phase chromatography according to the method of Ivin *et al.,* 1983. The HPLC system consisted of the following Waters equipment: Model 590 solvent delivery pump; WISP Model 710 B automatic sample injector; R410 differential refractive index detector; radial compression module RCM-100; Maxima 820 Chromatography Work Station. The column was a C18 Radial-PAK cartridge (5 μm particle size, 100 x 8 mm) fitted with a pre-column insert (C18 Guard-PAK) and pressurised in the RCM-100. The solvent, water, was vacuum filtered (0.45 μm, Millipore) and degassed prior to use. The injection volume was usually 10 μL and the flow rate constant at 0.5 mL min^{-1}. A two point calibration based on peak area integration was determined from duplicate injections of standard sugars covering the concentration range 50-1000 ppm on solids. The response for each standard sugar was shown to be linear over this range. A similar procedure was used for quantitative evaluation of the thawed hydrolysates.

3.2.8 Gel Filtration Chromatography

Protein purification was attempted and the apparent molecular weight of the dextranase was estimated by gel filtration chromatography, at 5°C, using the following markers: bovine serum albumin (MW 68 000) and ovalbumin (MW 43 500). Sepharose CL-6B was washed with distilled water to remove fines and equilibrated at 5°C with 0.05 M Tris buffer, pH 7.5, containing 0.15 M KCl. The column (2.5 x 30 cm) was packed under gravity. Between samples the column was washed exhaustively with the buffer until the eluent and feed buffer had the same absorbance at 280 nm.

The enzyme solution was dialysed against the column buffer prior to the application of 2.0 mL of the sample (containing a total A_{280} = 60) to the column. Fractions of approximately 2 mL were collected and weighed: the density of the buffer was taken as 1.00 in calculating elution volumes. The detection of proteins in the eluent was carried out by absorbance measurements at 280 nm. Elution volumes (V_e) were measured with respect to 0.2% (w/v) Blue Dextran 2000 which gave a measure of the void volume (V_o).

3.2.9 Ion Exchange Chromatography

CM- and DEAE-celluloses were prepared for chromatography by washing exhaustively with distilled water to removed "fines". Chromatography columns (1.8 x 15 cm) were packed under gravity. The columns were equilibrated at 5°C and washed exhaustively until the pH and A_{280} of the eluent were identical in value to those of the feed buffer. Linear gradient systems were used to elute the enzyme at a flow rate of approximately 1 mL min^{-1}.

3.2.10 Chromatofocusing (Pharmacia, 1982)

Polybuffer exchanger PBE 94 was equilibrated with the starting buffer (0.025M Tris-CH_3COOH, pH 8.3) and the column (1.0 x 40 cm) packed under gravity. The uniformity of the packing was confirmed using cytochrome c, a coloured and strongly basic (pI = 10.5) marker protein. A clear solution of the partially purified *C. gracile* dextranase (5 mL; 50 mg of protein) was loaded onto the chromatofocusing column. The proteins were eluted at a rate of 1 mL min^{-1} with polybuffer 96 (30%) and polybuffer 74 (70%) -CH_3COOH (pH 5.0) diluted 1:10.

Fractions of approximately 2 mL were collected and weighed: the density of the buffer was taken as 1.00 in calculating elution volumes. The detection of protein in the eluent was estimated by absorbance measurements at 280 nm. Enzyme activity and pH of aliquots were also measured. The results from the chromatofocusing run were utilised to determine the isoelectric point (pI) of the *C. gracile* dextranase following the method described by Sluyterman and Elgersma (1978) and Sluyterman and Wijdenes (1978).

3.2.11 Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed in polyacrylamide rods or slab gels using a Pharmacia vertical electrophoresis system at a constant voltage. All experimentation except staining was performed at 5°C. Vertical disc gel electrophoresis was carried out by the method of Inkerman *et al.* (1975a). Gels were formed in glass tubes 16 cm in length and 0.5 in internal diameter. Alternatively, vertical slab gels (4 mm thickness) were prepared using Pharmacia gel slab casting apparatus.

The gel solution (6% w/v) was prepared by the addition of reagents in the following order:

12 mL stock gel solution 20% (w/v) acrylamide and 0.6% (w/v) N,N'-methylene bisacrylamide;

4 mL 0.6 M tris-glycine buffer, pH 8.9 (0.2 M tris- 0.4 M glycine);

22.9 mL distilled water,

deaerate the solution under vacuum

1.0 mL ammonium persulphate (3.25% (w/v) - freshly prepared in water).

Polymerisation of the 6% gels was complete in about one hour at 5°C.

(i) Electrophoresis in the Presence of Sodium Dodecyl Sulphate

The following procedure was based on the method of Inkerman *et al.* (1975a). Bovine serum albumin, pepsin, chymotrypsinogen, haemoglobin were used as standard protein markers. Protein samples (2 mL, 1 mg mL^{-1}) were incubated at 37°C for 3 hours in 0.1 M phosphate buffer, pH 7.1, which contained 1% (w/v) sodium dodecyl sulphate (SDS). Thereafter, samples were dialysed against the same buffer.

Alternatively, protein samples were denatured by incubation for 5 min in 0.1 M at 100°C in phosphate buffer, pH 7.1, containing 1% (w/v) SDS and 1% 2-mercaptoethanol (ME).

Electrophoresis was carried out at a constant voltage (350 V), using 0.1 M phosphate as the anodic and cathodic buffer, until the dye front moved the length of the gel. Gels were fixed with TCA (10%, 10 min) prior to staining with coomassie brilliant blue (0.25% in 45% methanol and 9% acetic acid) overnight. Protein bands were visualised after destaining gels using methanol (45%): acetic acid (9%) in the Pharmacia destaining apparatus.

(ii) Gradient Polyacrylamide Gel Electrophoresis (Native PAGE)

Electrophoretic analysis of the native enzyme was performed by modification of the method of Inkerman *et al.* (1975b). Samples (200 µL) were prepared for electrophoresis by addition of sucrose (20% (w/v)) and bromophenol blue (20 µL, 0.025% (w/v)). The native protein (10 µL, 4.7 mg mL^{-1}) was loaded on to the gels

and electrophoresis carried out at a constant voltage (350 V), using the Pharmacia vertical electrophoresis apparatus. Proteins were visualised using the coomassie brilliant blue staining technique outlined above.

Detection of dextranase activity was attempted by incubating the gels in a Blue Dextran solution; however, no banding patterns were detected. Alternative activity staining methods of Mukasa *et al.* (1986) and Gabriel and Wang (1969) were also investigated without success.

To determine if all bands in the electrophoretogram of the *C. gracile* dextranase were active, gel segments were assayed after electrophoresis. The enzyme was loaded onto two slab gels and electrophoresed as described above. Three thin strips (one from the centre and one from each edge) were cut out of each slab, stained for protein and repositioned against the gel. The five sections of the gel corresponding to the protein bands were excised, mashed and placed in 1.0 mL citrate buffer (0.05 M, pH 5.0) to extract the enzyme. A Blue Dextran solution (3.0 mL, 0.4 %) was added to the gel extract and incubated at 60°C for 1 h. The reaction was stopped and the absorbance at 630 nm measured to detect activity.

Simplification of C. gracile Banding Pattern

Treatment with thiols: The *C. gracile* dextranase was dialysed against either 10 mM 2-mercaptoethanol or dithiothreitol (in 0.03 M tris-glycine buffer, pH 8.9) at 5°C as described by Dudman (1969) and Dudman and Zerner (1969). Thereafter, the samples were loaded onto the polyacrylamide gels, electrophorised and stained as outlined above.

Treatment with neuraminidase: An equal volume (50 μL) of neuraminidase (315 IU mL^{-1}) was added to a sample of *C. gracile* dextranase. Thereafter, the sample was incubated at 25°C for 20 h (Campbell *et al.,* 1973) prior to electrophoresis.

3.3 RESULTS AND DISCUSSION

3.3.1 Temperature Optimum

The temperature optimum of the *C. gracile* dextranase was found to be 55°C by the rapid Blue Dextran assay in the presence of 20 brix sucrose (Figure 2.5) and the micro-haze test substrate dextran T2000 (Figure 2.9). A similar figure was also obtained by the PAHBAH assay (an exo-activity method) (Figure 3.1).

The specific activity for the commercial *C. gracile* preparation was calculated using the above results (Table 3.1). As the figure is very high at 55°C, this has made the enzyme very competitive commercially. (The enzyme is used in raw sugar factories at 55 - 60°C.)

The *C. gracile* dextranase was also assessed by the micro-scale version of the haze test. The results using cane dextran (in deteriorated cane juice) as the substrate are given in Figure 3.2. Maximum activity was again found to be exhibited at 55°C. Thus, the same value for the temperature optimum was obtained using independent methodology and either Pharmacia or cane dextrans as the substrate.

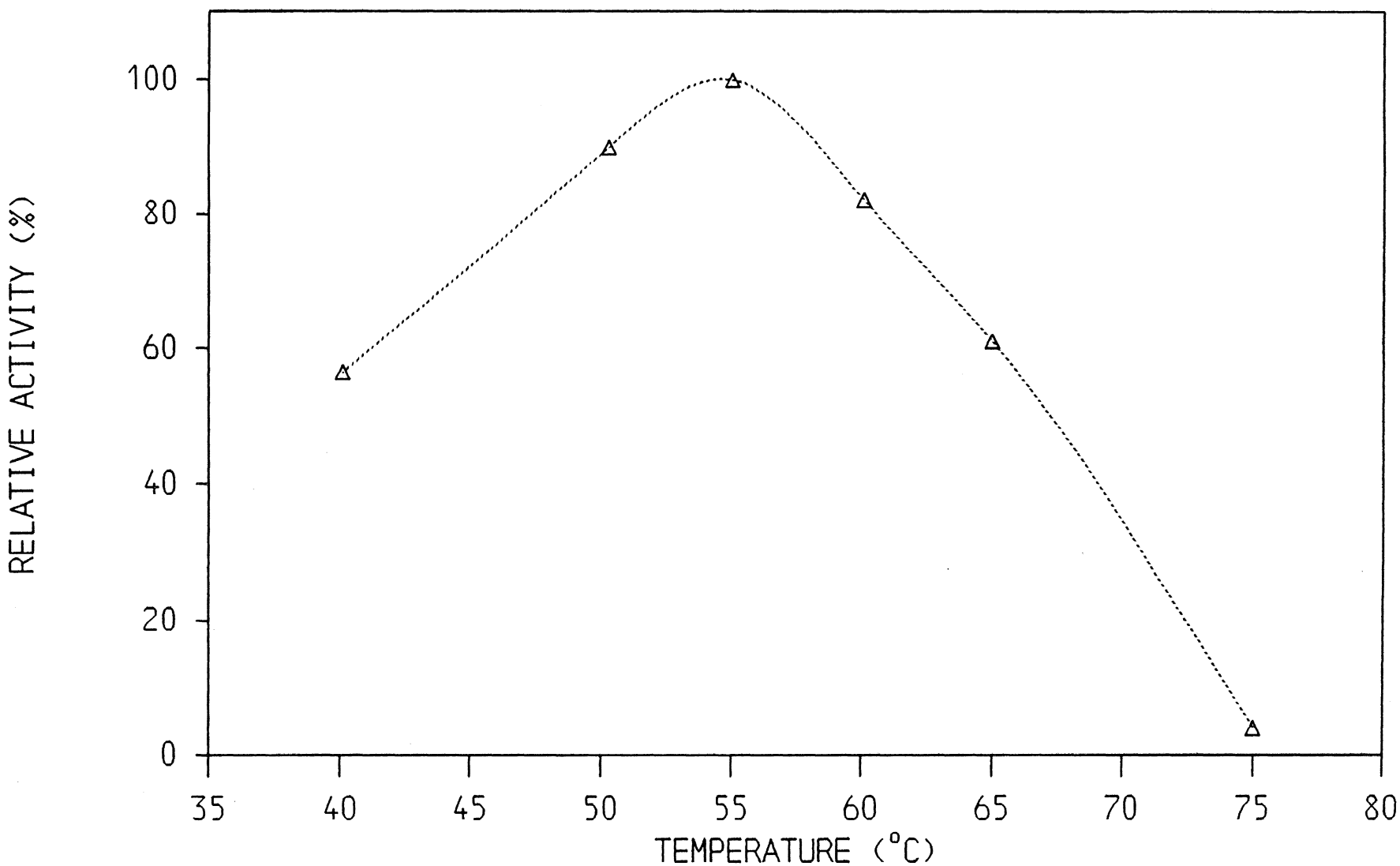


Figure 3.1 Temperature-activity profile for the *C. gracile* dextranase measured using the PAHBAH assay.

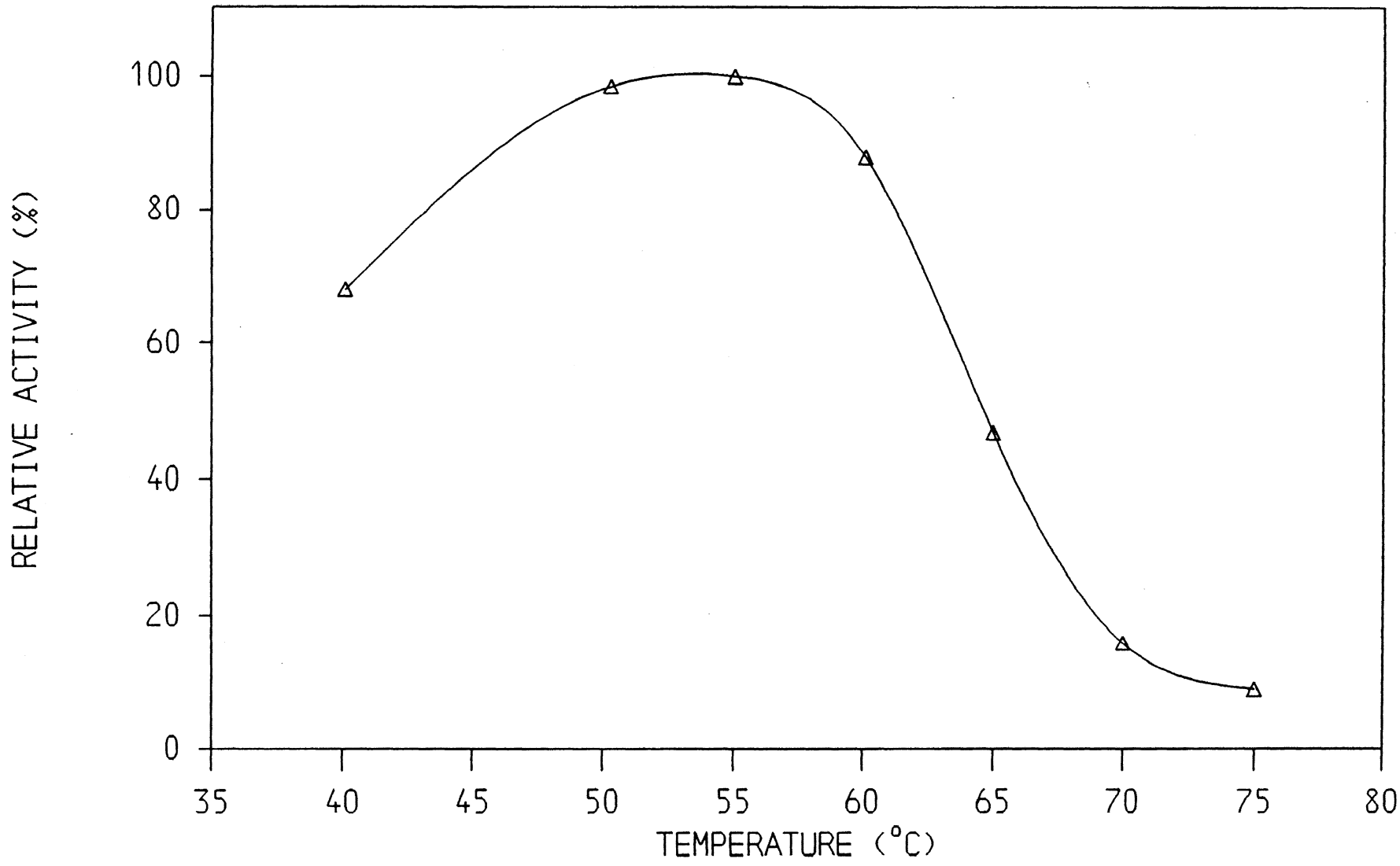


Figure 3.2 Temperature-activity profile for the *C. gracile* dextranase measured using the micro-haze test (cane dextran substrate).

TABLE 3.1. SPECIFIC ACTIVITY[a] OF THE COMMERCIAL PREPARATION OF THE *C. gracile* DEXTRANASE

Temperature (°C)	PAHBAH Assay (μmoles min^{-1} mg^{-1})	Micro-haze Assay[b] (ppm on brix min^{-1} mg^{-1})
55°C	2750	1.7×10^7
75°C	110	1.0×10^6

[a]Protein concentration in commercial enzyme samples ~4.2 mg mL^{-1}.

[b]Substrate was cane dextran in deteriorated cane juice.

This temperature optimum was 5-10°C lower than the result published by Hattori *et al.* (1981) using a viscometric method. However, the value of 55°C agrees with the figure obtained by these authors when assay conditions similar to factory conditions were used (Dr. S. Minato, private communications).

The data obtained by the various methods also suggest that the *C. gracile* dextranase exhibited significant activity above 65°C. These results should be treated with caution as they may be due to artifacts of the assay procedures. For example, in the assay mixture a time delay occurs before the added (cold) enzyme solution (5-200 μL) becomes equilibrated with the bulk of the hot assay substrate (~100 μL) solution. This would allow a small amount of product formation before the higher temperature is reached. Further there were practical problems associated with the maintaining of a constant high temperature in the small scale apparatus used in the assay procedures.

3.3.2 pH Optimum

The pH optimum for the *C. gracile* dextranase (at 60°C) was determined over the pH range 3-8 (Figure 3.3). Optimal activity for the enzyme was exhibited at 5.0. Similar profiles have been obtained using different assay methodologies.

3.3.3 Hydrolytic products of *C. gracile* dextranase

The products of exhaustive hydrolysis of cane dextran by *C. gracile* dextranase were separated by reverse phase HPLC (Brown and Inkerman, 1992). A typical chromatogram for cane dextran is shown in Figure 3.4. The order of elution as determined from retention times (RT) is buffer salts, glucose, isomaltose, isomaltotriose (when present). These are followed by the internal standard, stachyose, and higher MW isomaltodextrins.

The progress curve for the hydrolysis of dextran is given in Figure 3.5. No measurable enzymic degradation of isomaltose or stachyose was observed under the experimental conditions.

3.3.4 Other Catalytic Activity

The commercial preparation of the *C. gracile* dextranase was analysed for the presence of other enzymic activities (Table 3.2).

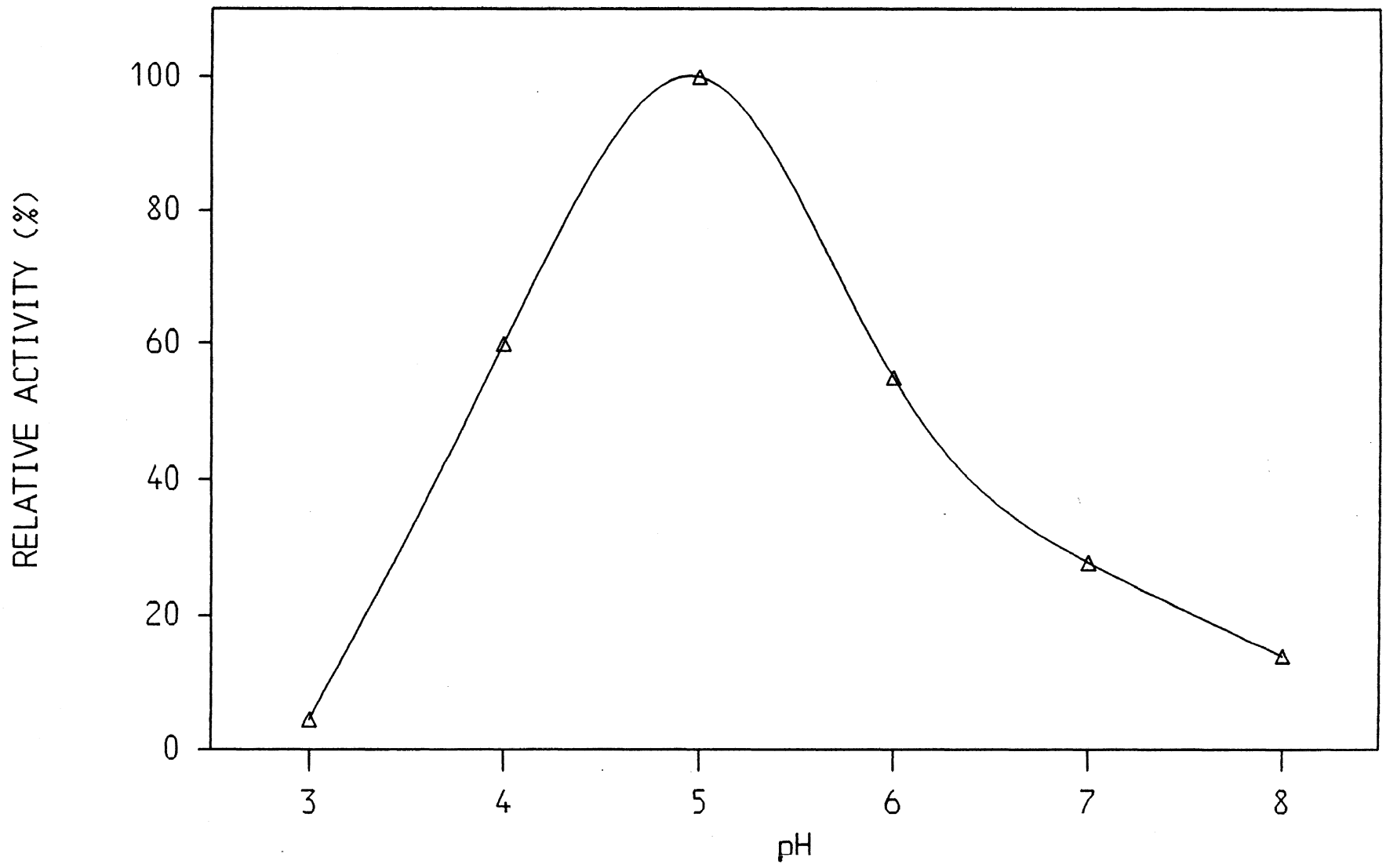


Figure 3.3 pH-activity profile for *C. gracile* dextranase at 60°C as measured by the Blue Dextran assay.

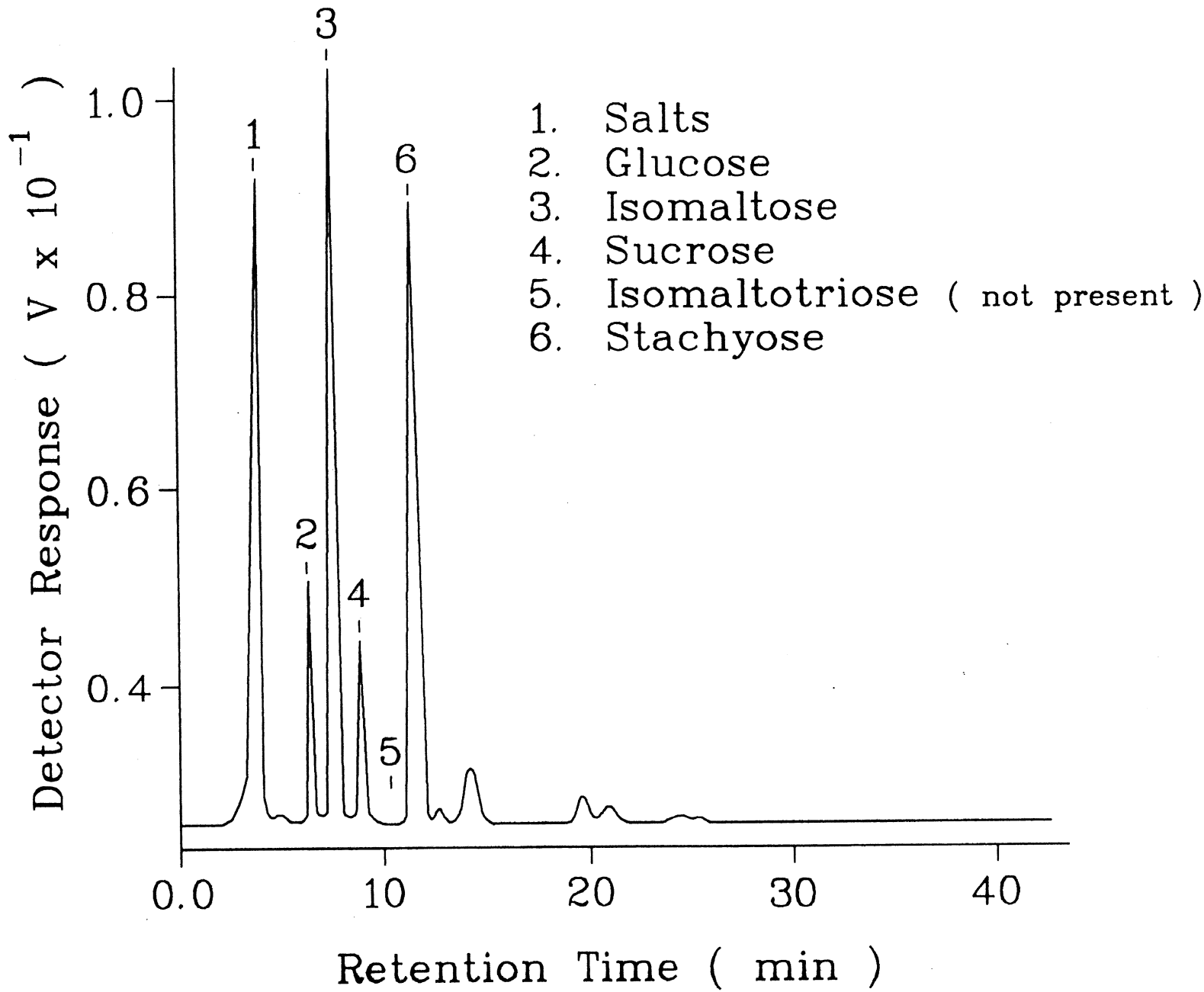


Figure 3.4 **Separation by HPLC of the sugars released from cane dextran after incubation with *C. gracile* dextranase.**

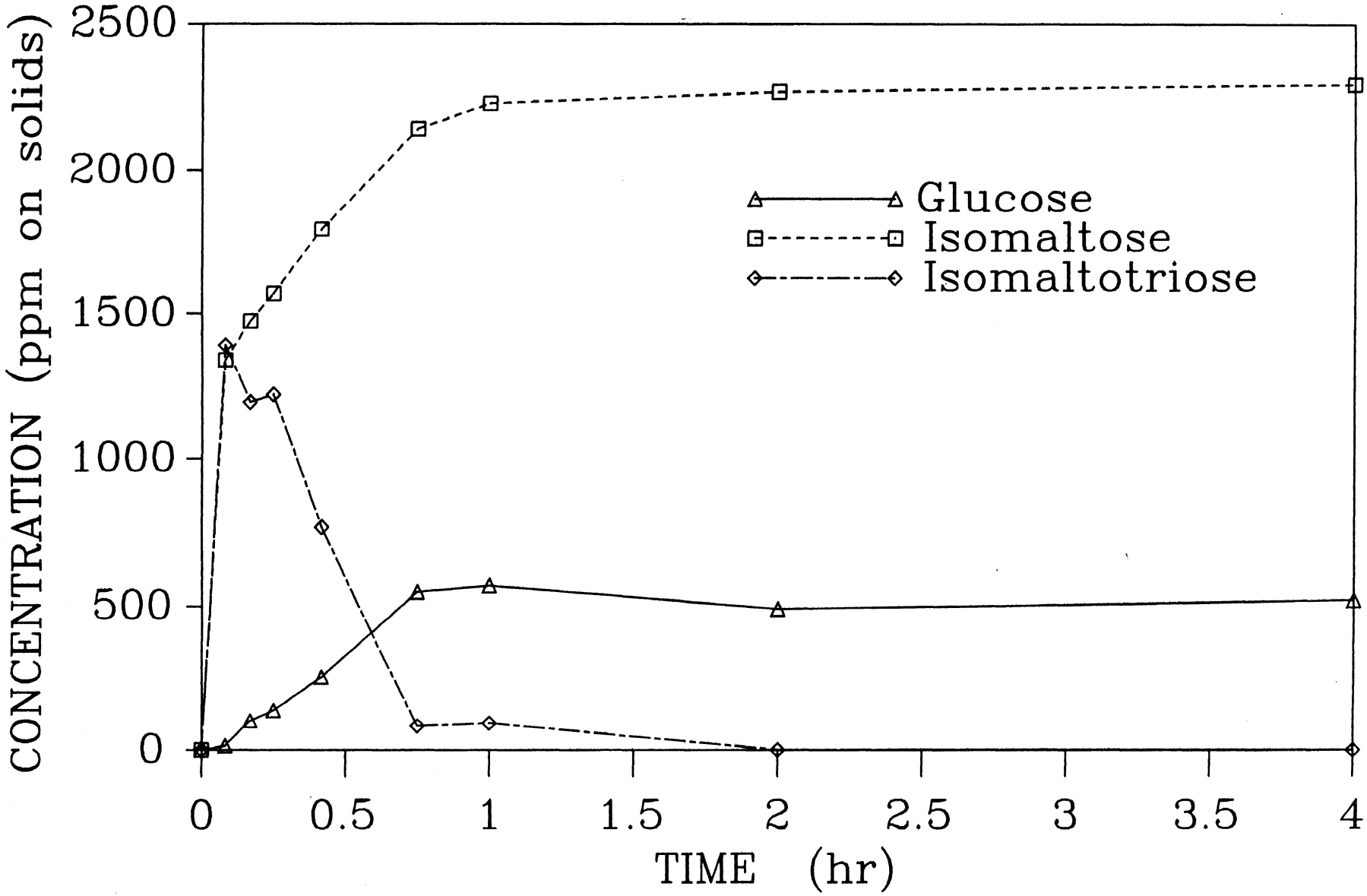


Figure 3.5 Progress curves for the *C. gracile* dextranase - catalysed hydrolysis of dextran.

TABLE 3.2 OTHER CATALYTIC ACTIVITIES PRESENT IN THE COMMERCIAL PREPARATION OF *C.gracile* DEXTRANASE

Impurity	Activity[a]
Amylase	Trace[b]
Invertase	Nil[c]
Pullulanase	Nil[c]
Cellulase	Trace[c]
Hemicellulase	Nil[c]
Arabinogalactanase	Trace[c]

[a]All tests carried out at pH 5.0. [b]Determined using a rapid α-amylase assay (Inkerman, unpublished). [c]Determined using the reducing sugar method of Dygert *et al.* (1965).

Commercial *C. gracile* dextranase also exhibits no hemicellulase, proteinase, ribonclease, aminopeptidase or carboxypeptidase activity (Dr. S. Minato, private communication). Thus, the commercial *C. gracile* dextranase preparation is relatively free of other carbohydrase and enzyme activites.

The enzyme-HPLC method (Brown and Inkerman, 1992) was utilised in additional studies to detect other enzymic activities in commercial preparations of *C. gracile* dextranase. At enzyme concentrations 10 times the levels used for dextran analysis, no other catalytic activity was detected against any of the other carbohydrates listed (*cf.* Hattori *et al.,* 1981), with the exception of potato starch from which glucose was released. Glucose was shown by HPLC analysis to be the sole product of enzymic hydrolysis of starch. This exo-activity was later found to be exhibited by all the partially separated electrophoretic variants of the *C. gracile* dextranase. Under the same conditions, a chromatographically pure dextranase

from *Penicillium sp.* was also found to exhibit a similar exo-activity against potato starch (Brown and Inkerman, unpublished). This data suggests that these dextranases may exhibit real catalytic against terminal α,1$\rightarrow$4 glycosidic bonds.

3.3.5 Electrophoresis of the Commercial *C. gracile* Dextranase

(i) Electrophoresis in the Presence of Sodium Dodecyl Sulphate

A sample of the commercial dextranase (as supplied by the manufacturer) was electrophoresed to determine its state of purity. Initially, the sample was denatured prior to electrophoresis by incubation with SDS (1%) at 37°C. The resulting pattern (a broad diffuse band) was indicative of a very impure protein or incomplete dissociation of the protein during sample preparation. Subsequently, electrophoresis was repeated using a dextranase sample which had been incubated at 100°C with both SDS (1%) and ME (1%).

Using these conditions, the preparation appeared as a single protein band possessing an average MW of 73 000 ± 4000 (Figure 3.6). A number of determinations were carried out with different protein loadings.

(ii) Polyacrylamide Gel Electrophoresis (Native)

Electrophoretic analysis of the native enzyme was performed on a commercial preparation of the *C. gracile* dextranase. At least five distinct protein

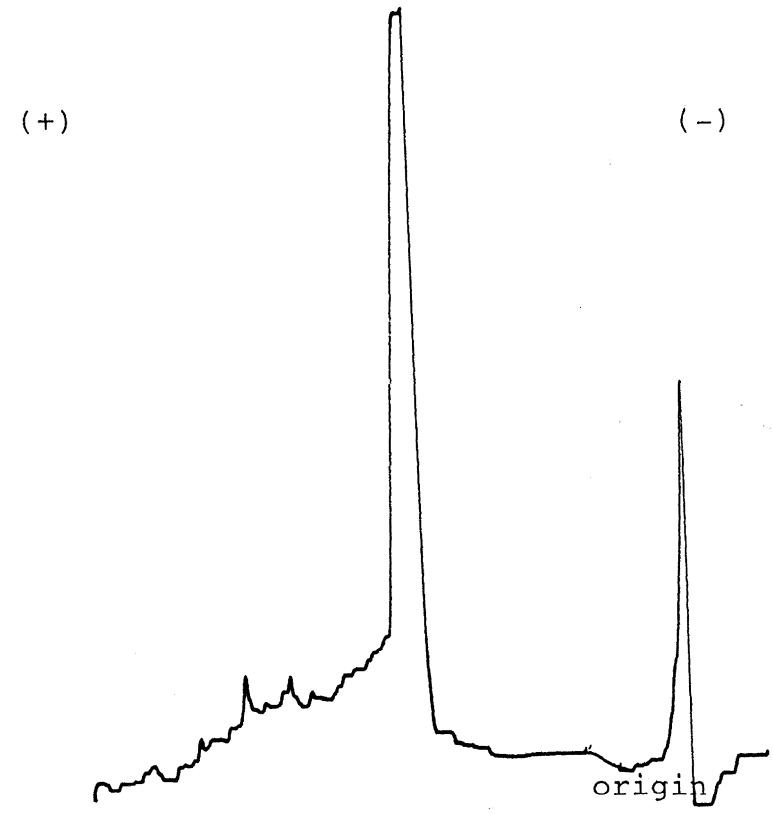


Figure 3.6 Densitometric trace of a polyacrylamide gel electrophoretogram of the commercial preparation of the *C. gracile* dextranase (in the presence of SDS and ME).

bands were visible after staining with coomassie brilliant blue. Figure 3.7 shows a densitogram of the relative positions and intensities of the bands.

Attempts were made to simplify the banding pattern obtained for the native *C. gracile* dextranase. One technique investigated was the dialysis of the enzyme in a thiol (10 mM of 2-mercaptoethanol or dithiothreitol) prior to electrophoresis (Dudman, 1969; Dudman and Zerner, 1969). However, the number and relative positions of the protein bands did not change after this treatment (Figure 3.8).

An alternative technique for simplification of electrophoretic patterns of enzymes investigated was treatment with neuraminidase (Campbell *et al.,* 1973). Again the number and relative positions of the protein bands in the electrophoretogram were not altered compared to those of the native *C. gracile* dextranase.

To determine if all five bands detected after electrophoresis of the native *C. gracile* enzyme were active dextranases, gel segments were extracted and assayed for activity using a Blue Dextran assay. The results are presented in Table 3.3. All bands were found to be active against the Blue Dextran substrate with the greatest activity being found in the most intense central band.

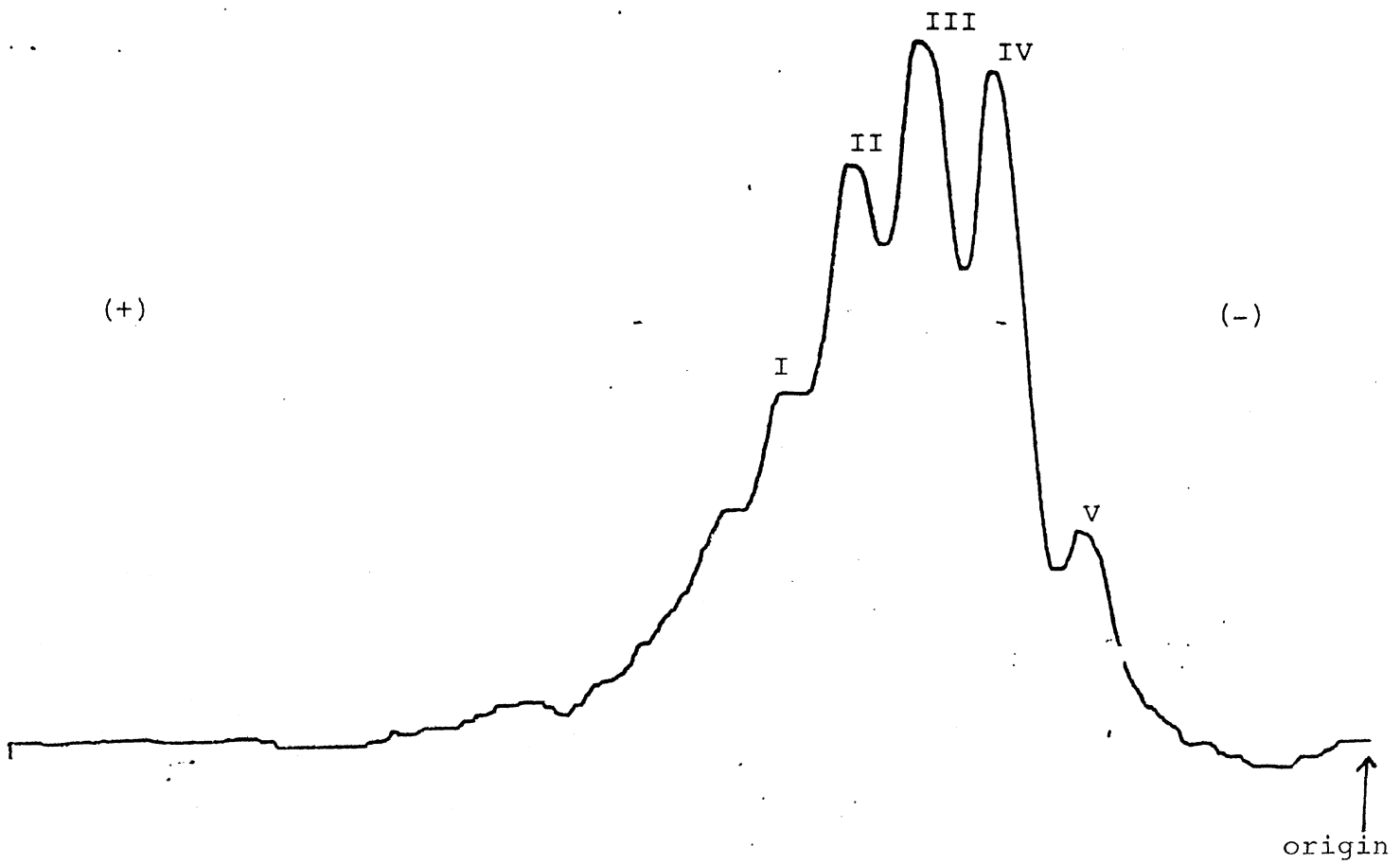


Figure 3.7 **Densitometric trace of a polyacrylamide gel electrophoretogram of the commercial preparation of the *C. gracile* dextranase**

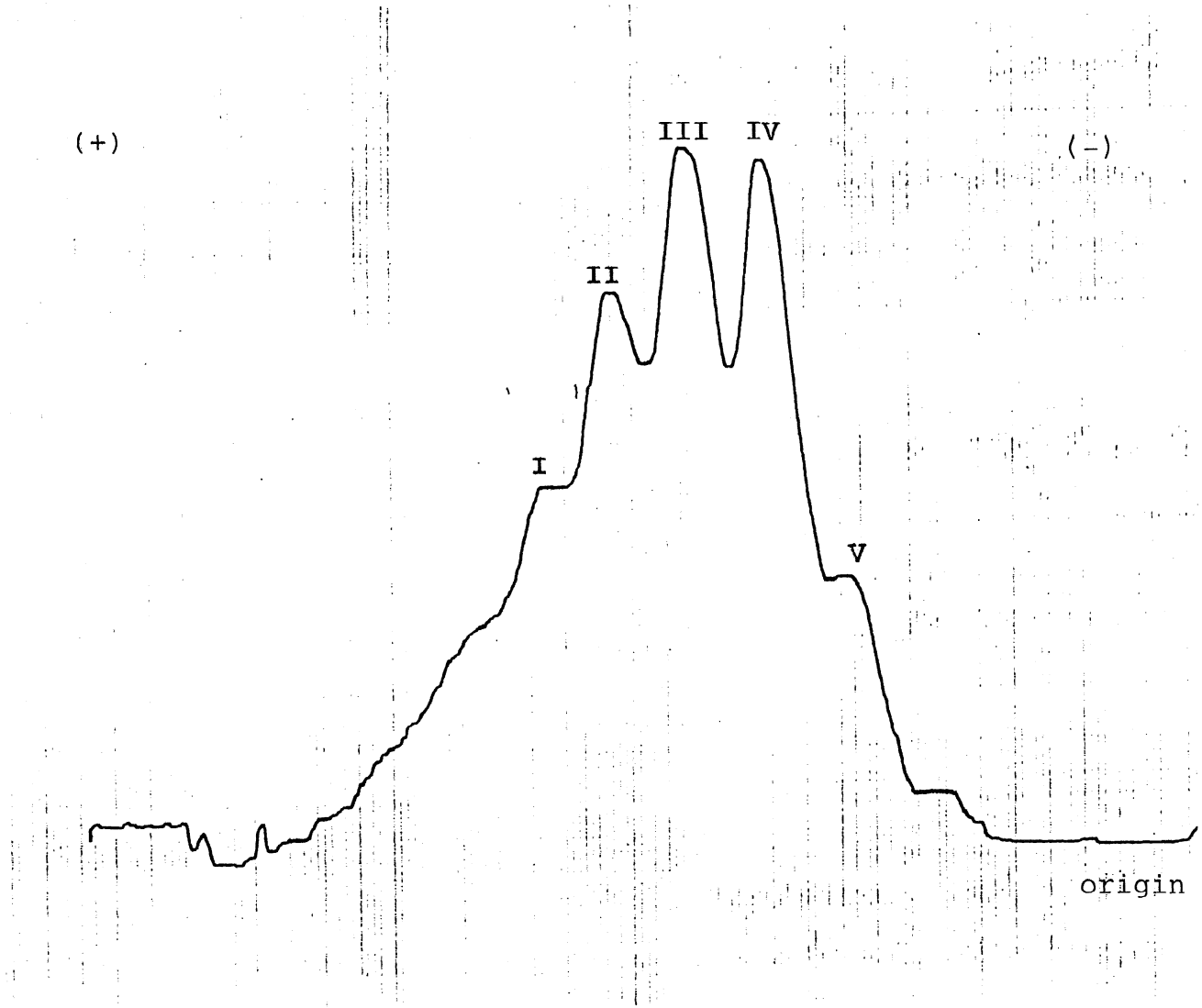


Figure 3.8 Densitometric trace of a polyacrylamide gel electrophoretogram of the *C. gracile* dextranase after thiol treatment.

TABLE 3.3 ACTIVITY[a] OF INDIVIDUAL BANDS OF THE NATIVE *C. gracile* DEXTRANASE.

Section Number	A_{630}		
	Gel 1	Gel 2	Average
1	0.927	0.971	0.949
2	0.996	1.069	1.033
3	1.012	1.106	1.059
4	0.980	1.092	1.036
5	0.940	1.052	0.996

[a]Activity measured using the Blue Dextran assay.

3.3.6 Electrophoresis of the 'Purified' *C. gracile* Dextranase

(i) Electrophoresis in the Presence of Sodium Dodecyl Sulphate

The 'purified' *C. gracile* dextranase (supplied by Dr. S. Minato) was incubated at 100°C with SDS (1%) and ME (1%). The sample was electrophoresed using SDS polyacrylamide gel electrophoresis. A single protein band was observed on all gels (Figure 3.9). The molecular weight for the enzyme was found to be 72 800 ± 3 000. Hattori *et al.* (1981) previously reported values of 71 000 and 77 000 for two purified forms of the *C. gracile* dextranase.

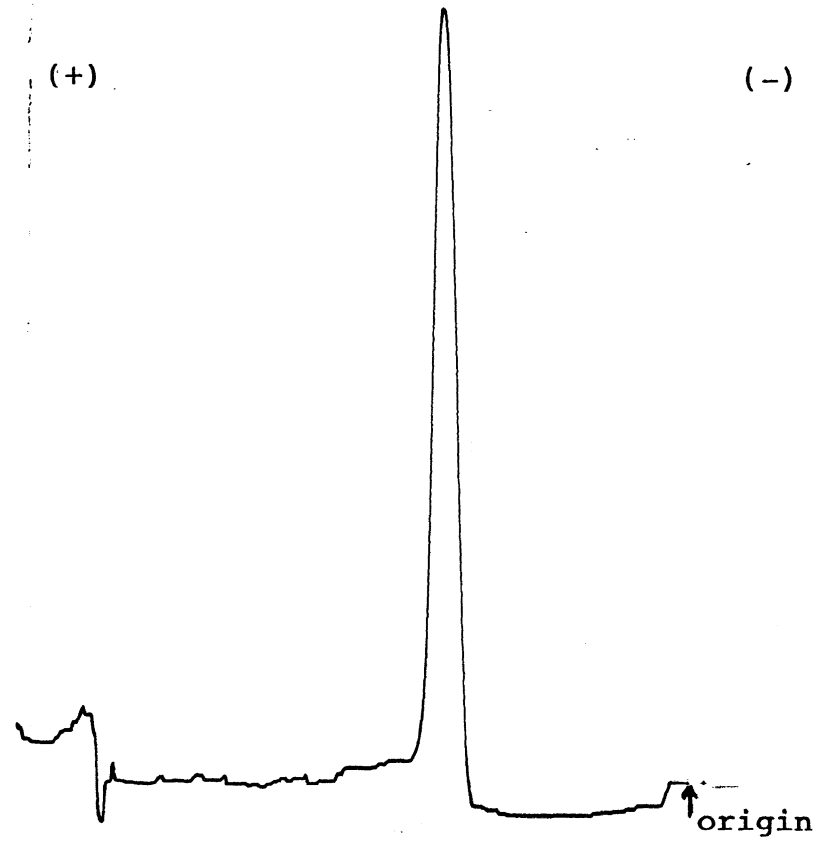


Figure 3.9 Densitometric trace of a polyacrylamide gel electrophoretogram of a 'Pure' preparation of the *C. gracile* dextranase (in the presence of SDS and ME).

(ii) Polyacrylamide Gel Electrophoresis (Native)

Electrophoretic analysis of the native enzyme was performed on the 'purified' *C. gracile* dextranase. Three distinct protein bands were visible after staining with coomassie brilliant blue. Figure 3.10 gives a densitogram showing the relative positions and intensities of the bands. These bands correspond to band numbers II, III and IV found in the commercial preparation of the *C. gracile* dextranase.

3.3.7 U.V. Absorption Spectrum

The purity of the commercial *C. gracile* dextranase (as supplied by the manufacturer) was also assessed by measurements taken of the UV absorption spectrum. The spectrum is shown in Figure 3.11. The A_{280}/A_{260} was taken as a measure of the purity of the protein (Warburg and Christian, 1941).

The statistical average for this ratio for pure proteins is 1.75 - 1.80. A value approaching this would demonstrate that the preparation consisted of mainly protein. The A_{280}/A_{260} value for the commercial *C. gracile* preparation was 1.6 (after appropriate correction was made for scattered light). The A_{280}/A_{260} value could be increased to 1.76 simply by exhaustive dialysis of the commercial preparation of the *C. gracile* dextranase. This value indicates that the sample contained a high level of protein.

Using the extinction coefficient for dextranase ($A^{1\%}_{1cm}$ = 19.0 at 280 nm) determined by Hattori *et al.* (1981), the protein concentration in the preparation was

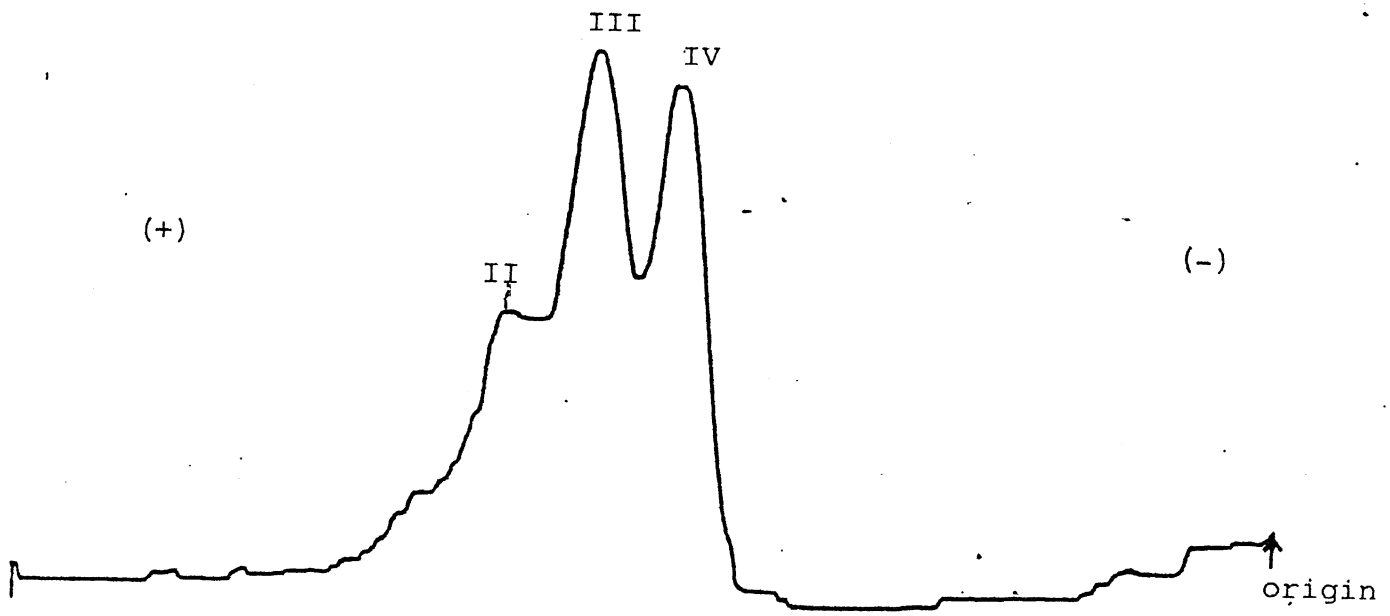


Figure 3.10 Densitometric trace of a polyacrylamide gel electrophoretogram of the 'purified' *C. gracile* dextranase.

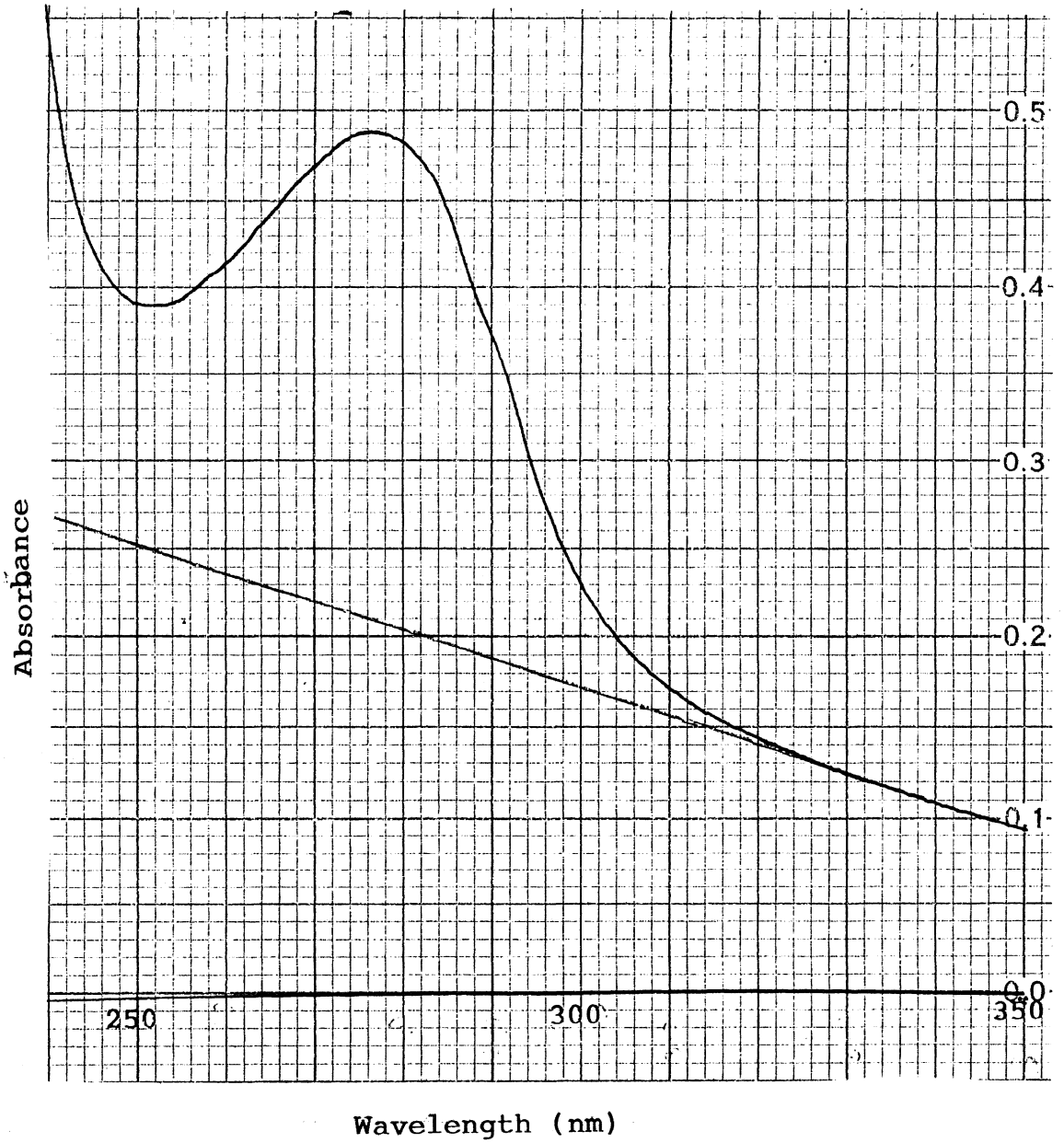


Figure 3.11 UV spectrum of the *C. gracile* (diluted 1/25 in 0.05 M citrate buffer, pH 5.0).

calculated to be 3.7 mg mL^{-1}. A similar value (4.2 mg mL^{-1}) for the protein concentration in the commercial *C. gracile* dextranase was determined using the Biorad assay.

3.3.8 Gel Filtration Chromatography

Preliminary investigations to develop a purification procedure for the *C. gracile* dextranase were carried out using gel filtration chromatography. The chromatography medium initially chosen was Sephadex G-200. However, the dextranase hydrolysed the gel even at the low temperature (5°C). Another medium, Sepharose CL-6B was therefore selected as the separation medium. No separation of protein components in the *C. gracile* dextranase was observed as the sample eluted from the column as a single Gaussian peak (Figure 3.12).

To check if separation of proteins had occurred, polyacrylamide gel electrophoresis was carried out on the peak fractions collected from the Sepharose column (Figure 3.13). The densitometric trace of the electrophoretogram confirmed that no separation of the bands in the native *C. gracile* dextranase was achieved.

In addition to protein separation, gel filtration may be used for molecular weight estimation. The technique was therefore utilised for the determination of the apparent molecular weight of the *C. gracile* dextranase preparation. Calibration of the column was carried out with proteins of known molecular weight. Using the

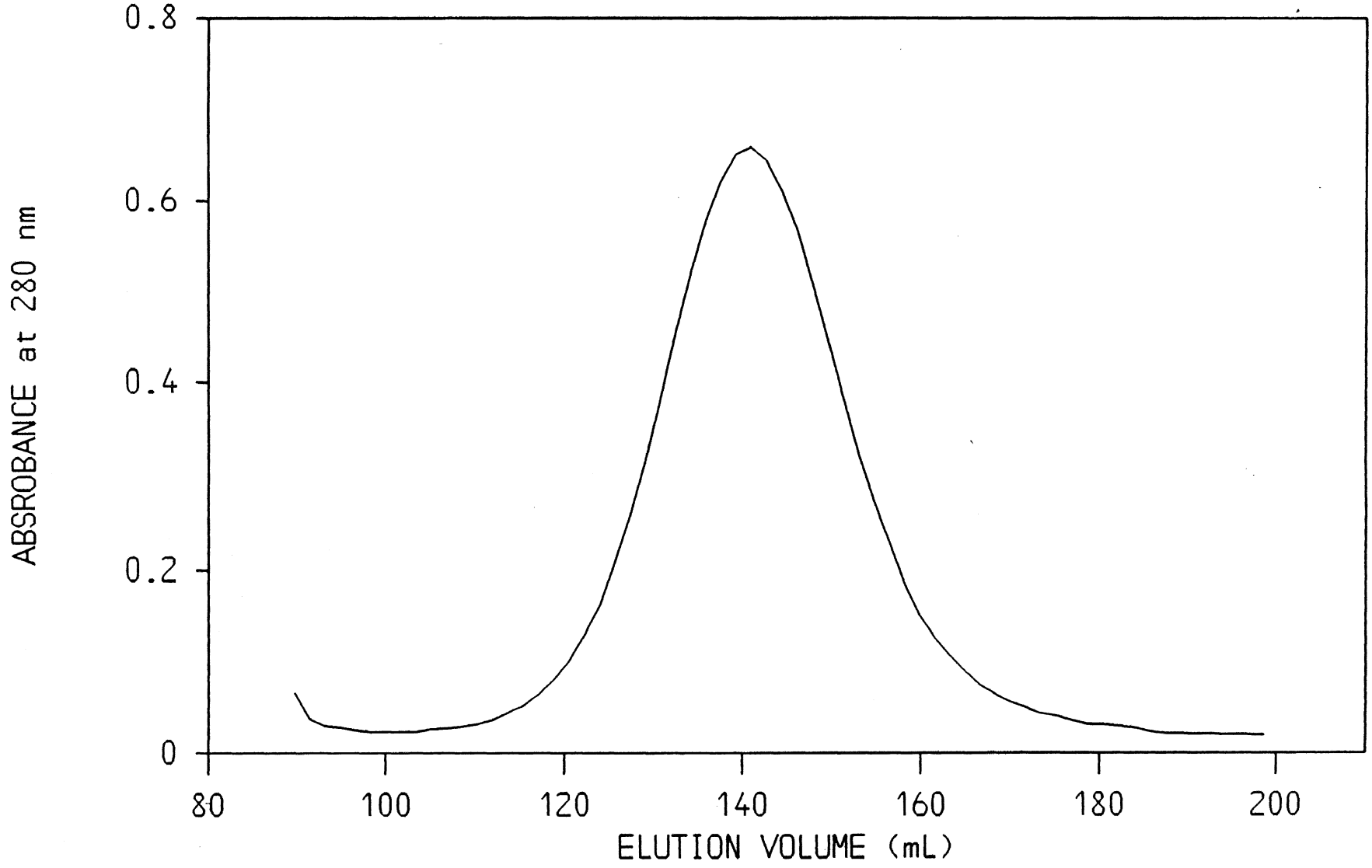


Figure 3.12 Elution profile for *C. gracile* dextranase on Sepharose CL-6B

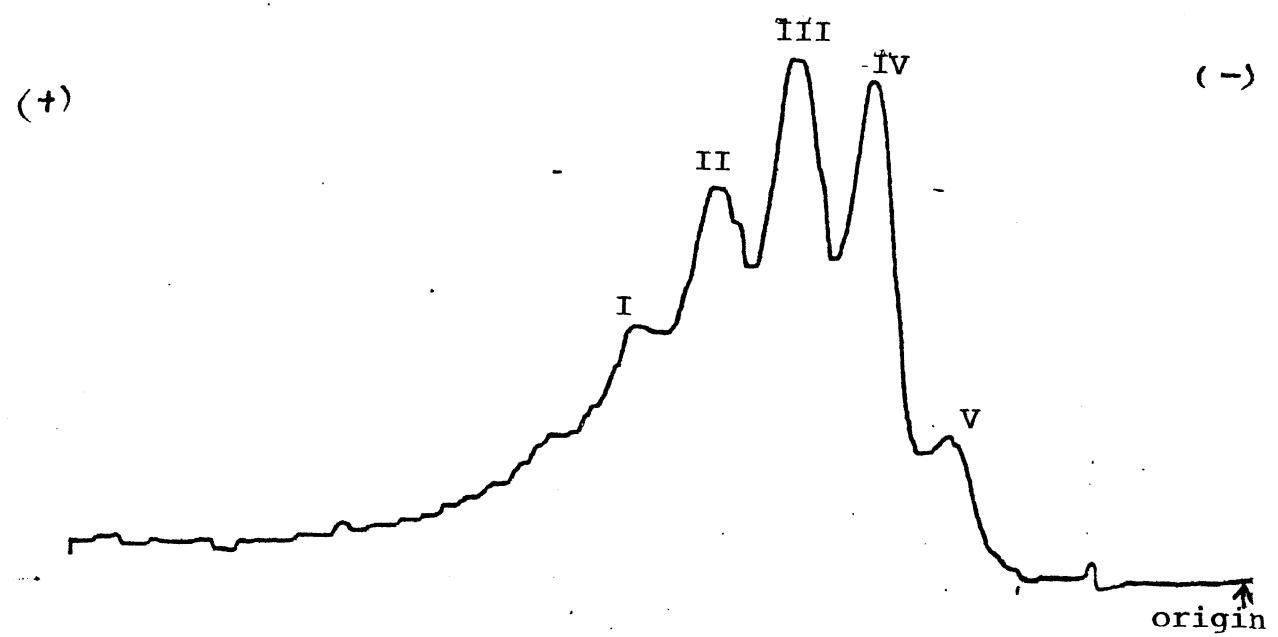


Figure 3.13 Densitometric trace of a polyacrylamide gel electrophoretogram of the 'peak fraction' obtained from the Sepharose CL-6B column.

method described by Inkerman (1973) the apparent molecular weight for the dextranase was calculated to be 31 000.

This value was less than half that obtained using the SDS/PAGE methodology. The reason for the difference may be explained if the *C. gracile* dextranase was a glycoprotein and/or bound to the support medium. Therefore, the carbohydrate content of the enzyme preparation was determined.

3.3.9 Covalently Bound Carbohydrate

An exhaustively dialysed sample of the *C. gracile* dextranase was assayed using the phenol-sulphuric acid assay (Dubois *et al.*, 1956). The total carbohydrate level in the protein was found to be about 10 per cent *i.e.* 10 mg carbohydrate was detected in a sample containing 100 mg protein. This level of carbohydrate would be expected to influence the molecular weight determination by gel filtration chromatography.

3.3.10 Ion Exchange Chromatography

Hattori *et al.* (1981) used sequential chromatography on CM- and DEAE-cellulose columns to purify the *C. gracile* dextranase. Ion exchange chromatography was carried out in an attempt to separate the protein bands in the commercial preparation of the *C. gracile* dextranase.

(i) CM-Cellulose Chromatography

A sample of the enzyme was dialysed against the column buffer (0.01 M acetate, pH 5.5; Hattori *et al.,* 1981). A 3.5 mL aliquot containing 20 mg protein was loaded on to the column and eluted using a linear salt (0-0.2 M NaCl) gradient. The resulting elution profile is depicted in Figure 3.14. Most of the protein was eluted early in the profile indicating that it had not bound to the column. For this chromatographic run, the molarity of the buffer employed was low and the pH was close to the pI of the protein. The experiment was repeated using a higher buffer concentration and slightly lower pH. The enzyme sample and CM-cellulose column were equilibrated with 0.05 M acetate buffer (pH 5.0). A 3.5 mL aliquot was applied to the column and washed with 80 mL of the start buffer. The bound enzyme was eluted with a linear salt gradient (0-0.5 M sodium acetate). The data is given in Figure 3.15 indicates that the protein had bound to the column under these conditions with most of the protein being eluted well after the start of the linear salt gradient. However, no separation of the protein bands was achieved with only a single Gausian peak being detected.

(ii) DEAE-Cellulose Chromatography

An enzyme sample was dialysed against the column buffer (0.025 M Tris, pH 8.7). A 4.5 mL aliquot containing 30 mg protein was applied to the column which was washed with starting buffer. The bound enzyme was eluted with a linear salt gradient (0 - 0.5 M NaCl). The elution profile is presented in Figure 3.16. Under the conditions employed, no separation of the protein bands was achieved. Hence, the separation of commercial *C. gracile* dextranase preparation using ion exchange chromatography as reported by Hattori *et al.* (1981), was not able to be repeated in this work using their procedures or modifications thereof. As the protein was able

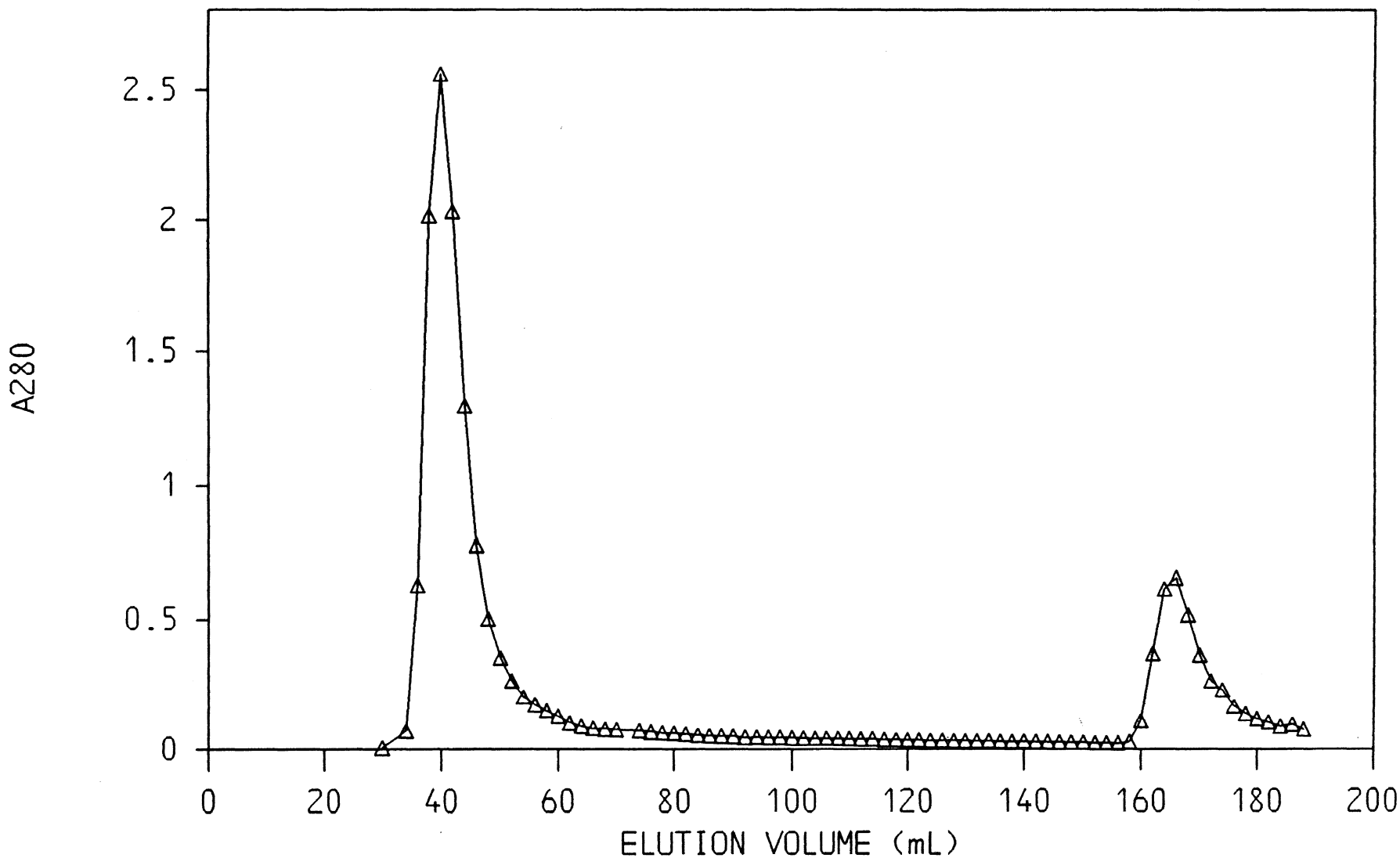


Figure 3.14 CM-cellulose chromatography of *C. gracile* dextranase

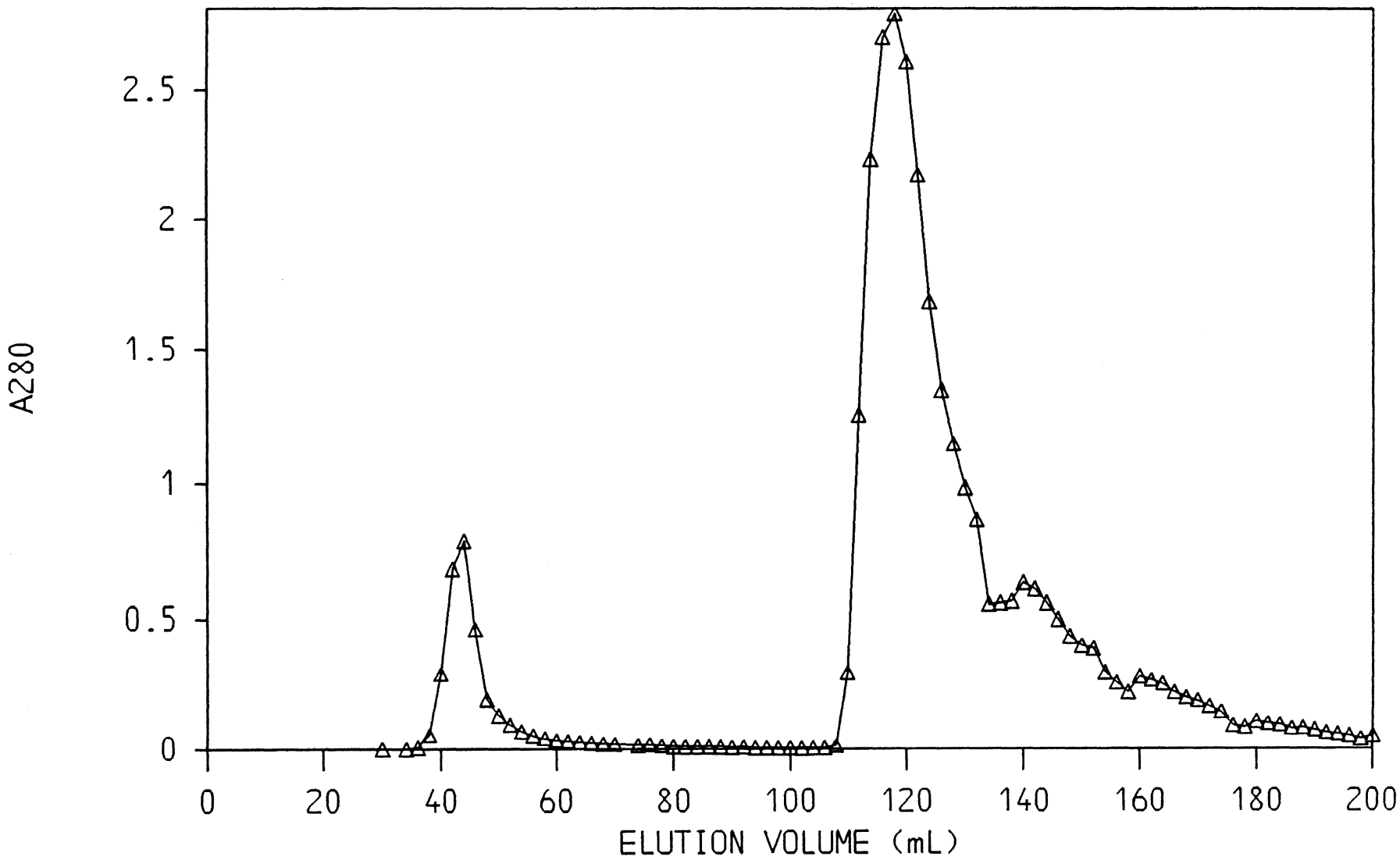


Figure 3.15 CM-cellulose chromatography of *C. gracile* dextranase.

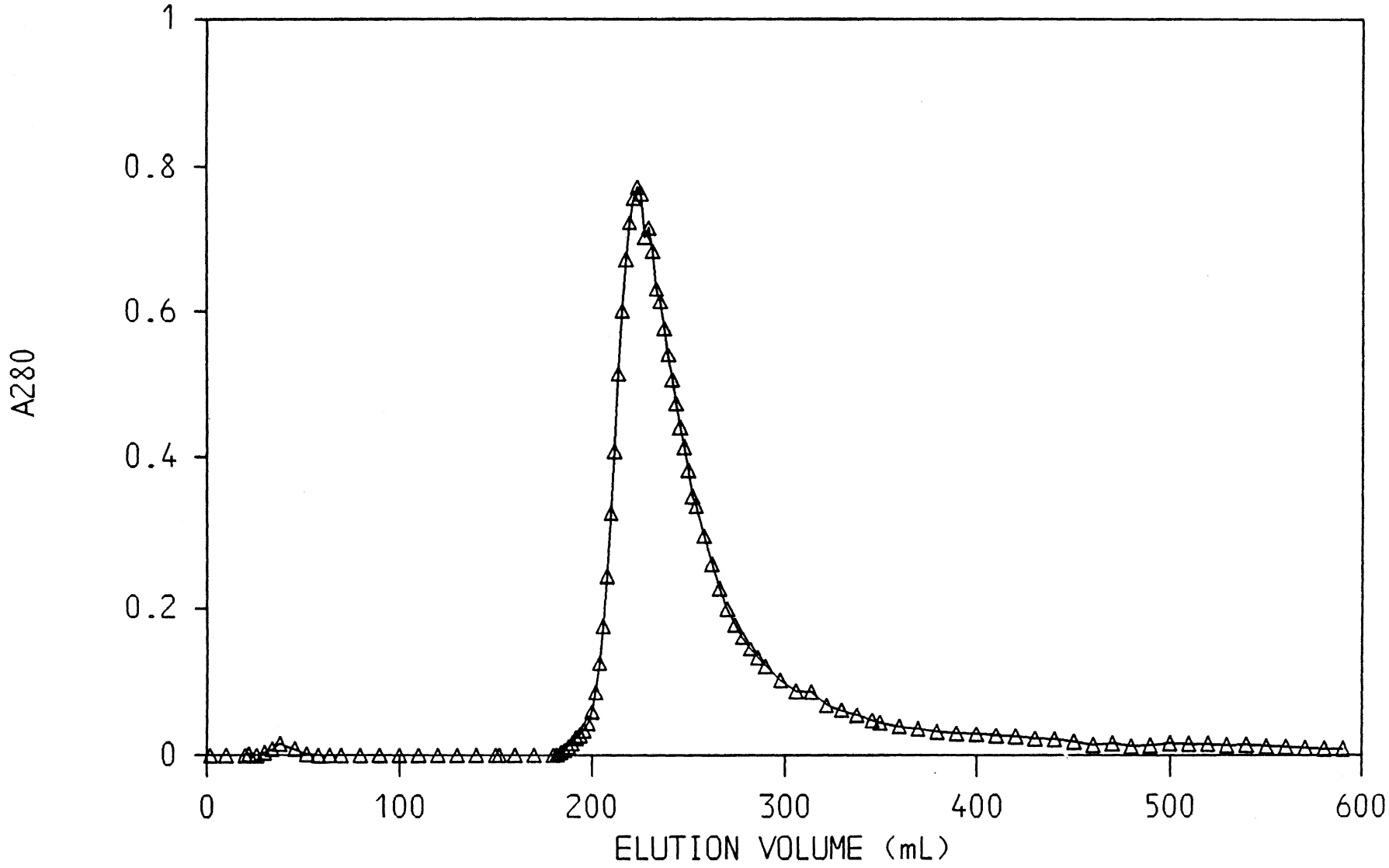


Figure 3.16 DEAE-cellulose chromatography of *C. gracile* dextranase.

CHAPTER 4

Sources of Microbial Dextranases

ACKNOWLEDGEMENT

The author wishes to acknowledge that the microbial investigations described in this chapter were carried out by co-workers involved in the high temperature dextranase project. The isolation and screening of microorganisms for dextranase-producers, the optimisation of conditions for growth and enzyme production by the bacteria and fungi, and the production of crude culture filtrates (using either batch or continuous culture techniques) was carried out by the following personnel at their respective research organisations: Miss L.M. Cox and Dr. M.W. Dawson (Sugar Research Institute), Miss S.R. Lucas and Dr. P.A. Inkerman (formerly Sugar Research Institute), Dr. B. Patel (Griffith University) and Dr. C. Wynter (Griffith University/University of Queensland).

A preliminary report on aspects of microbial screening for the project "Development of a High Temperature Dextranase" was presented at a poster session at the 1990 Australian Biotechnology Conference (Lucas *et al.*, 1990).

4.1 INTRODUCTION

The background and development of the techniques utilised for the isolation and screening of the dextranase producing microorganisms will not be detailed in this thesis. However, for continuity and completeness the microbial aspects of this

research project are presented in summarised form. The inclusion of the microbial research was considered necessary to give an insight into the sources of the thermostable dextranases under study. The basic approach used by the microbiologists was to attempt to find in nature a microorganism (or several microorganisms) which produced a dextranase with a temperature and pH optima of 75°C and 5.0, respectively. Initial studies were directed towards developing specific methodology for the screening, isolation and growth of dextran-hydrolysing microorganisms. Particular emphasis was placed on the thermophiles *i.e.* those microorganisms which grew at high temperatures, in particular, in the range 55 to 75°C.

4.2 EXPERIMENTAL

4.2.1 Sources of Microbial Dextranases

Isolates from Culture Collections

Bacterial and fungal isolates were obtained from a number of culture collections such as the American Type Culture Collection (ATCC), the United States Department of Agriculture (NRRL), the National Collection of Industrial and Marine Bacteria (NCIB), the University of Queensland (Department of Microbiology, UQM) and the Sugar Research Institute (SRI) Culture Collections.

Isolates from Factory Process or Environment.

Soil, water, gravel and bagasse were sampled in and around the sugar

factory at sites where temperatures were greater than 45°C. Cane field soils were also sampled. Samples were collected from within the mud filters, incubator and other points throughout the process, in particular near steam lines.

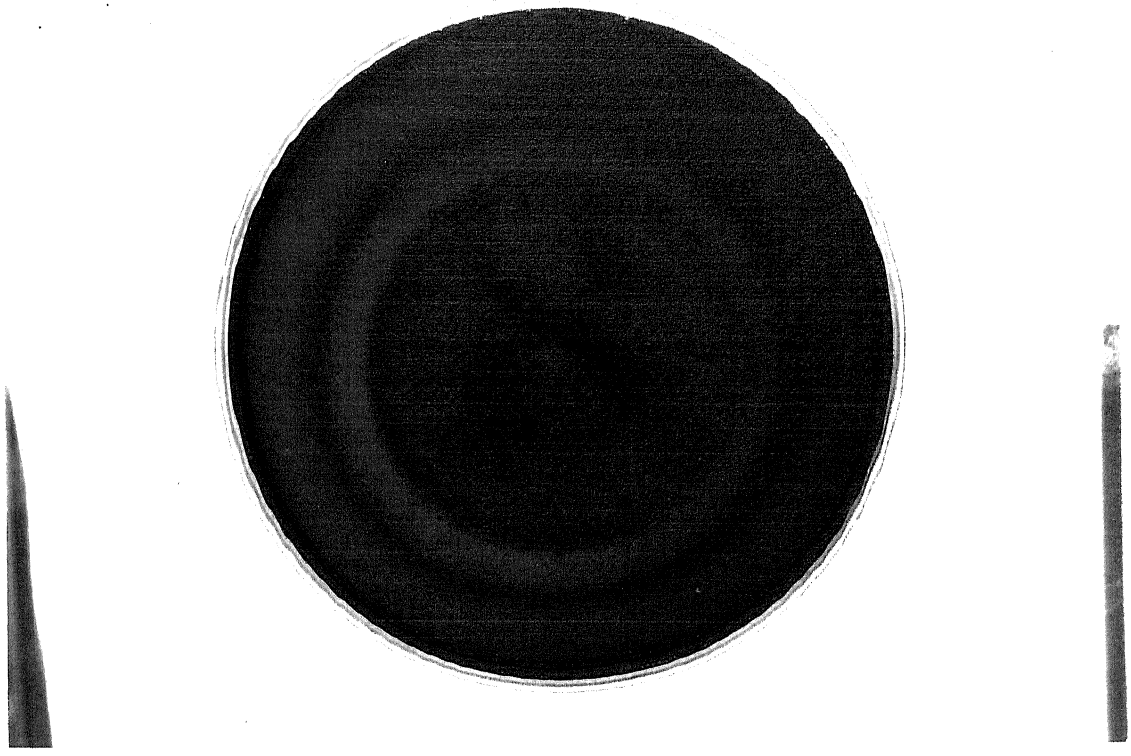
Isolates from Thermal Springs and Artesian Bores.

A number of water samples were collected from artesian bores by co-workers from Griffith University. Also, screening for thermophilic isolates previously collected from thermal springs was carried out to find producers of thermostable dextranases.

4.2.2 Screening of Dextranase Producers

Initially the screening procedure of Mencier (1972) was modified to detect microbial species capable of growing at high temperatures using dextran as a carbon source. This procedure involved the direct inoculation of Blue Dextran agar (BDA) plates with dilutions of the liquid or solid samples. Plates were incubated at temperatures ranging from 30 to 75°C (at 75°C special precautions were required to ensure agar did not dry out during incubation). Development of a clearing zone around growing colonies was indicative of dextranase production at the incubation temperature. Production of the enzyme was found to be proportional to the size of the clearing zone (or lighter halo) detected around the colony (Figure 4.1). Incubation was carried out under both aerobic and anaerobic conditions.

(a)



(b)

Figure 4.1 Clearing zones, indicating dextran hydrolysis, developed on blue dextran agar plates. (a) Example of a dextranase-producing fungal isolate. (b) Example of a dextranase-producing bacterial isolate.

In addition, an enrichment process (Veld Kamp, 1970) in liquid broth was carried out prior to streaking the samples onto the BDA plates. In this instance, aliquots of the diluted liquid or solid samples were incubated in a liquid enrichment media (containing dextran T2000, yeast, tryptone and mineral salts) at temperatures of 45 to 75°C for up to 21 days. Fresh subcultures were taken from the enrichment media every three days. Aliquots of the broths were placed on BDA plates and incubated over a range of temperatures. The production of dextranase activity was detected as described above.

In the case of strict anaerobic isolates, particular precautions were necessary to avoid exposure of the samples to aerobic conditions during microbial manipulations. A technique for screening strict anaerobes for production of thermostable dextranases was developed which utilised a refinement of the above enrichment media where the dextran T2000 was replaced by Blue Dextran (Wynter *et. al.,* 1992). This method was found to give a reliable estimate of dextranase production by measuring the absorbance of the dye soluble in 70 % ethanol. The amount of soluble dye correlated with microbial growth and also dextranase activity (measured by the reducing sugar assay). This method was utilised for the screening of water samples from thermal sources which appeared to contain dextranase-producing bacteria, from which growth on BDA plates was not achieved (Wynter *et al.,* 1992).

4.2.3 Selection and Purification of Isolates.

The criteria used to select isolates for further study was based on the highest temperature at which clearing zones developed, the speed of development of the clearing zones and the size of the clearing zones relative to the colony size.

Standard microbial techniques were employed for the purification of bacterial and fungal isolates. Where possible, successive subculturing on BDA plates was employed to obtain pure microbial cultures.

Alternatively, isolates which did not grow on BDA plates were grown to purity in liquid culture by serial dilution. These isolates were shown by phase-contrast microscopy to be a pure strain. The attainment of pure microbial cultures was essential prior to carrying out experiments to determine the parameters required for optimal growth and subsequently the conditions for maximum dextranase production.

4.2.4 Preparation of Crude Extracellular Culture Fluid

Pure strains of the dextranase producing isolates were cultured by either batch or continuous techniques to produce sufficient quantities of the thermostable dextranase for assay and characterisation. Liquid cultures were prepared under the selected conditions and growth temperatures. Culture vessels were inoculated with a standard volume of an actively growing inoculum (*i.e.* less than 24 hr old). When the optical density ($OD_{600\ nm}$ for aerobes and OD_{660} for anaerobes) of the inoculated broth was no longer increasing or was in excess of 0.8, the liquid cultures were harvested. Centrifugation at 5°C, 30 min and 20 000*g* produced a cell free extract.

The dextranase activity of the crude preparation was assayed directly (where possible) or concentrated prior to assay.

4.3 RESULTS AND DISCUSSION

4.3.1 Isolates from culture collections

Approximately 100 bacterial and fungal isolates were obtained from existing culture collections. Strains selected were known to possess at least one of the following characteristics: produced thermostable dextranases, were thermophiles, produced thermostable cellulases and/or were isolated from thermophilic environments. Isolates which exhibited substantial growth or dextranase production on BDA were selected for further investigations (Table 4.1).

American Type Culture Collection

Two strains of the fungus *C. gracile* were obtained from ATCC (Strain No. 16153 and 60154). Both strains produced dextranase activity at 30°C on BDA, but neither grew at 55°C. At 30°C, large clearing zones were observed within two days. Several thermophilic fungi and bacteria were also acquired from the ATCC. The fungal isolates exhibited optimum growth temperatures in in the range 26 to 45°C, while, for the bacteria, the range was 55 to 70°C (up to 40°C above that for *C. gracile*). The results reported in Table 4.1 confirm that the new fungal isolates did not produce a dextranase which exhibited a temperature optimum above that of the *C. gracile* dextranase. Furthermore, no bacterial isolate was selected which produced a dextranase under the culture conditions employed.

TABLE 4.1. SELECTED STRAINS OF BACTERIA AND FUNGI OBTAINED FROM CULTURE COLLECTIONS SCREENED FOR PRODUCTION OF THERMOSTABLE DEXTRANASES.

Organism	Incubation Temperature (°C)	Dextranase Produced
Chaetomium gracile ATCC 16153	30	Yes - 55°C
Chaetomium gracile ATCC 60154	30	Yes - 55°C
Pseudomonas mixta UQM 733	30	Yes - 30°C
Cellovibrio mixtus UQM 1666	30	Yes - 30°C
Cellovibrio mixtus UQM 2601	30	No
Bacillus coagulans UQM 264	30	No
Chaetomium indicum ATCC 48386	30	Yes - 55°C
Chaetomium spinosum ATCC 48389	30	No
Chaetomium thermophilum var. *coprophilum* ATCC 16451	45	No
Chaetomium thermophilum var. *coprophilum* ATCC 28076	45	Yes - 55°C
Chaetomium thermophilum var. *coprophilum* ATCC 58195	45	Yes - 55°C
Chaetomium thermophilum var. *dissitum* ATCC 16452	45	No
Chaetomium thermophilum var. *dissitum* ATCC 28077	45	No
Chaetomium thermophilum var. *dissitum* ATCC 58196	45	No
Chaetomium thermophilum ATCC 58420	45	No
Chaetomium thermophilum var. *thermophilum* ATCC 58136	37	Yes - 55°C
Chaetomium virescens ATCC 32319	37	Yes - 55°C
Chaetomium luteum ATCC 34113	37	Yes - 55°C
Bacillus sp. ATCC 31199 (patented strain)	55	No
Clostridium thermohydrosulfuricum ATCC 33223	65	No
Thermoanaerobacter ethanolicus ATCC 31550 (patented strain)	55	No
Thermus flavus ATCC 33923	70	No
Bacillus stercorarium NCIB 11754		No
Bacillus steareothermophilus NRRL 3880		No
DP17 (Uni. NSW)	65	Yes - 65°C

University of Queensland (Microbiology Department) Collection

Four bacterial strains were obtained from the University of Queensland (Department of Microbiology, UQM) for investigation. No strains grew at 55°C, while only 733 and 1666 produced dextranase (clearing zones on BDA plates) at 30°C.

Sugar Research Culture Collection

The Sugar Research Institute maintains a microbial culture collection containing isolates which have been collected from the sugar factory and surrounding environment. Fourteen strains of fungi already grown to pure culture were selected from the SRI culture collection. All but one of the strains were originally isolated from bagasse heaps (exposed for some time to temperatures as high as 70°C). The one exception was isolated from a sugar mill cooling tower. These isolates were grown on BDA plates to determine if they possessed the ability to produce dextranase. Thirteen of the strains grew on BDA either at 30°C or 55°C. No clearing zones were observed for any of the strains.

University of New South Wales Collection

During the period 1980-1982, a similar project was carried out at the University of NSW (Teh, 1982) to research microbial dextranases with temperature optima of the order of that operating in the raw sugar factory *i.e.* 75°C. The source of the isolates were compost samples which were placed in an enrichment medium at 65°C for 48 hours prior to screening. Colonies which produced zones of dextran hydrolysis (clearing) were purified by successive plating on BDA plates. A total of nine isolates which appeared to differ from each other in morphology and cultural behaviour were obtained. The most thermostable dextranase was produced by the isolate DP17. A pure culture of this isolate was kindly supplied to SRI by Dr. Tribe (University of New South Wales) and grown in liquid culture under the recommended conditions.

4.3.2 Isolates from the Factory Process or Environment

Initial samples were obtained from sites in and around the sugar factory where temperatures exceeded 45°C. A total of 45 strains were isolated which grew on BDA plates at 30, 45 or 55°C. However, no strains grew or produced clearing zones at 75°C. Further samples were obtained from within the mixed juice incubator (normal operating temperature 75°C) and from the mud filters (operating temperatures of up to 80°C). Isolates were obtained which grew up to 65°C, but no clearing zones were obvious under aerobic or anaerobic conditions.

A novel screening procedure was devised in an attempt to selectively screen for dextranase producers. A solution of dextran and mineral salts was allowed to drip continuously onto a gauze covered cotton wool swab placed in a wire basket (designated 'dextran trap'). This, in turn, was situated within the steam from a factory steam outlet line at temperatures of 65-70°C for 2-4 days. The swabs were removed and placed directly into enrichment media or alternatively inoculated onto BDA plates. The mixed cultures obtained were inoculated both onto fresh BDA plates and into enrichment media. Isolates were obtained which grew and gave clearing at temperatures of in excess of 60°C.

Over 300 strains were isolated using these methods of which approximately 60 appeared suitable for further investigation (Table 4.2). Some of the strains which exhibited dextranase activity at the higher temperatures were also cultured in liquid broth to enable quantitative analysis to be carried out.

TABLE 4.2. STRAINS OF BACTERIA AND FUNGI ISOLATED FROM THE FACTORY PROCESS OR ENVIRONMENT EXAMINED FOR PRODUCTION OF THERMOSTABLE DEXTRANASES

Isolate Number	Type	Source	Temperature of Isolation (°C)	Optimum Growth Temperature (°C)	Optimum Dextranase Production Temperature (°C)
SRI 2082	bacterium	Mill Mud	30	30	
SRI 2083	bacterium	Mill Mud	30	30/45	
SRI 2084		Mud Filter	55	55	
SRI 2085	bacterium	Incubator	55	55	55
SRI 2086		Mud Filter	55	45	
SRI 2087	bacterium	Incubator	30	30	30
SRI 2088	bacterium	Mud Filter	30	30	30
SRI 2089	bacterium	Mud Filter	30	30	
SRI 2090	bacterium	Mud Filter	30	30	
SRI 2091	bacterium	Mud Filter	30	30	30
SRI 2092	bacterium	Mud Filter	30	30	
SRI 2093	bacterium	Mud Filter	30	30	
SRI 2094	bacterium	Mud Filter	30	30	
SRI 2095	bacterium	Mud Filter	30	30	
SRI 2096	bacterium	Mud Filter	30	30	
SRI 2097	bacterium	Mud Filter	30	30	
SRI 2098		Incubator	30	30	30
SRI 2099		Incubator	30	30	30
SRI 2100	fungus	Contaminant	30	30	30
SRI 2101	bacterium	Incubator	30	30	30
SRI 2102	bacterium	Artesian bore	55-68	68	
SRI 2103	bacterium	Artesian bore	55-68	68	
SRI 2104	bacterium	Artesian bore	55-68	68	
SRI 2105	bacterium	Mud Filter	55	45	55
SRI 2106	bacterium	Mud Filter	55	45	45
SRI 2107	bacterium	Mud Filter	55	45	45
SRI 2108		Incubator	45	45	45
SRI 2109	bacterium	Juice	30	30	
SRI 2110	bacterium	Juice	30	30	
SRI 2111		Incubator	30	30	30
SRI 2112	bacterium	Incubator	30	30	
SRI 2113	fungus	Contaminant	30	30	55
SRI 2114		Mud Filter	45	45	45
SRI 2115	bacterium	Bagasse	45	45	45
SRI 2116	bacterium	Mud Filter	55	45	55
SRI 2117		Mud Filter	45	45	45
SRI 2118	bacterium	Mud Filter	45	45	50
SRI 2119		Incubator	45	45	45
SRI 2120	bacterium	Incubator	45	50	55
SRI 2121	bacterium	Incubator	30	30	
SRI 2122	bacterium	Mud Filter	55	55	55
SRI 2123	bacterium	Incubator	30	30	
SRI 2124	bacterium	Mud Filter	30	55	55
SRI 2125	bacterium	Dextran Trap	65	65	65/70
SRI 2126	bacterium	Mud Filter	60	60	60/70
SRI 2127	bacterium	Dextran Trap	65	65	65/70
SRI 2128	bacterium	Dextran Trap	65	65	65/70
SRI 2129	bacterium	Dextran Trap	65	65	65
SRI 2130	bacterium	Dextran Trap	65	60	60
SRI 2131	bacterium	Dextran Trap	60	65	65
SRI 2132	bacterium	Dextran Trap	65	65	65
SRI 2133	bacterium	Dextran Trap	65	65	65
SRI 2134	bacterium	Dextran Trap	65	65	65
SRI 2135	bacterium	Dextran Trap	65	65	65
SRI 2136	bacterium	Dextran Trap	65	65	65
SRI 2137	bacterium	Dextran Trap	65	60	60
SRI 2138	bacterium	Dextran Trap	60	60	60
SRI 2139	bacterium	Mud Filter	60	60	65

4.3.3 Isolates from Thermal Springs and Artesian Bores.

A number of water samples (60) were collected from artesian bores in Western Queensland by co-workers at Griffith University. About 30 anaerobic thermophilic bacteria and six facultative anaerobes were found to exhibit dextranase activity at 70°C or above. Thermophilic isolates previously obtained from New Zealand thermal springs were also screened for aerobic and anaerobic high-temperature dextranase producers. About 14 of these samples have been found to contain microorganisms which grew and produced clearing zones on Blue Dextran agar at 68°C. The most promising were selected for further investigation and subsequent characterisation (Table 4.3). These strains were selected based on the relative amounts of colour change which occurred in the liquid growth medium used for strict anaerobic isolates (Wynter *et al.*, 1992).

The first pure culture isolated from a thermal water supply which produced a thermostable dextranase (AB11A) was obtained from an Australian artesian bore. Subsequently, a better producer of thermostable dextranases was found to be another anaerobic thermophile (RT364) isolated from a New Zealand hot spring.

TABLE 4.3. SOURCES OF THE BEST STRAINS OF BACTERIA ISOLATED FROM THERMAL WATER SAMPLES EXAMINED FOR PRODUCTION OF THERMOSTABLE DEXTRANASES.

Isolate Number	Source	Temperature of Isolation (°C)
AB8	Australian Artesian Bore, 4893 A	
AB9	Australian Artesian Bore, 4893 B	
AB10	Australian Artesian Bore, 4164 A	75
AB11A	Australian Artesian Bore, 4164 B	70
AB11B	Australian Artesian Bore, 4164 C	
AB14	Australian Artesian Bore, 4165 C	
AB15	Australian Artesian Bore, 4165 D	
AB17	Australian Artesian Bore, 3034 B	70
AB18	Australian Artesian Bore, 3034 C	
AB20	Australian Artesian Bore, 3034 E	
AB24	Australian Artesian Bore, 5143 D	
AB30	Australian Artesian Bore, 376	53
AB39	Australian Artesian Bore, 17263C	
AB41	Australian Artesian Bore, 17263E	
AB51	Australian Artesian Bore, 69436B	
AB55	Australian Artesian Bore, 4381 A	
AB50	Australian Artesian Bore,	
RT69	Hot spring, Rotorua, New Zealand	
RT56	Hot spring, Rotorua, New Zealand	
RT44	Hot spring, Rotorua, New Zealand	
RT8A	Hot spring, Rotorua, New Zealand	
RT8C	Hot spring, Rotorua, New Zealand	
RT364	Hot spring, Rotorua, New Zealand	76
RT51	Hot spring, Rotorua, New Zealand	
RT4	Hot spring, Rotorua, New Zealand	
STER	*Bacillus stearothermophilus* ?	
CT61	*Clostridium thermocellum* 61	
14A	Hot spring, Rotorua, New Zealand	
B4	India	

CHAPTER 5

Characterisation of Thermostable Dextranases

5.1 INTRODUCTION

A number of dextranase producing microorganisms were isolated using the microbial screening techniques outlined in Chapter 4. However, for many of the isolates only preliminary screening on BDA plates was carried out. Further investigation of these strains was discontinued as it was found that the isolate either did not grow or give clearing on the BDA plates at higher temperatures *i.e.* above 55°C.

Only microorganisms which produced a dextranase (clearing zones on BDA plates) at elevated temperatures were selected for further study. Optimal growth conditions were determined and the purified strains were cultured in liquid broth. This enabled the production of sufficient extracellular dextranase for partial physico-chemical characterisation. However, it must be noted that assessment of the dextranases in this section is based on the characteristics exhibited by the crude enzymes.

Since the aim of the project was to obtain a dextranase which could be used in the sugar factory at 75°C, the initial parameter determined on each of the crude dextranases was the temperature optimum. Preparations which appeared to be of highest thermal stability were assessed by several of the assay methods to determine the effects of different conditions. Techniques were used to obtain a rapid

(Blue Dextran or micro-Blue Dextran assay) or sensitive (the reducing sugar method) assessment of relative activity of the dextranases. Thereafter, a meaningful estimate of the enzymes performance under factory conditions was determined using the micro-haze test (Chapter 2).

Other properties important for commercial application of an enzyme are the pH optimum, the amount of enzyme produced under the culture conditions (*i.e.* yield or activity per mL of culture broth) and the specific activity of the dextranases. These parameters were determined on a limited number of preparations from the more promising microbial isolates and were compared to those of the commercial dextranase currently used in Australian raw sugar factories *i.e.* the *C. gracile* dextranase.

5.2 EXPERIMENTAL

5.2.1 Reagents

All chemicals were the best available analytical grade reagents and were supplied by Ajax Fine Chemicals (Auburn, Australia) unless otherwise noted. Denatured and absolute ethanol (CSR Limited, Sydney, Australia) was used as supplied or aqueous solutions were prepared by dilution (v/v) with distilled water. UltraPure ammonium sulphate (Enzyme grade) was obtained from Bethesda Research Laboratories (Gaitersburg, MD).

The standard sucrose (starch and dextran free) was obtained from CSR Limited (Sydney, Australia). Commercial dextrans (MW 2×10^6) T2000 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The sucrose and dextrans were used without further purification.

High purity glucose and *p*-hydroxybenzoic acid hydrazide (analytical grade) were purchased from Sigma Chemical Co. (St. Louis, MO).

Potato starch was purchased from BDH Chemicals Limited (Poole, England). Pullulan ($\bar{M}_n$ 8.6 x 10^4) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan).

Acid-washed celite and mixed ion-exchange resin were prepared as outlined in Chapter 2.

Purified cane dextrans were isolated from the first-expressed juice extracted from deteriorated sugar cane billets by repeated precipitation with alcohol (50 %, v/v). The precipitate was lyophilized and stored at approximately 20°C and 10-15 per cent moisture.

Dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11) isolated from *Chaetomium gracile* was supplied as a partially purified liquid preparation (in 50 % (v/v) glycerol) by Miles-Kyowa (Japan). The commercial enzyme preparation was stored at 4°C and used as supplied for activity assays. However, because of the high activity exhibited by this enzyme, dilution with buffer (pH 5.0, 0.05 M citrate) was carried out prior to assay.

The thermostable α-amylase, Termamyl 120L, was purchased from Novo Industri, Denmark. The liquid preparation is a product of a selected strain of *Bacillus licheniformis*.

5.2.2 Preparation of deteriorated Juices and Syrups.

Burnt cane billets were collected during late October from a Mackay factory and stored in a large bin at ambient temperature for ten days. After crushing, the deteriorated cane in a laboratory mill, the first expressed juice (pH 4.0, 25 brix) was clarified in the laboratory. The resultant "cloudy" juice was concentrated to 72 brix (pH 6.5) in the SRI experimental pan and stored at 5°C. Deteriorated juices and syrups were prepared as required by dilution with distilled water to 18 and 70 brix, respectively.

5.2.3 Preparation of Concentrated Extracellular Culture Fluid

Crude cell-free culture extracts were prepared as described in Chapter 4. The protein present in the cell free extract was concentrated up to 100-fold by precipitation with ammonium sulphate. A gently stirred extracellular culture extract was slowly bought to 80 % saturation by the addition of solid ammonium sulphate. Once the salt had dissolved, the solution was allowed to stand for 1 hour prior to centrifugation at 20 000*g* for 30 min at 5°C. The resultant precipitate was redissloved in a minimum volume of the buffer (0.05 M citrate, pH 5.0) to achieve maximum concentration. This crude dextranase solution was dialysed exhaustively against the same buffer at 5°C. To ensure that an optically 'clean' liquid enzyme preparation was obtained for assays, the dialysate was centrifuged at 5°C for 30 min at 20 000*g* (Brown and Inkerman, 1992).

5.2.4 Measurement of Dextranase Activity

Dextran solutions and buffers were prepared as described in Chapter 2. The dextranase activity of the crude culture broths or their concentrates was determined by one or more of the methods outlined in Chapter 2 *i.e.* the Blue Dextran assay, the PAHBAH Assay, the micro-Blue Dextran assay or the micro-haze method (using either purified dextran T2000 or deteriorated raw cane juice as the substrate).

5.2.5 Measurement of Protein

The concentration of protein in the crude culture extracts was determined using the standard Biorad protein assay (Bradford, 1976).

5.2.6 Determination of Impurities

The PAHBAH assay was modified to determine the presence of other hydrolytic activities in the crude extracts. The substitution of the dextran substrate with either starch (0.1%) or pullulan (0.4%) was used to detect the presence of amylase and pullulanase activity in the crude enzyme preparations.

5.3 RESULTS AND DISCUSSION

5.3.1 Isolates from Culture Collections

American Type Culture Collection

Two strains of the fungus *C. gracile* were obtained from ATCC (Strain No. 16153 and 60154). Both strains produced dextranase activity at 30°C on BDA, but

neither grew at 55°C. One of the strains (ATCC 60154) was grown in liquid culture at 30°C for 5 days prior to harvest. The growth medium was centrifuged and the cell free supernatant concentrated about 30-fold with ammonium sulphate. The resulting extract was assayed by the Blue Dextran assay in the presence of sucrose (20 brix). The data obtained for the temperature-activity profile is presented in Figure 5.1. The relative activity of the commercial *C. gracile* dextranase was also determined to provide a comparison of the dextranases from the different strains of the same fungus.

The limited number of data points (3) used to graph the results for ATCC strain No. 60154 resulted in a broad curve with an optimum of about 55°C. In comparison, the commercial enzyme exhibited as much narrower temperature-activity profile with an optima of 55°C.

Other isolates, namely thermophilic fungi and bacteria (see Table 4.1 in Chapter 4), were obtained from the ATCC and screened for the production of thermostable dextranases. Liquid cultures of the *Chaetomium* isolates (Table 5.1) were prepared and the concentrated extract assayed for dextranase activity. Extracts from about half the isolates exhibited dextranase activity. None of the *Chaetomium* isolates produced a dextranase which exhibited a temperature optimum above that of the *C. gracile* dextranase. The dextranase obtained from *Chaetomium virescen* was the most active and exhibited residual activity at 75°C.

University of Queensland (Microbiology Department) Collection

Four bacterial strains were obtained from the University of Queensland a (Department of Microbiology) for investigation (see Table 4.1). No strains grew at 55°C and only two produced dextranase (clearing zones on BDA plates) at 30°C.

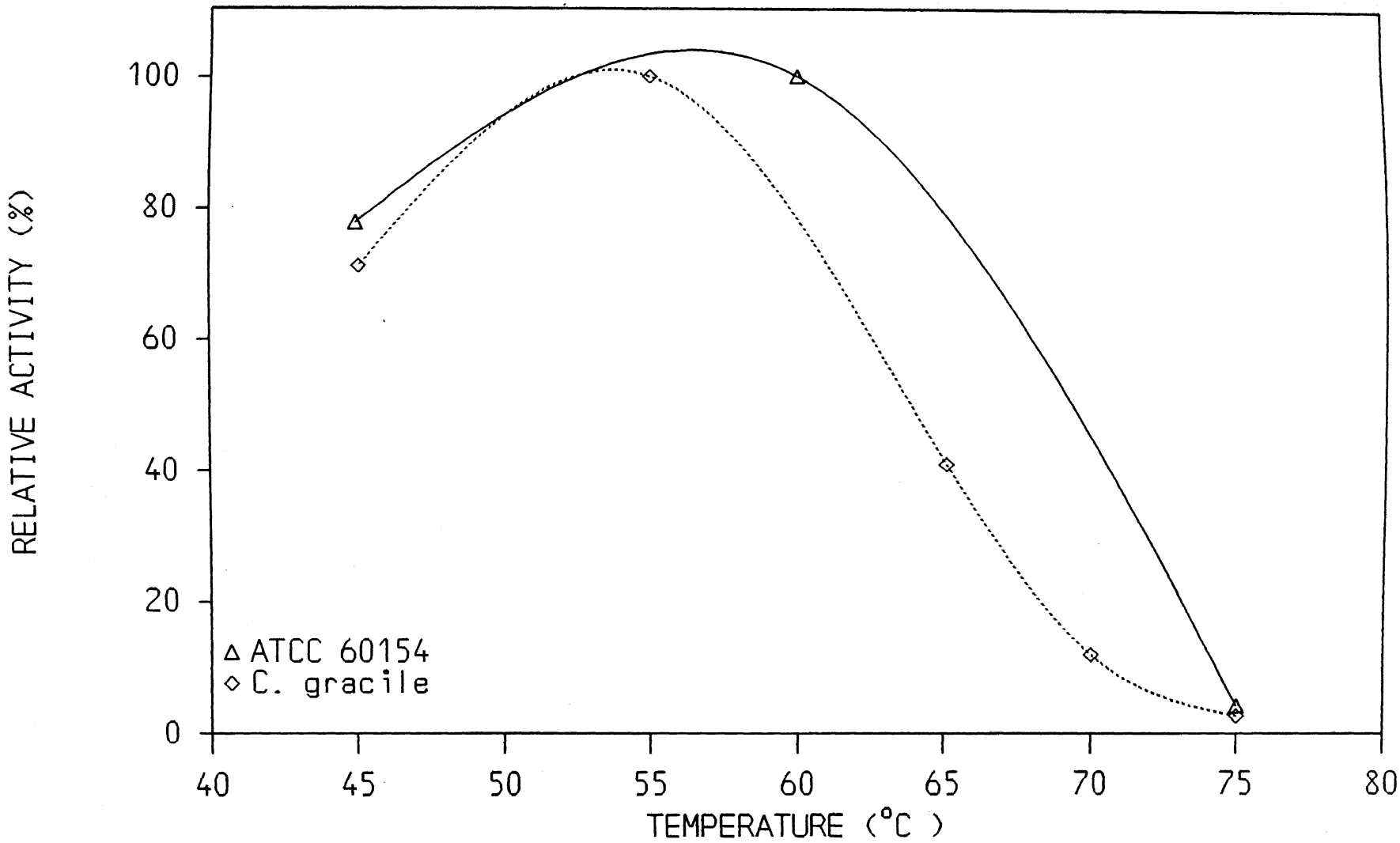


Figure 5.1 **Temperature-activity profiles for the dextranase in the concentrated culture extract from ATCC Strain No. 60154 and the commercial *C. gracile* dextranase as measured by the Blue Dextran assay (pH 5.0, 30 min, 20 brix sucrose).**

TABLE 5.1. ACTIVITY[a] OF THE CRUDE DEXTRANASES PRODUCED BY STRAINS OF BACTERIA AND FUNGI FROM THE ATCC SCREENED FOR THE PRODUCTION OF THERMOSTABLE DEXTRANASES.

ATCC Isolate	Initial Volume (mL)	Final Volume (mL)	Protein (mg mL^{-1})	Temperature (°C)			
				55.0[b]	55.0[c]	65.1[c]	75.0[c]
Chaetomium thermophilium var *COPROHPILUM* ATCC 58195	745	10.0	1.26	20.1	19.2	2.60	0
Chaetomium thermophilium var *COPROHILUM* ATCC 16451	740	11.0	0.58	0			
Chaetomium thermophilium var *COPROHILUM* ATCC 28076	660	10.0	0.83	27.5	28.8	8.4	0.65
Chaetomium thermophilium var *DISSITUM* ATCC 28077	320	5.0	1.26	0			
Chaetomium thermophilium var *DISSITUM* ATCC 58196	320	6.0	1.13	0.39			
Chaetomium thermophilium var *DISSITUM* ATCC 16452	345	8.0	3.06	0			
Chaetomium virescen ATCC 32319	240	5.5	0.80	High OR	1257	114	0
ATCC 32319		15.0		High OR	>10000	3837	117
Chaetomium thermophilium var *thermophilium* ATCC 58136				215			
Chaetomium luteum ATCC 34113				78			
Chaetomium indicum ATCC 4838	190	13.0		High OR	23	26	8
Chaetomium spinosum ATCC 48389	160	14.0		0			

[a]Activity of the dextranases in concentrated extract determined by the PAHBAH assay. [b]Initial value determined at 55°C, 30 min, pH 5.0 using 200 µL extract. [c]Active extracts were reassayed over the range 55-75°C, 30 min, pH 5.0 using 10-20 µL extract. OR, out of range.

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Chaetomium spinosum ATCC 48389	160	14.0		0			

[a]Activity of the dextranases in concentrated extract determined by the PAHBAH assay. [b]Initial value determined at 55°C, 30 min, pH 5.0 using 200 µL extract. [c]Active extracts were reassayed over the range 55-75°C, 30 min, pH 5.0 using 10-20 µL extract. OR, out of range.

Sugar Research Institute Culture Collection

Unidentified fungi isolated from bagasse heaps were grown on BDA plates to determine if they possessed the ability to produce dextranase. Growth was obtained for most of these strains on BDA plates (two at 30°C; others at 55°C). However, no clearing zones were observed for any of the isolates.

University of New South Wales Collection

A similar project (Teh, 1982) was carried out at the University of New South Wales (1980-1982) to research microbial dextranases with temperature optima of the order of that operating in the raw sugar factory *i.e.* 75°C. The temperature-activity profiles of the dextranases produced by the isolates were determined using an assay procedure which measured the reduction in viscosity of a solution of synthetic cane juice containing a commercial dextran (T2000). This method measured endo-dextranase activity. The most thermostable dextranase was found to be produced by isolate DP17 which exhibited a pH optimum of 6.0, and a temperature optimum of 70°C. Significantly, it retained about 70 per cent of its activity at 75°C (Teh, 1982). An attempt was made to measure the temperature-activity profile of the dextranase present in a cell-free liquid culture extract of DP17 using the alcohol haze test. However, the level of dextranase activity present in the culture broth was too low to cause a reduction in haze formation; indicating that prior concentration would be necessary in order to measure its activity. The optimum temperature for the concentrated dextranase preparation was found to be 5°C lower by the alcohol-haze method (*i.e.* 65°C) compared to that obtained using the viscometric assay, even though both assays give a measure of endo-dextranase activity (Teh, 1982).

A pure culture of DP17 was grown in liquid broth under conditions recommended by Teh (1982). The cell-free broth was concentrated about 12-fold to a protein concentration of 10.2 mg mL^{-1}. The dextranase activity was determined at a number of temperatures by the PAHBAH assay and by the micro-haze test. A comparison of the profiles is given Figure 5.2. Temperature optima of approximately 64 and 68°C were obtained by these exo- and endo-dextranase methods, respectively. The values for the temperature optima were similar to those previously obtained by Teh (1982), but fall short of the target optimum of 75°C for this project.

The residual activities measured at 75°C were about 5 and 20 per cent as determined by the micro-haze test and PAHBAH assay, respectively. These values were significantly less than the 70 per cent reported by Teh (1982). In addition, the specific activity (determined using the PAHBAH assay) for the concentrated crude extract from DP17 was very low (0.21 μmole glucose min^{-1} mg^{-1} of protein at the optimum temperature, 65°C) compared to a value of 2.7×10^3 μmole glucose min^{-1} $mg^{-1}$of protein for the commercial preparation of partially purified *C. gracile* dextranase at its optimum.

5.3.2 Isolates from the Factory Process or Environment

Within a raw sugar factory there are numerous sites where the process is operated at temperatures greater than 45°C. These high temperature sites were chosen as sample collection points for screening of dextranase-producing microorganisms.

Isolates from the Mud Filter

The mud filter was selected as a sampling site because it is exposed to temperatures of up to 88°C for short periods about 8 times per hour *viz.* approximately 10 min. A number of strains which gave clearing on BDA plates at various temperatures were isolated from the mud filter. On the basis of their ability to produce relatively large clearing zones on BDA plates at 55°C, four of the isolates were selected from 25 strains for more detailed study. These were SRI Strain Nos. 2116, 2120, 2140 and 2126.

SRI Strain No. 2116

SRI strain No. 2116 was isolated from a Mackay factory (Marian Mill). The isolate was grown in liquid culture (930 mL) and the cell-free broth was concentrated with ammonium sulphate about 70-fold. The temperature profile for the concentrated extract from this strain measured by the micro-Blue Dextran assay has been presented in Chapter 2 (Figure 2.7). The temperature-activity profile determined using the PAHBAH assay is given in Figure 5.3. Optimum activity was exhibited at

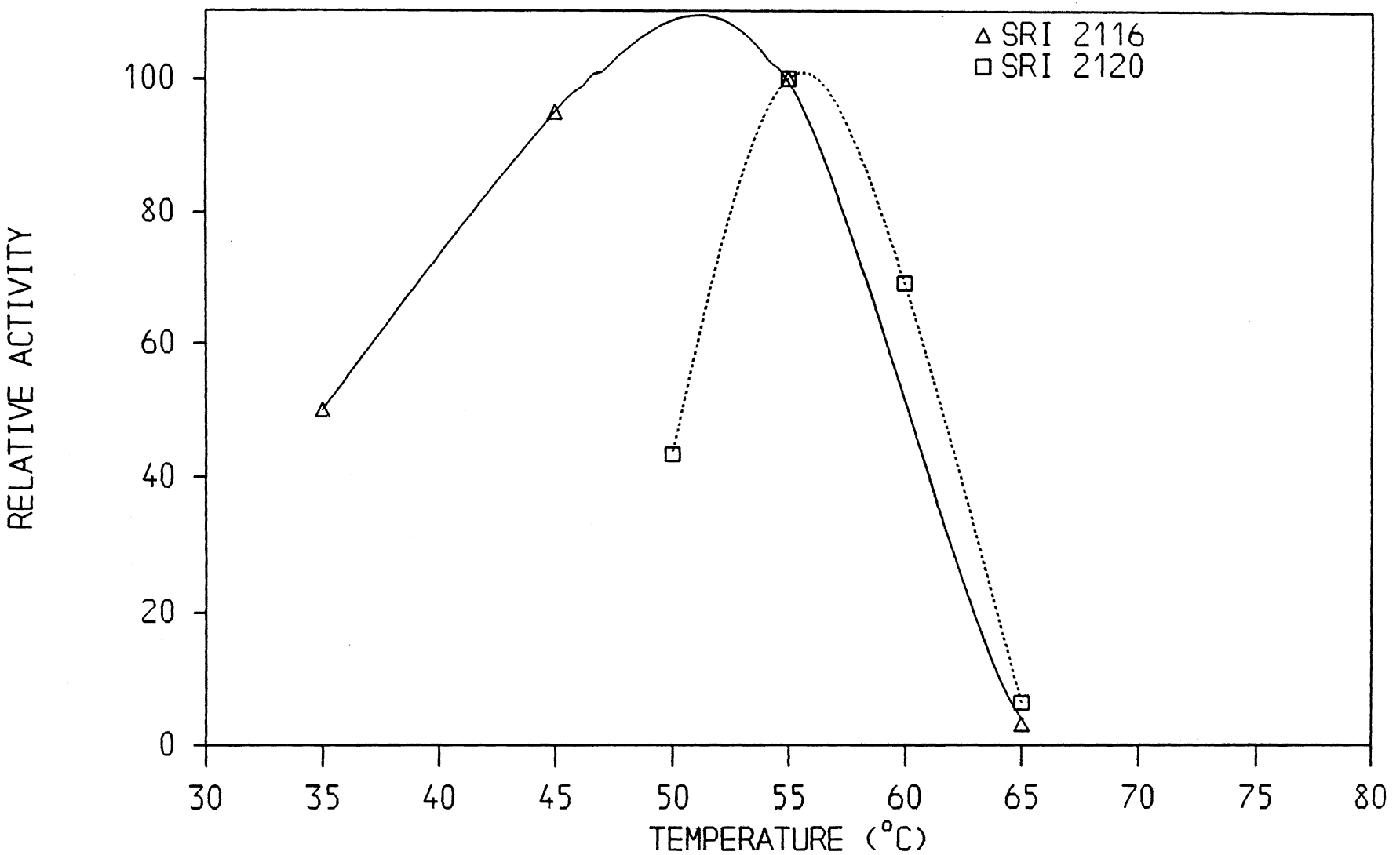


Figure 5.3 Temperature-activity profiles for dextranases produced by SRI 2116 and SRI 2120 determined using the PAHBAH assay (pH 5.0, 30 min).

50°C with less than 5 per cent residual activity in the temperature range 65 to 75°C. The temperature profile shows that the dextranase produced by this isolate is not as thermostable as the *C. gracile* dextranase. Therefore, investigations on other properties of the SRI 2116 dextranase were not carried out as it was not considered a suitable source for a thermostable dextranase.

The pH-activity data for this isolate is presented in Table 5.2. The optimal pH (5.0) is comparable to that exhibited by the *C. gracile* dextranase and conditions pertaining in the raw sugar factory.

TABLE 5.2 pH-ACTIVITY DATA FOR SRI STRAIN NO. 2116[a].

Parameter	pH			
	4.3	5.2	6.2	7.1
Relative Activity	1	100	93	45
Activity (nmoles min^{-1} mL^{-1})	5.2	399	371	179

[a]Activity measured by the PAHBAH assay (15 min, 45°C).

SRI Strain No. 2120

A second isolate (SRI strain No. 2120) from Marian Mill was chosen for more detailed study. A liquid culture of the isolate was prepared (650 mL) and concentrated about 40-fold. The temperature optimum for the concentrated dextranase preparation was determined using the reducing sugar method (Figure 5.3) . Maximum activity was observed at 55°C with less than 10 per cent residual

activity at 65°C. Overall, the temperature-activity profile was narrow compared to dextranases from other isolates. This enzyme was also considered unsuitable as its thermostability did not exceed that of the *C. gracile* dextranase.

The pH-activity data again compares favourably to that obtained for the commercial *C. gracile* dextranase with the pH optimum being around 5.5 (Table 5.3).

TABLE 5.3. pH-ACTIVITY DATA FOR SRI STRAIN NO. 2120[a]

Parameter	pH			
	4.3	5.2	6.2	7.2
Relative Activity	0	92	100	42
Activity (nmoles min^{-1} mL^{-1})	0	58	63	27

[a]Activity measured by the PAHBAH assay (15 min, 45°C).

SRI Strain No. 2140 and 2126

The remaining two strains of aerobic bacteria selected from the mud filter isolates for further assessment were obtained from a different source in the Mackay district (Racecourse Mill). SRI Strain No. 2140 and 2126 were grown in liquid culture and the crude extracts were concentrated to give a final protein level of 5.7 mg mL^{-1} and 11.2 mg mL^{-1}, respectively. The micro-haze method was used to determine the temperature optima of the dextranases (Figure 5.4).

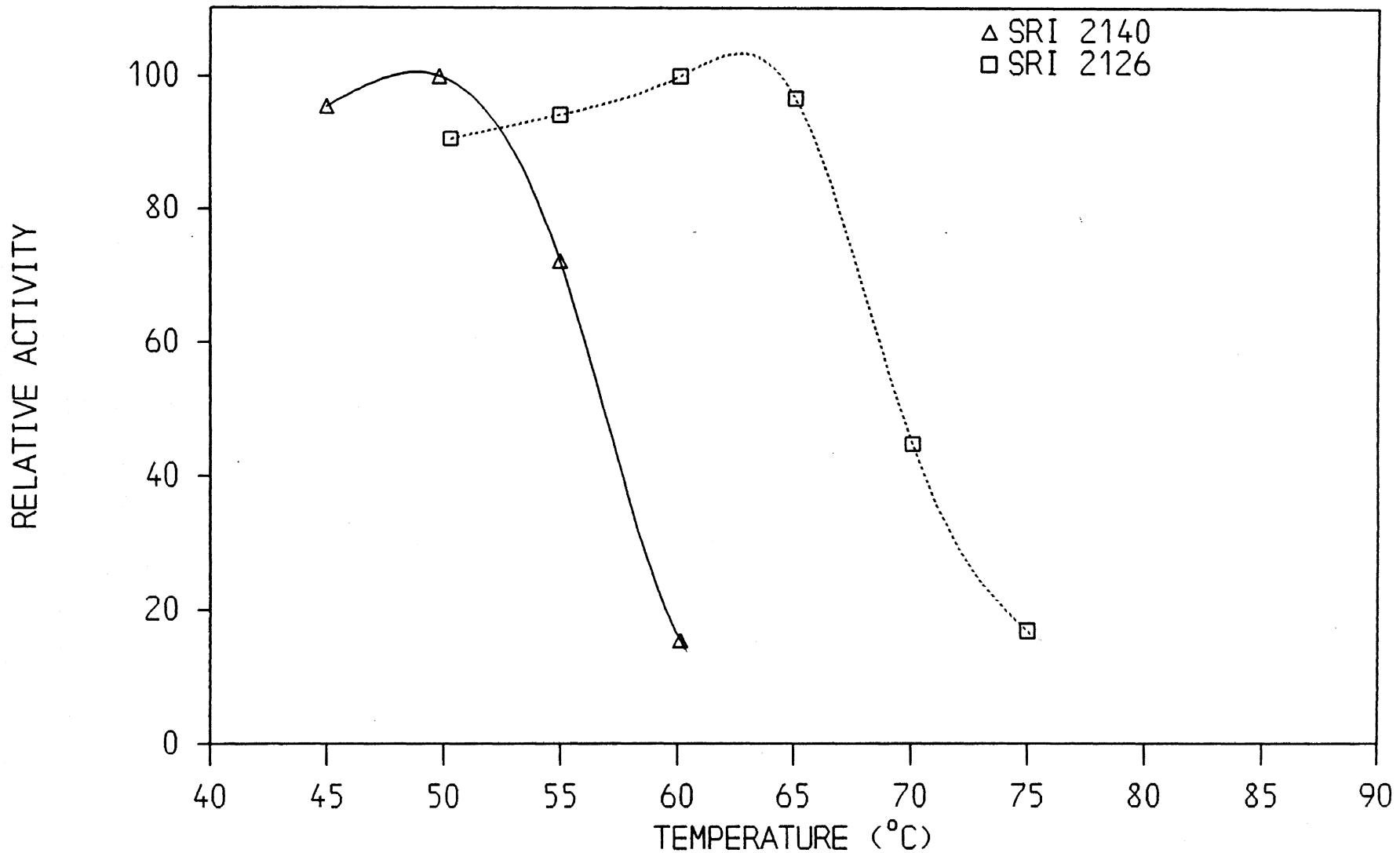


Figure 5.4 **Temperature-activity profiles for dextranases produced by SRI 2140 and SRI 2126 determined by the micro-haze test (pH 5.0, 30 min).**

A low optimum (less than 50°C) was obtained for SRI 2140 enzyme while the dextranase from SRI Strain No. 2126 proved to be more promising. The latter enzyme exhibited high activity over a broad range of temperatures and an optimum at about 62°C, perhaps indicating the presence of more than one dextranase. Almost 20 per cent residual activity was retained at 75°C. Therefore, of the dextranases studied to date the enzyme from SRI 2126 appears to be the most promising as it exhibits a broad temperature-activity profile with the highest temperature optimum (62°C) and greatest residual activity at 75°C. Again, no further work was carried out on this enzyme, as the temperature optimum of 62°C was considered too low.

Isolates from Factory Incubators

Under normal factory operating conditions the mixed juice incubator is maintained at 75°C. Twelve strains which produced clearing on BDA plates were isolated from an operating factory incubator. On the basis of its ability to produce dextranase activity at 55°C and the relative size of the clearing zone, one isolate (SRI Strain No. 2085) was selected for more detailed study.

SRI Strain No. 2085

This isolate was grown in liquid culture (690 mL) and the cell free extract concentrated about 26-fold. Using the PAHBAH assay, the temperature-activity profile of the crude concentrated dextranase was determined (Figure 5.5). Optimum activity was exhibited at 55°C, the same temperature as that obtained for the commercial *C. gracile* dextranase (also shown in Figure 5.5). However, at higher

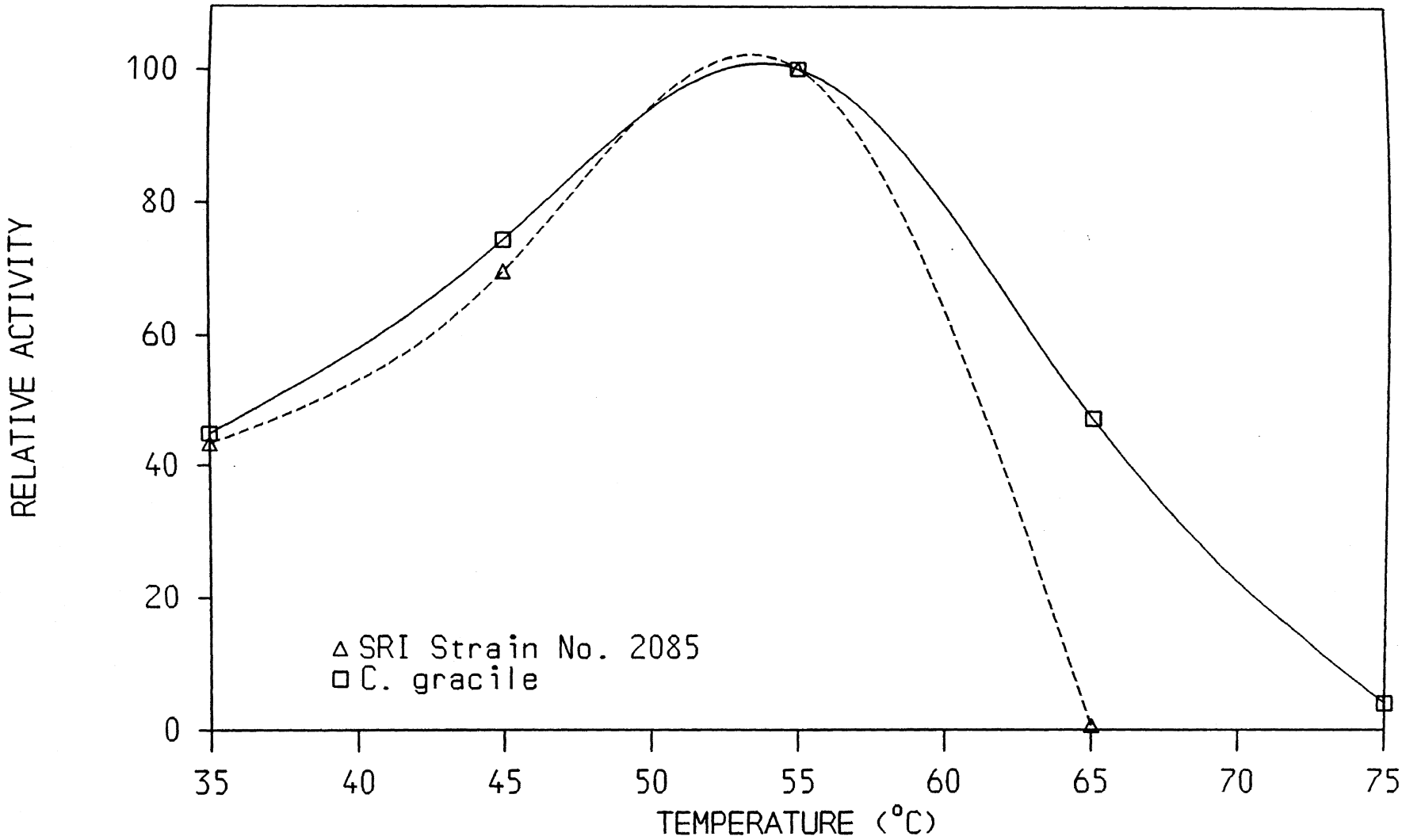


Figure 5.5 Temperature-activity profile for the dextranase produced by SRI 2085 measured using the PAHBAH assay (pH 5.0, 30 min).

temperatures, the dextranase from this isolate exhibited less thermal stability than the commercial dextranase with a complete loss of activity at 65°C or greater.

The concentrated extract was also assayed at several different pH values to determine the optimal pH for the activity of the dextranase present in the liquid extract. The data for the pH profile is presented in Table 5.4. The pH optimum was approximately 5.5 as similar to isolates described previously and for the *C. gracile* dextranase.

TABLE 5.4 pH-ACTIVITY DATA FOR SRI STRAIN NO. 2085[a].

Parameter	pH			
	4.2	5.2	6.2	7.1
Relative Activity PAHBAH Assay	0.3	100	100	49
Activity (nmoles min^{-1} mL^{-1})	0.3	181	182	89

[a]Activity measured by the PAHBAH assay (15 min, 45°C).

Isolates from the in situ Dextran Enrichment Procedure

A novel enrichment procedure was devised for isolation of thermophilic dextranase-producing microorganisms (described in Chapter 4). Screening was carried out with the 'dextran trap' placed within the steam released from a factory outlet line (at temperatures of greater than 65°C). A number of isolates exhibited dextranase production at a temperature of 65°C on BDA plates. Two were selected

for further characterisation. The selection criteria was based on the rate at which clearing zones developed around colonies and the relative size of the clearing zones.

SRI Strain No. 2125

A large scale batch culture of liquid broth (~17 L) was prepared and inoculated with the isolate, SRI 2125, grown under optimal conditions. After removal of the cell mass, the extract was concentrated approximately 400-fold to a final protein concentration of 14.7 mg mL^{-1}. The temperature-activity profile was determined by both the PAHBAH method and micro-haze test (using cane dextran as the substrate). The results are depicted graphically in Figure 5.6 and Figure 5.7, respectively. The optimum determined by both methods was of the order of 61-64°C. This is 6-9°C higher in value than the *C. gracile* enzyme. Significantly, the enzyme exhibited appreciable residual activity (about 40%) at 75°C compared to the *C. gracile* enzyme.

SRI Strain No. 2128

A bulk liquid preparation (~6 L) of the crude extracellular dextranase from the SRI Strain No. 2128 was prepared and the cell-free supernatant concentrated approximately 400-fold to a protein concentration of 4.7 mg mL^{-1}. The optimum temperature was determined using the PAHBAH assay (Figure 5.8) and the micro-haze test (Figure 5.9) using both dextran T2000 and cane dextran, respectively, as the substrates.

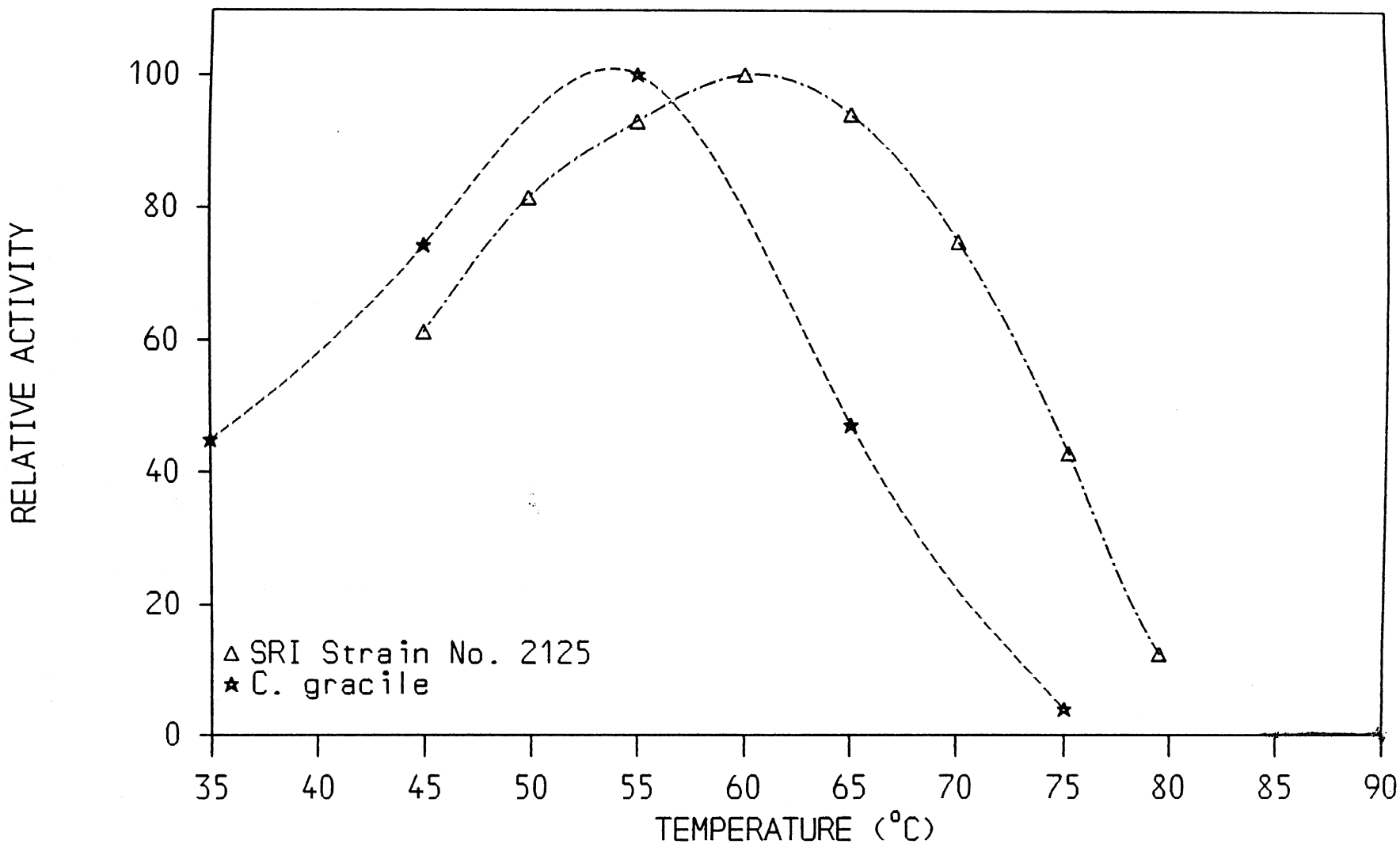


Figure 5.6. **Temperature-activity profile for the dextranase produced by SRI 2125 measured using the PAHBAH assay (pH 5.0, 30 min).**

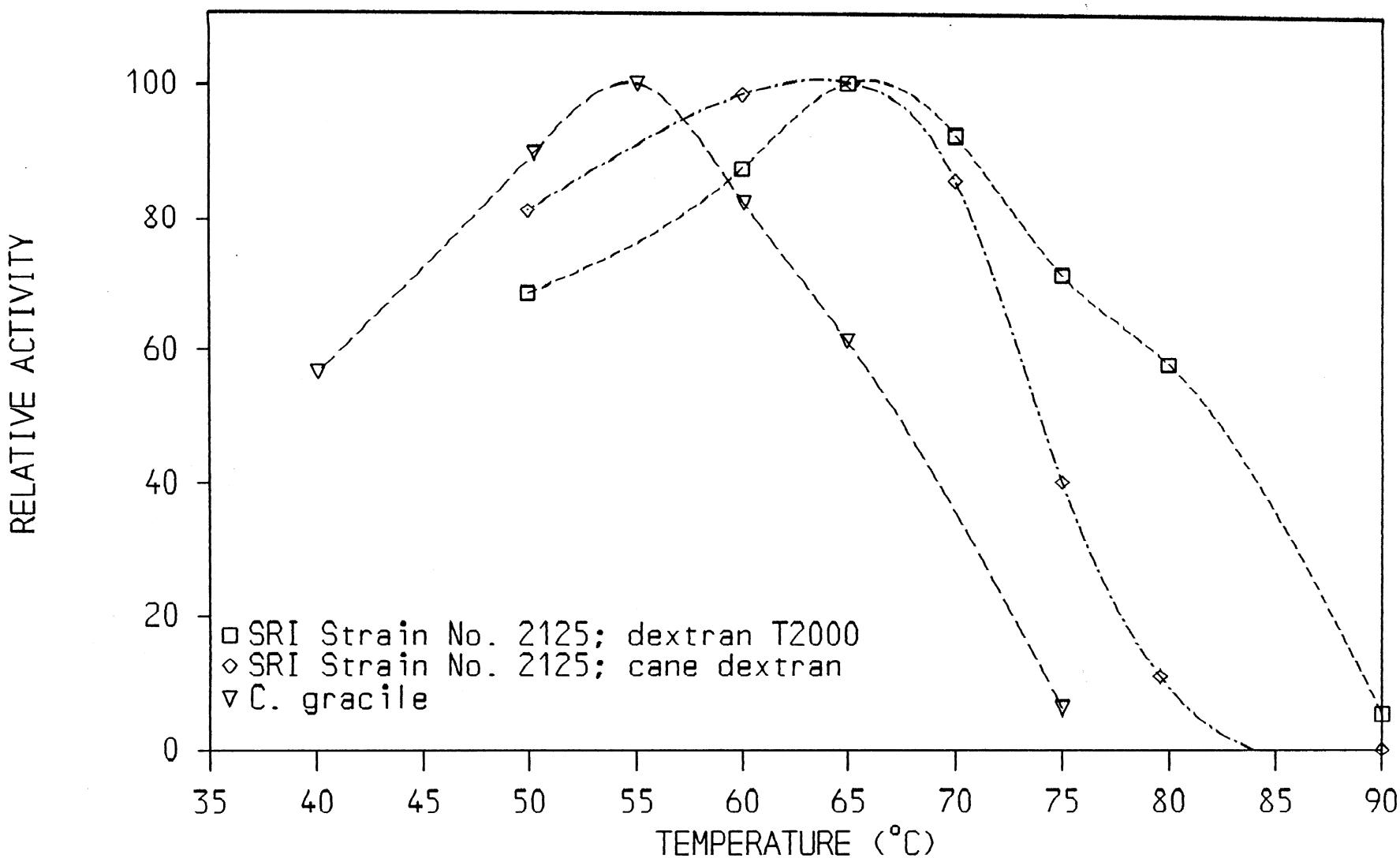


Figure 5.7 **Temperature-activity profiles for the dextranase produced by SRI 2125 determined using the micro-haze test (pH 5.0, 30 min).**

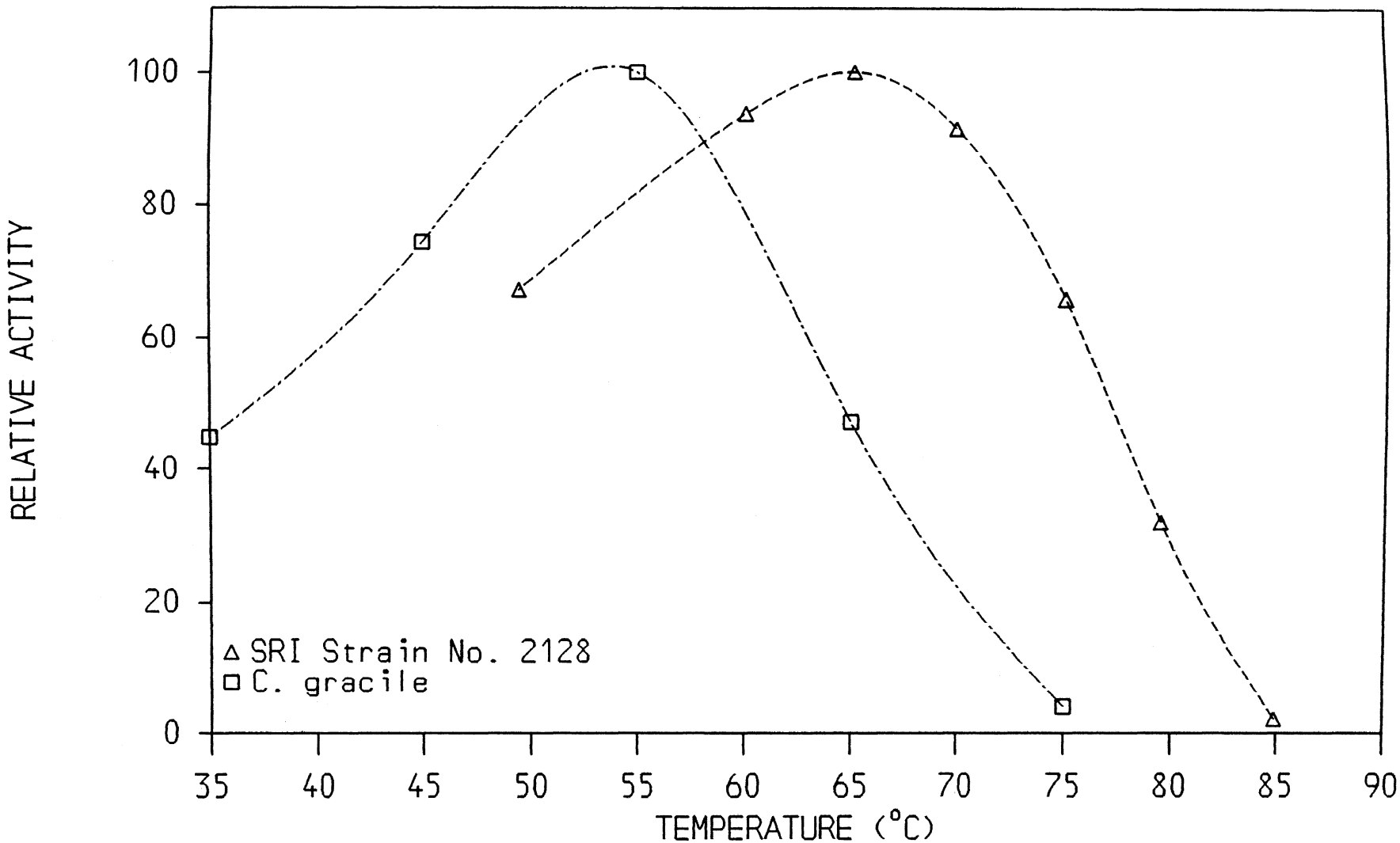


Figure 5.8. Temperature-activity profile for the dextranase produced by SRI 2128 measured using the PAHBAH assay (pH 5.0, 30 mins).

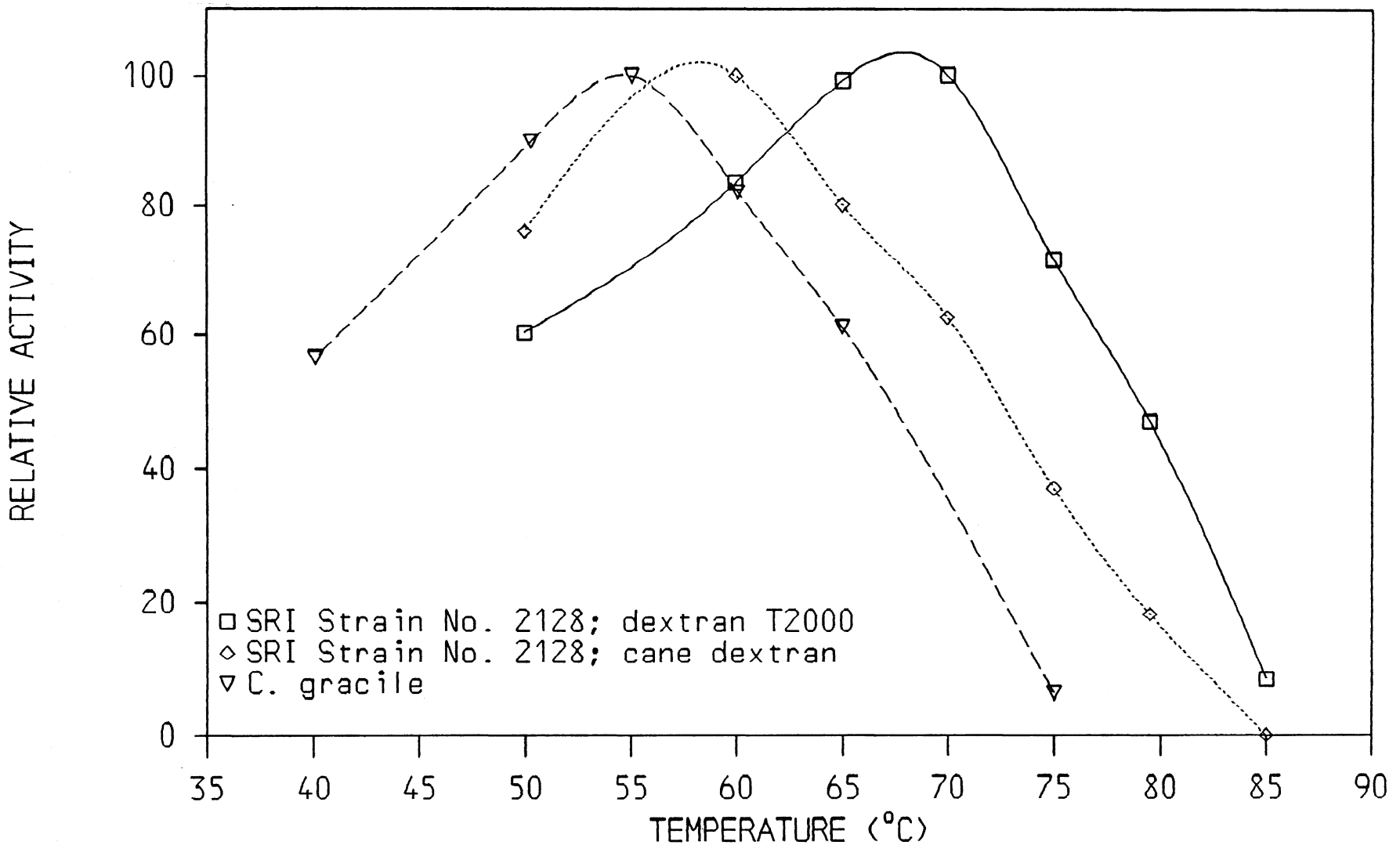


Figure 5.9 **Temperature-activity profiles for the dextranase produced by SRI 2128 determined using the micro-haze test (pH 5.0, 30 min).**

The temperature optimum as measured by the PAHBAH assay was found to be 65°C. Further, this crude enzyme retained significant activity (70%) at 75°C. With regard to the micro-haze methods different optima were determined for the two different substrates, *viz.* 61 and 67°C using cane dextran and T2000 dextran respectively. The reason for this difference is not known. Further this difference was not observed with the *C. gracile* dextranase (see Chapters 2 and 3).

The thermostable properties of the crude dextranase preparations from both SRI Strain No. 2125 and SRI 2128 indicated that these enzymes would be worthy of further study.

5.3.3 Isolates from Thermal Springs and Artesian Bores

Thermal water sources have previously been sampled in search of strains of microorganisms which produce other thermostable enzymes such as amylases and pullulanases (Plant *et al.*, 1987).

A number of aerobic and anaerobic thermophilic bacteria isolated from artesian bore water on thermal springs were found to grow on dextran and produce dextranase activity (see Table 4.3). AB11A grew well and produced dextranase at 68°C in an enriched dextran media with low tryptone (0.2%) and yeast (0.05%). In contrast, the best growth at 68°C in the enriched dextran media with high tryptone (1.0%) and yeast (0.3%) was obtained from isolate RT364 (Wynter *et al.*, 1992). Broth cultures of both these isolates were prepared for assessment and partial characterisation.

Isolate AB11A

AB11A was the first purified isolate from a thermal water supply which was demonstrated to produced a thermostable dextranase. A concentrated cell-free extract of the dextranase produced by AB11A was prepared by Dr. C. Wynter at the Griffith University. This preparation, containing a final protein concentration of 2.2 mg mL^{-1}, was dispatched to SRI for comparative analysis by the different assay procedures. The temperature-activity profile was determined using the PAHBAH assay (Figure 5.10) and the micro-haze test (Figure 5.11) using both dextran T2000 and cane dextran as the substrate.

The optimum determined by the reducing sugar method was 68°C with about 80 per cent of the activity retained at 75°C. Using the micro-haze test, the maximal temperature for dextranase activity with either substrate was also found to be approximately 68°C. Under simulated factory conditions, the enzyme from this isolate exhibited a broad temperature activity profile with high levels (> 90 per cent) of activity from 60 to 70°C with about 50 per cent residual activity at 75°C.

Isolate RT364

The thermophilic anaerobic isolate, RT364, was isolated from a water sample obtained from a New Zealand thermal spring. This strain produced the highest amount of dextranase activity under anaerobic conditions (Wynter *et al.*, 1992). Therefore, RT364 was selected for further study and subsequent characterisation.

Three concentrated cell-free liquid preparations of the dextranase produced by this isolate were prepared from either batch or continuous culture (Dr. C. Wynter,

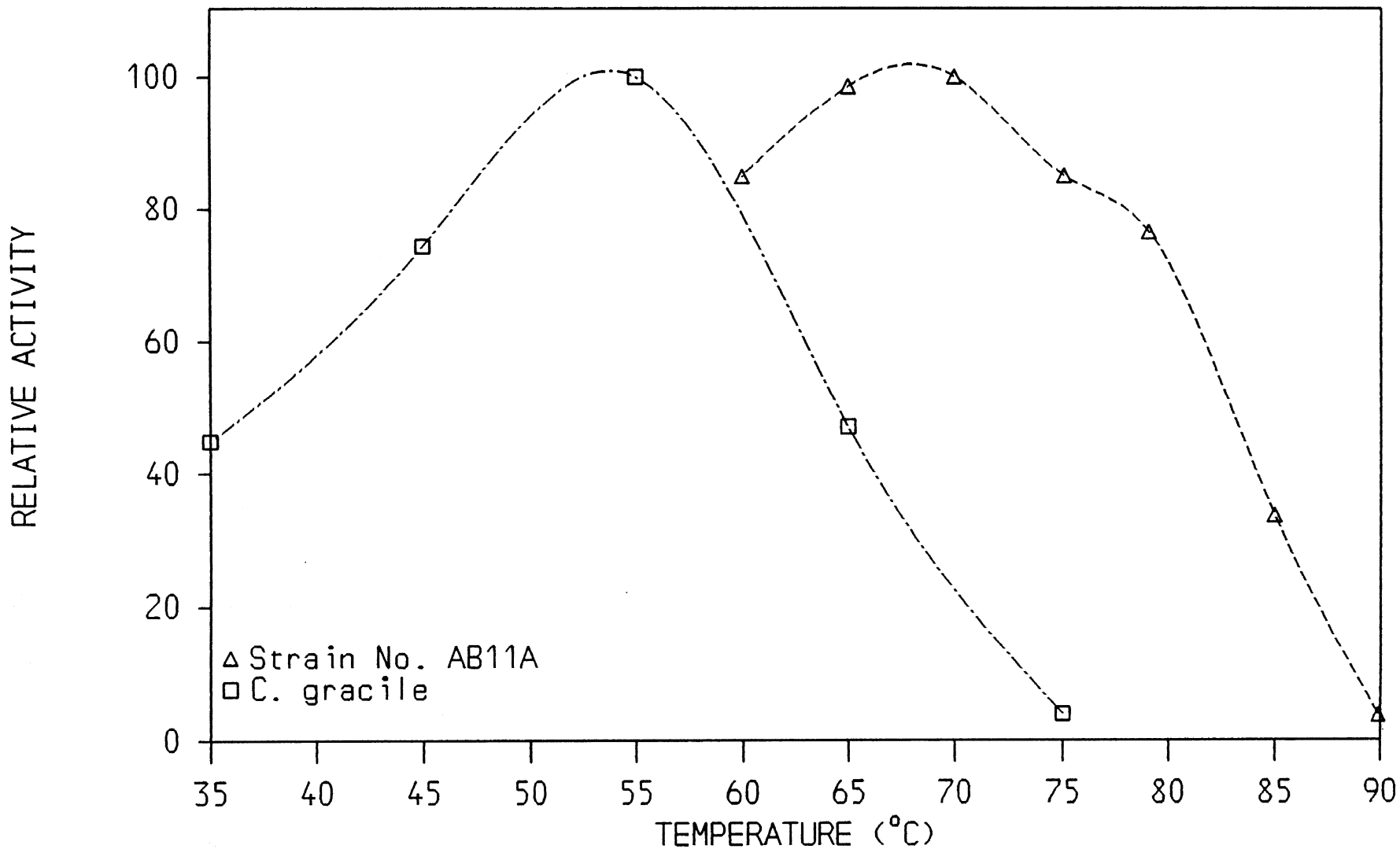


Figure 5.10 Temperature-activity profile for the dextranase produced by AB11A measured using the PAHBAH assay (pH 5.0, 30 min).

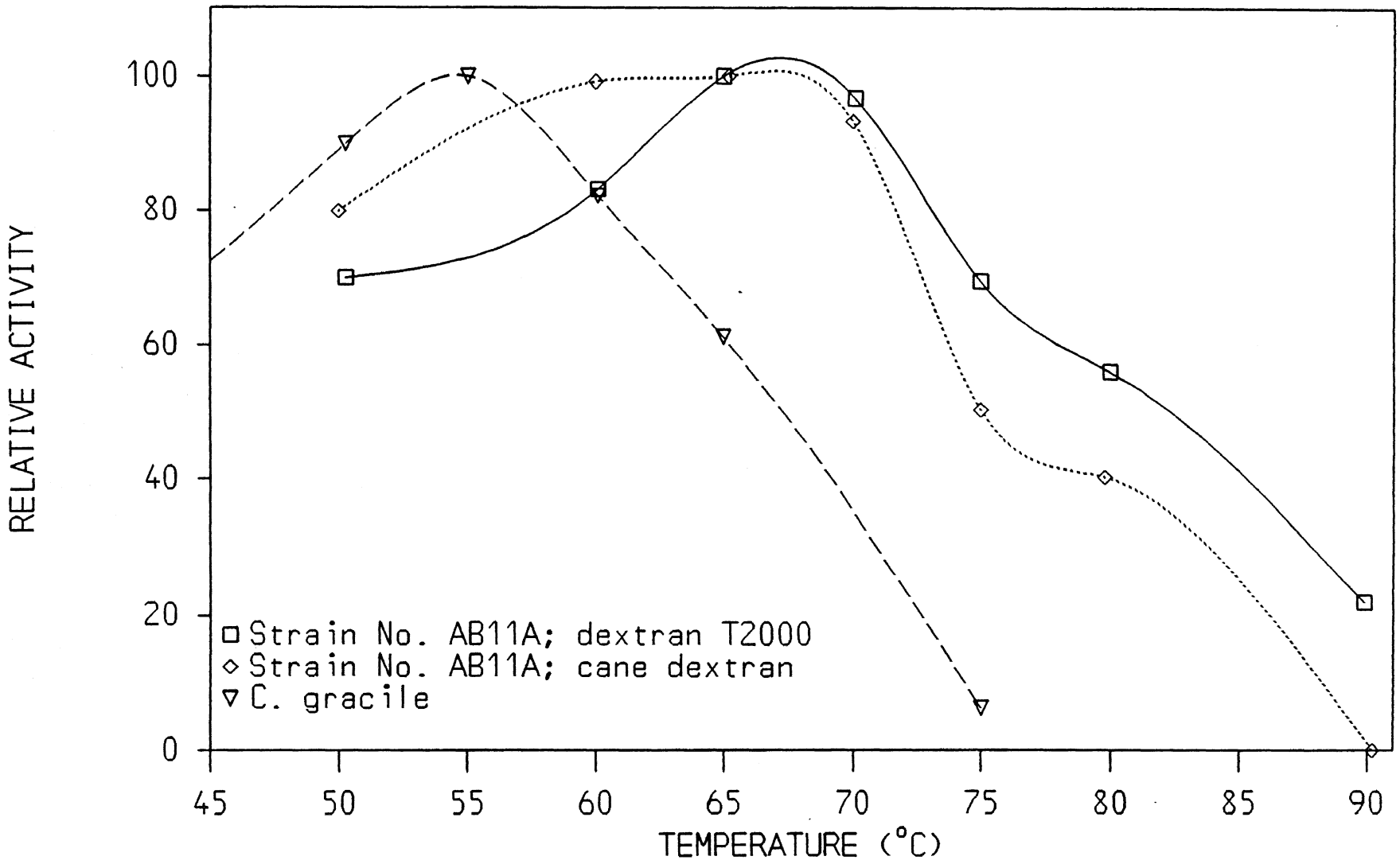


Figure 5.11. Temperature-activity profiles for the dextranase produced by AB11A determined using the micro-haze test (pH 5.0, 30 min).

Griffith University) and forwarded to SRI for activity assays. All samples were assayed over a range of temperatures using the PAHBAH assay (Figure 5.12) and the micro-haze test (Figure 5.13) using dextran T2000 as the substrate.

Some variation was exhibited in the temperature-activity profile from preparation to preparation. More significantly, there was a large difference in optimal temperature determined by the reducing sugar assay (about 75°C) and the micro-haze test (approximately 65°C). It was reasoned that large differences in the temperature optimum might indicate that the crude preparations contained multiple forms of the dextranase or different dextranases *i.e.* endo- and exo-enzymes, with different temperature optima. Purification of the dextranases from the crude preparation and characterisation of the purified enzyme(s) would be required to investigate this possibility (see Chapter 6).

The activity and protein concentrations of the three RT364 preparations were different from batch to batch (Table 5.5). Changes in the composition of the concentrated extract were probably due to variations in the culture conditions employed for microbial growth (Dr. C. Wynter, private communication)

TABLE 5.5 ACTIVITY OF THE RT364 DEXTRANASE PREPARATIONS

Preparation	Protein (mg mL^{-1})	Temp (°C)	PAHBAH assay (nmoles $min^{-1}mL^{-1}$)	Temp (°C)	Micro-haze test (ppm/bx $min^{-1}mL^{-1}$)
No. 1	4.9	70	236	65	2680
No. 2	2.25	75	1540	65	13067
No. 3	1.65	75	687	65	7637

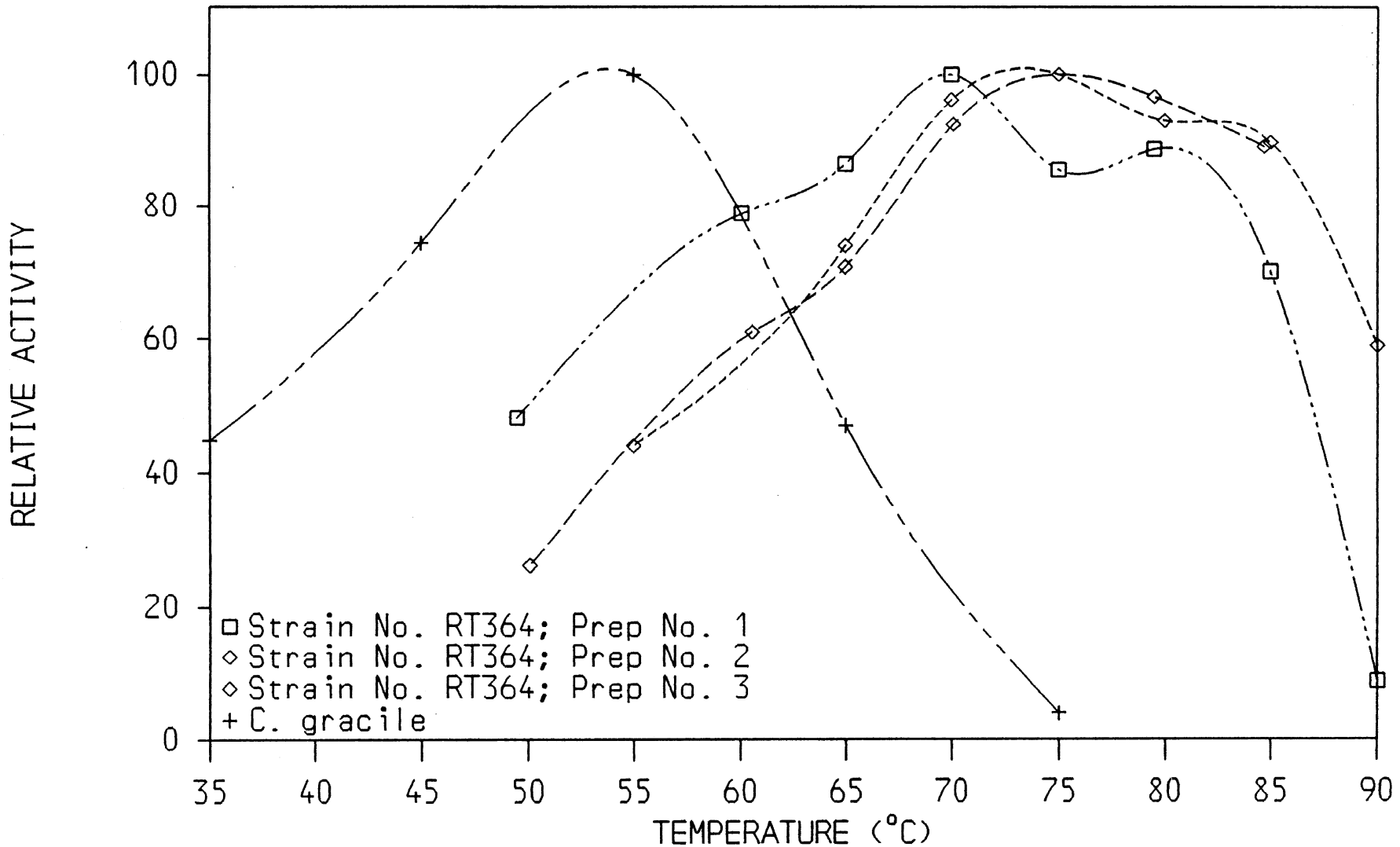


Figure 5.12 Temperature-activity profiles for the dextranase produced by RT364 (preparation 1,2, and 3) determined using the PAHBAH assay (pH 5.0, 30 min).

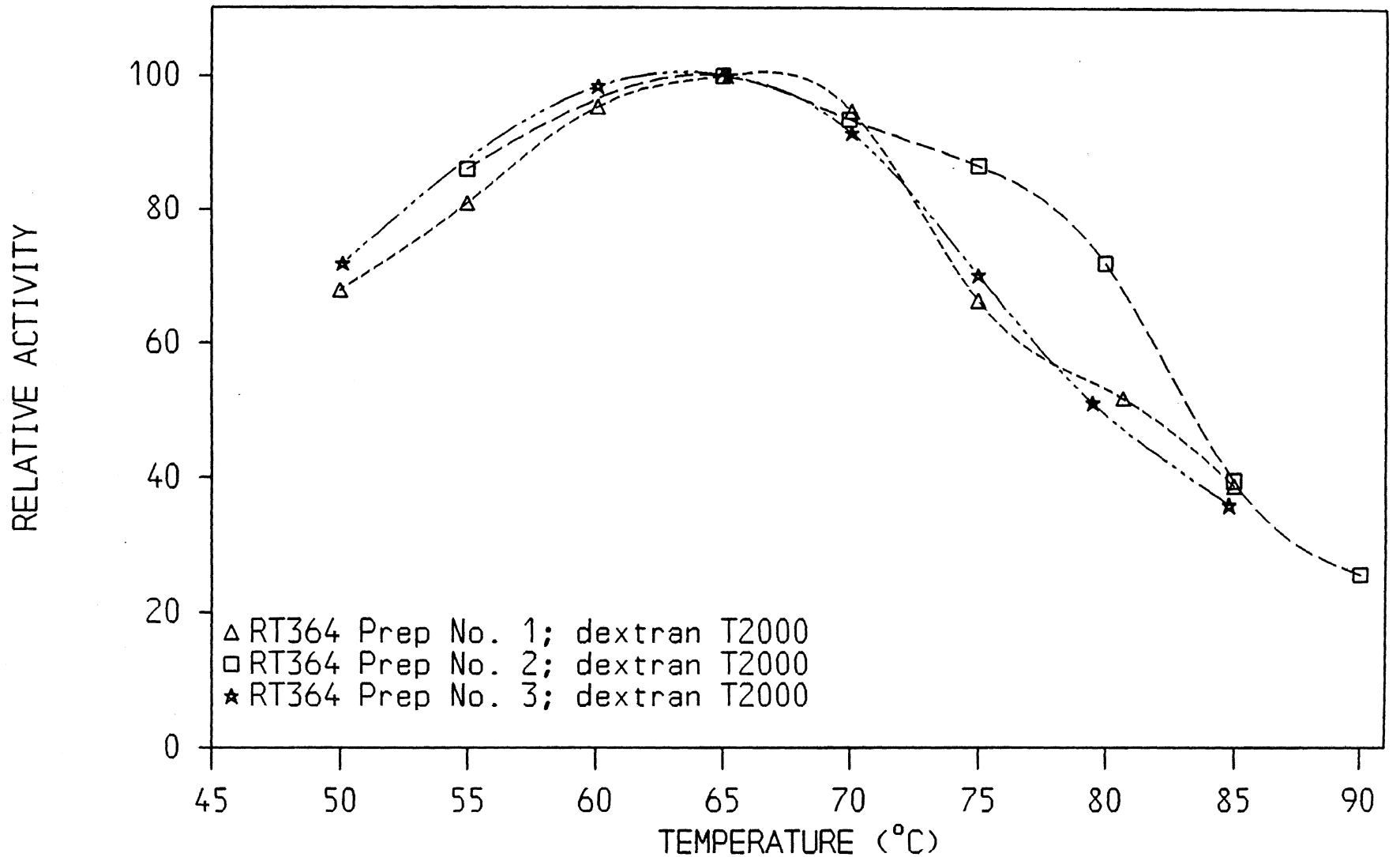


Figure 5.13. Temperature-activity profiles for the dextranase produced by RT364 (preparation 1,2, and 3) determined using the micro-haze test (Dextran T2000, pH 5.0, 30 min).

The most active sample (Preparation No. 2) was assayed to determine its temperature-activity profile under simulated factory conditions (Figure 5.14). The optimum was found to be between 65 and 70°C and hence significantly less than determined by the PAHBAH assay and below that sought for application in sugar factories. An optimum of 65°C was confirmed by determination of the temperature-activity profile using an alternative endo-dextranase activity assay, the micro-Blue Dextran method (Figure 5.15). Nevertheless. the optimum is 10°C higher than that exhibited by the *C. gracile* dextranase. In addition, considerable activity (about 80%) was retained at 75°C. Thus, of all the enzymes isolated and studied to date, the dextranase from RT364 possessed the best thermostable characteristics.

The concentrated extract (Preparation 2) was also assayed at several different pH values to determine the optimal pH. The pH-activity profile is presented in Figure 5.16. The pH of greatest activity was found to be 5.5. This value is comparable to that exhibited by the commercial preparation of *C. gracile* and to conditions pertaining to the use of commercial dextranase in the raw sugar factory.

5.3.4 Comparison of Properties of Thermostable Dextranase(s).

Temperature Optima

A comparison of the crude dextranases extracted from the microbial isolates based on the temperature optimum determined using different liquid assays is given in Table 5.6. Results from the PAHBAH assay indicated that an isolate which produced a dextranase with the desired thermostability was identified *i.e.* RT364. However, when results from the micro-haze test (particularly those determined under

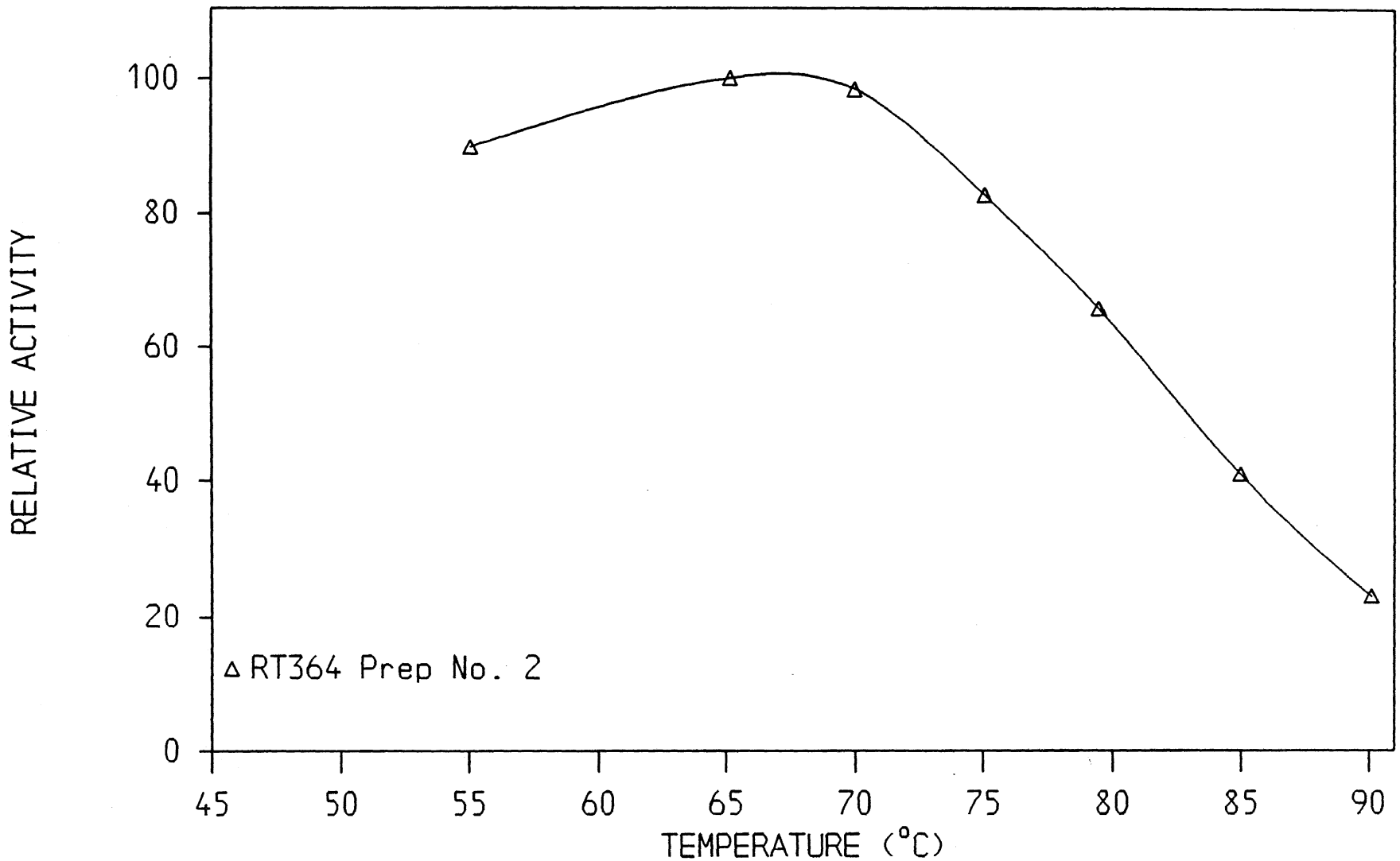


Figure 5.14. Temperature-activity profile for the dextranase produced by RT364 (preparation 2) determined using the micro-haze test (cane dextran, pH 5.0, 30 min).

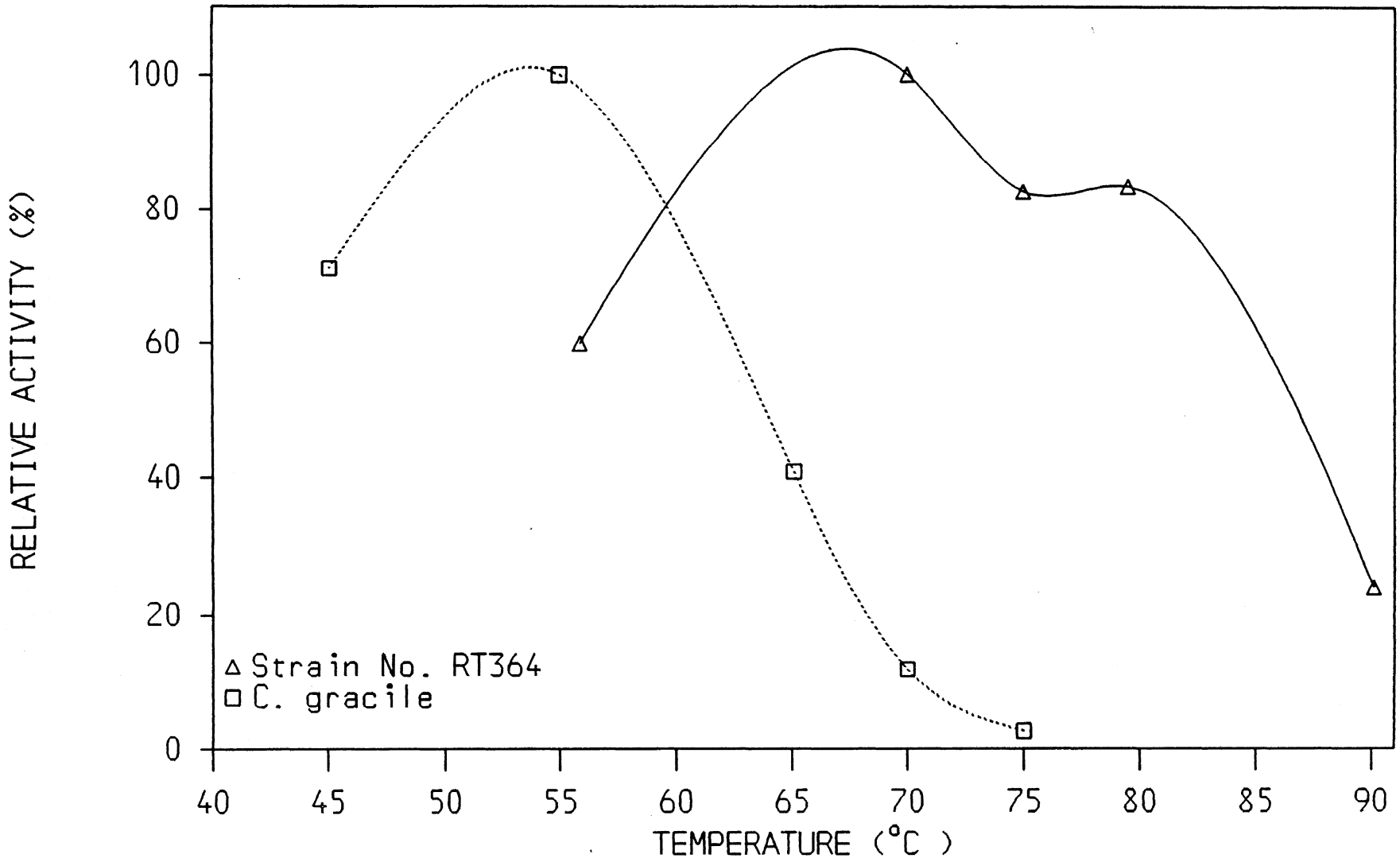


Figure 5.15. Temperature-activity profile for the dextranase produced by RT364 (preparation 2) as measured by the micro-Blue Dextran assay (pH 5.0, 30 min, 20 brix sucrose).

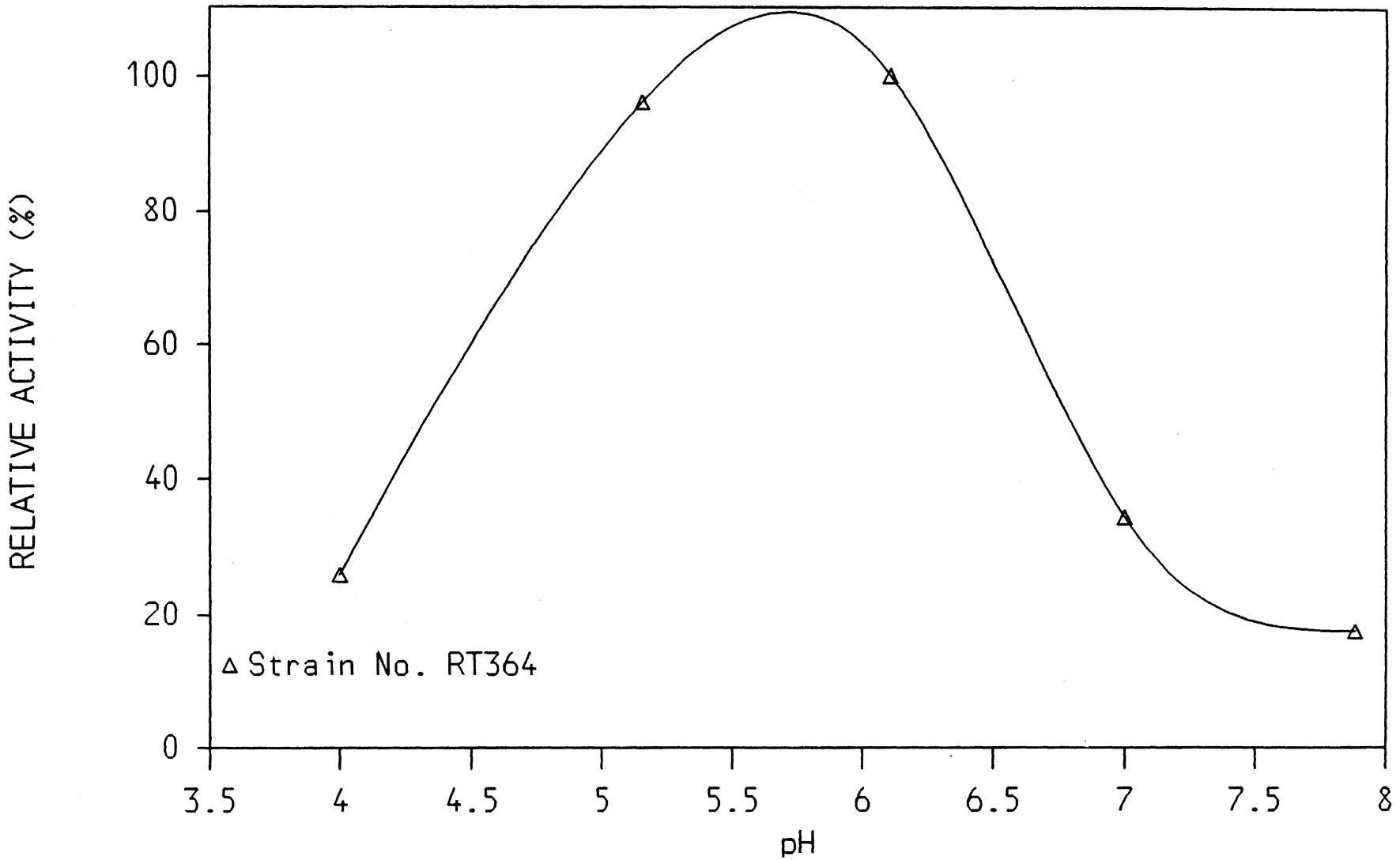


Figure 5.16. pH activity profile for the dextranase produced by RT364, determined using the micro-haze test (T2000, 75°C, 30 min).

simulated factory conditions) were also included in the assessment, several isolates produced an enzyme which possessed maximal activity at about the same temperature *i.e.* 65°C. None of the dextranases exhibited optimal activity at 75°C under simulated factory conditions; though, four isolates (SRI Strain No. 2125, SRI Strain No. 2128, AB11A and RT364) retained significant residual activity at 75°C (Table 5.7). Under simulated factory conditions, these values range from 35 to 70 per cent. The result for the crude extracts was encouraging and indicated that all four isolates were worthy of further study.

Microbial Productivity

Another factor of significance for commercial potential of an enzyme is the productivity of the microbial source. However, the limitations of low productivity of the thermophilic microorganisms may be overcome using genetic manipulations i.e. cloning and expression of the dextranase.

A comparison of the dextranase activity recovered from the culture media for a number of isolates is given in Table 5.8. Similar calculations for AB11A could not be made using the available data.

TABLE 5.6 TEMPERATURE OPTIMA OF THE DEXTRANASES FROM SELECTED ISOLATES

Isolate Number	Temperature Optimum (°C)		
	PAHBAH Assay	Micro-Haze Test (Dextran T2000)	Micro-Haze Test (Cane Dextran)
C. gracile	55	55	55
DP17	64	68	
SRI 2116	50		
SRI 2120	55		
SRI 2140		48	
SRI 2126		61	
SRI 2085	55		
SRI 2125	61	66	64
SRI 2128	65	67	58
AB11A	68	68	68
RT364	75	65	67

TABLE 5.7 RESIDUAL ACTIVITY (%) FOR THE DEXTRANASES FROM SELECTED ISOLATES AT 75°C.

Isolate Number	Residual Activity (%) at 75°C		
	PAHBAH Assay	Micro-Haze Test (Dextran T2000)	Micro-Haze Test (Cane Dextran)
C. gracile	4	6	9.5
DP17	5	21	
SRI 2116	1		
SRI 2120	-		
SRI 2140		0	
SRI 2126		17	
SRI 2085	0		
SRI 2125	4	71	35
SRI 2128	66	72	40
AB11A	85	70	50
RT364	100	70-80	70

TABLE 5.8 DEXTRANASE ACTIVITY[a] RECOVERED EXTRA-CELLULARLY FROM LIQUID CULTURE OF THE ISOLATESa.

Isolate Number	Activity at Temperature (°C)										
	35	45	50	55	60	65	70	75	80	85	90
DP17				0.096	0.13	0.16	0.179	0.095		0.0097	0.0042
SRI 2116	0.00145	0.0027		0.0029		0.0009		0.0003			
SRI 2120			0.028	0.065	0.045	0.004					
SRI 2085	0.020	0.032		0.046		0.0003					
SRI 2125 (x 10^4)		4.2	5.55	6.34	6.75	6.34	5.05	2.90	0.83	0.49	0.17
SRI 2128 (x 10^4)			4.74		6.5	7.04	6.43	4.66	2.27	0.15	
RT364								0.044[b]			

[a]Activity (nmoles min^{-1}) per mL of culture media (determined by the PAHBAH assay:30 min; pH 5.0). [b]Values ranged from 0.004 to 0.129.

The most productive isolates were DP17 and RT364. The maximal activity produced extra-cellularly in liquid culture by these isolates were 10 to 200 times greater than all the other isolates. Further, the productivity of each of the isolates may be improved if culture conditions were optimised to achieve maximum enzyme production. To date, the conditions have been selected on the basis of maximum cell growth.

Specific Activity in the Crude Culture Extract

The specific activities of the dextranase produced in the crude culture extracts were determined using both the PAHBAH assay and the micro-haze test. The data is presented in Tables 5.9 and 5.10. The highest specific activities were

TABLE 5.9 THE SPECIFIC ACTIVITY[a] OF THE CULTURE FILTRATES AS DETERMINED BY THE PAHBAH ASSAY.

Isolate Number	Specific Activity at Temperature (°C)									
	45	50	55	60	65	70	75	80	85	90
DP17			0.11	0.149	0.183	0.206	0.109	0.011	0.005	
SRI 2125	0.0133	0.0177	0.020	0.0214	0.0203	0.016	0.0094	0.0026	0.0016	0.00053
SRI 2128		0.043		0.059	0.063	0.058	0.042	0.020	0.0013	
AB11A				0.105	0.121	0.123	0.105	0.105	0.094	0.042
RT364 No. 1 Preparation			0.023		0.038	0.042	0.049	0.041	0.043	0.0042
RT364 No. 2 Preparation				0.302		0.507	0.658	0.684	0.636	0.404
RT364 No. 3 Preparation		0.11		0.255	0.295	0.385	0.416	0.402	0.371	

[a]Specific activity expressed as µmole glucose released min^{-1} mg^{-1} protein.

TABLE 5.10 THE SPECIFIC ACTIVITY[a] OF THE CULTURE FILTRATES AS DETERMINED BY THE MICRO-HAZE TEST.

Isolate Number	Specific Activity at Temperature (°C)									
	45	50	55	60	65	70	75	80	85	90
SRI 2140 (T2000)	2306	2411	1740	377	357					
SRI 2126 (T2000)	1049	1092	1159	1120	520	119	94			
SRI 2125 (T2000)	69.5		88.7	101.4	93.6	72.2	58.5		5.2	
SRI 2125 (Cane)		71.6		86.7	88.2	75.5	35.6	9.7		0
SRI 2128 (T2000)	94.5		130	155	156	112	74.0	13.4		
SRI 2128 (Cane)		117.4		154.4	123.8	97.0	57.5	28.3	0	
AB11A (T2000)		508		603	726	702	503	408		161
AB11A (Cane)		405		503	507	473	257	205		0
RT364 Prep No. 1 (T2000)		376	448	523	547	519	357	285	211	57
RT364 Prep No. 2 (T2000)			5033		5807	5436	5031	4185	2304	1507
RT364 Prep No. 2 (Cane)			4897		5438	5343	4496	3579	2236	1271
RT364 Prep No. 3 (T2000)		3328		4560	4629	4236	3255	2378	1679	

[a]Specific activity expressed as dextran hydrolysed (ppm on brix) min^{-1} mg^{-1} protein.

determined using the PAHBAH assay exhibited by the enzymes from RT364 (Preparations No. 2 and No. 3) and DP17. In contrast, the dextranase isolated from RT364 (Preparation No. 1) was found to have a relatively low specific activity. In the case of RT364, this batch to batch variation is a reflection of the different culture conditions employed (*i.e.* batch versus continuous culture) and other parameters such as media composition. The low specific acitivites in the liquid culture preparations may have been due to the inclusion of larger amounts of other extracellular proteins during concentration of the culture extract.

The results for specific activity of the crude dextranase preparations were also calculated from the micro-haze data (Table 5.10) and reflected a similar trend *e.g.* the RT364 preparation No. 2 exhibiting the highest value.

Impurities in Crude Extracts

The crude concentrated extracts from the above four isolates were checked for the presence of other carbohydrase activities. In this regard, the contaminating activities of greatest relevance within a sugar factory are amylase and pullulanase activities. The relative activities of these impurities are listed in Table 5.11 together with the corresponding results for the partially purified commercial *C. gracile* dextranase.

TABLE 5.11 RELATIVE ACTIVITY AGAINST DEXTRAN, STARCH AND PULLULAN FOR SELECTED THERMOSTABLE PREPARATIONS[a].

Dextranase Source	Dextran	Starch	Pullulan Source
C. gracile (200μL)	100	1.5	0
SRI 2125 (50μL)	100	32	75
SRI 2128 (50 μL)	100	20	63
RT364 (5μL)	100	20	14
AB11A (50μL)	100	11	13

[a]Activity determined using the PAHBAH Assay; 65°C, pH 5.0; 30 min. Dextran T2000 (0.4%); Starch (potato, 0.1%); and Pullulan (0.4%).

Significant levels of amylase activity were present in all the crude concentrated extracts. Furthermore, very high levels of pullulanase activity were measured in the crude preparations from the two SRI isolates (*i.e.* in excess of 60 % relative to the dextranase activity).

Selection of Most Promising Thermostable Dextranase

A comparison of the above parameters resulted in the selection of the RT364 isolate as the most promising source for a thermostable dextranase. For example, this isolate produced an extracellular dextranase(s) which exhibited: the highest temperature, optimum determined by the PAHBAH assay; a high temperature optimum determined by the micro-haze test (using dextran T2000 and cane dextran

as substrates); significant (70-100 per cent) residual activity at 75°C (determine under all assay conditions); one of the highest levels of productivity in liquid culture; and a specific activity 3 - 30 times higher than the dextranases from other isolates in the crude preparations (determined by either the PAHBAH assay or the micro-haze test).

Further investigations on the thermostable dextranases from the other isolates were not continued due to either low microbial productivity, low temperature optima or low specific activity of the crude culture extract.

Thermal stability of RT364 dextranase

The thermal stability of the dextranase produced by RT364 isolate was determined at 65°C, 75°C and 85°C (measured by the micro-haze test). The results are given in Figure 5.17. Activity losses at 65°C and 75°C were 30% and 40% respectively, over a 24 hour period. At 85°C, significant activity (65%) was lost over a 2 hour period.

The average time the enzyme would be exposed at 75°C in a sugar factory incubator is 20-30 min. Thus, under these conditions the RT364 dextranase would be expected to retain about 85 per cent of its activity. In comparison, the *C. gracile* dextranase currently used retains approximately 80 per cent of its activity at temperatures of 55-60°C employed during use of this enzyme.

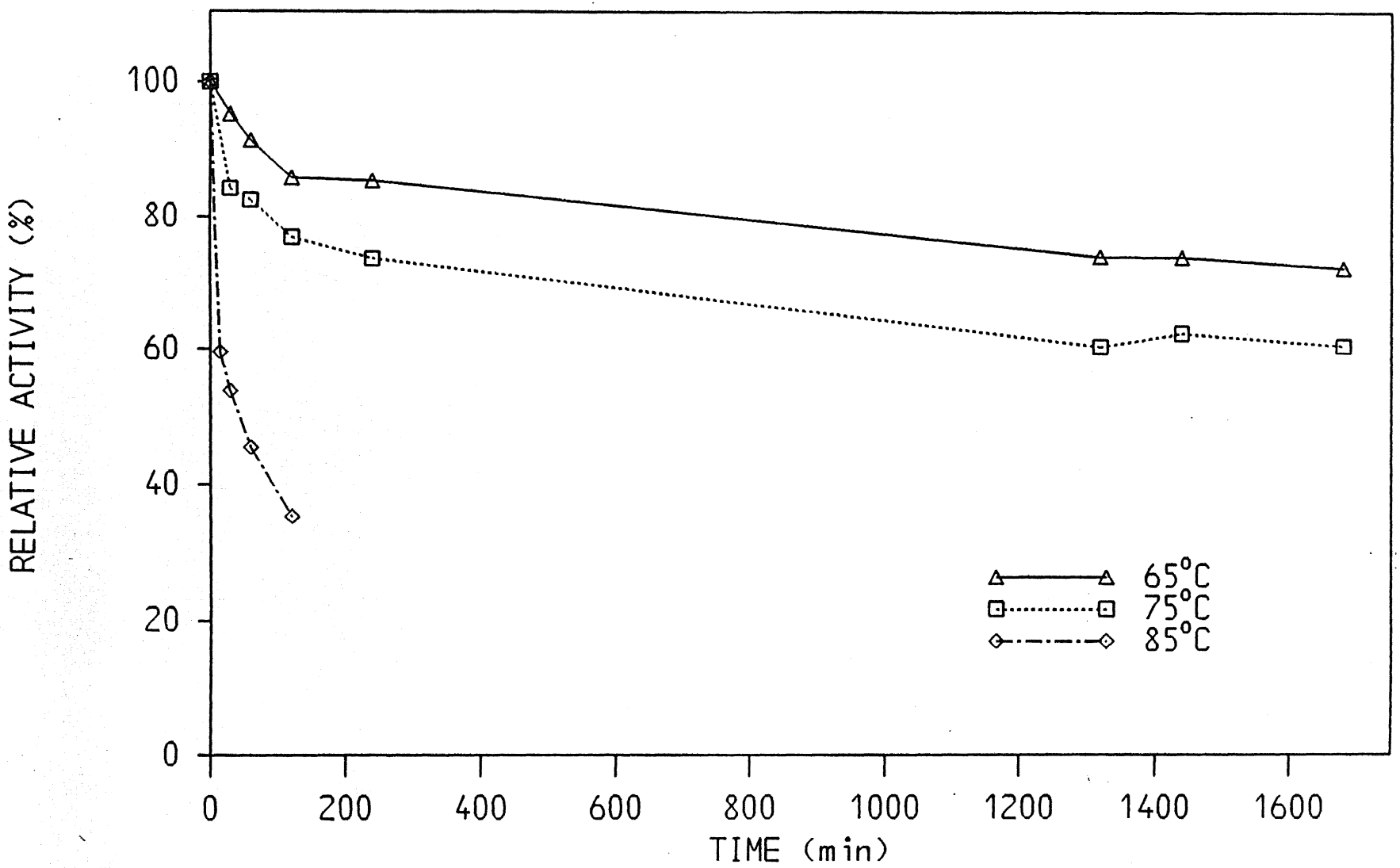


Figure 5.17 Thermostability of the dextranase produced by RT364 as measured by the micro-haze test (pH 5.0).

CHAPTER 6

Purification of a Thermostable Dextranase from a Crude Cell-Free Extract

6.1 INTRODUCTION

The thermostable dextranase preparations described in this thesis to date have been in the form of crude extracts from the fermentation media of the respective microorganisms. All enzymes were extra-cellular dextranases expressed by the microorganisms into the culture broth during fermentation. A comparison of the properties of the dextranases from the isolates examined in Chapter 5 led to the selection of the RT364 dextranase as the most promising for commercial application.

The work described in this chapter relates to the purification of dextranase from the large-scale laboratory preparations of crude cell-free extracts produced by isolate RT364. Several techniques of protein purification were investigated for their suitability for use in a purification protocol for the RT364 dextranase.

Once developed, this protocol would have the potential to be used for the purification of the other promising thermostable dextranases. Activity and protein assays were used to monitor the change in purity and specific activity after each step in the procedure. Thereafter, the physico-chemical characteristics of the partially purified or purified enzymes were determined. Initial studies were directed

toward temperature optima using assays specific for both endo- and exo-dextranase activity. Thereafter, gel filtration chromatography and polyacrylamide gel electrophoresis were utilised for estimation of the apparent molecular weight, while the isoelectric point was determined using chromatofocusing.

6.2 EXPERIMENTAL

6.2.1 Reagents

All chemicals were the best available analytical grade reagents as supplied by Ajax Fine Chemicals (Auburn, Australia) unless otherwise noted. Denatured ethanol (CSR Limited, Sydney, Australia) was used as supplied. UltraPure ammonium sulphate (Enzyme grade) was obtained from Bethesda Research Laboratories (Gaitersburg, MD).

High purity glucose, *p*-hydroxybenzoic acid hydrazide (analytical grade), molecular weight markers for sodium dodecyl sulphate (SDS) gel electrophoresis and individual proteins (yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, chymotrypsin and cytochrome c) for standardising gel filtration chromatography and chromatofocusing were purchased from Sigma Chemical Co. (St. Louis, MO).

Pre-cast polyacrylamide gels (HYLINX microgels and GSS-120 10-20 % linear gradient SDS mini gels), TTS Tris-Tricine-SDS buffer (pH 7.5), TEB Tris-EDTA-Borate buffer (pH 8.3) and Gradipure stain (a refined coomassie blue G250 stain) were obtained from Gradipore Limited (Sydney, Australia). Polybuffer (Type 74) and polybuffer exchanger (Type 94) were obtained from Pharmacia Fine Chemicals (Úppsala, Sweden).

Potato starch was purchased from BDH Chemicals Limited (Poole, England). Pullulan ($\bar{M}_n$ 8.6 x 10^4) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan).

Standard sucrose (starch and dextran free) was obtained from CSR Limited (Sydney, Australia). Commercial dextrans (MW 2 x 10^6) T2000 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The sucrose and dextrans were used without further purification. Sephadex G-100 and the gel filtration medium, Sepharose CL-6B, was also obtained from Pharmacia.

6.2.2 Preparation of Crude Extracellular Culture Fluid

The RT364 isolate was grown in batch culture (either in bottles or in a fermenter) under strict anaerobic conditions. At the end of the exponential growth phase (determined by OD_{660} measurements) the cells were separated from the broth by centrifugation (5°C, 30 min, 15 000*g*).

The protein present in the supernatant was concentrated by precipitation with ammonium sulphate. A gently stirred culture extract was slowly bought to 80 % saturation by the addition of solid ammonium sulphate. Once the salt had dissolved, the solution was allowed to stand for 1 hour prior to centrifugation at 20 000*g* for 30 min at 5°C. The resultant precipitate was redissolved in a minimum volume of buffer to achieve maximum concentration. The crude dextranase solution was then dialysed exhaustively against the same buffer at 5°C. The dialysate was centrifuged (5°C, 30 min, 20 000*g*) prior to assay.

6.2.3 Measurement of Dextranase Activity

The dextranase activity was determined by one or more of the methods outlined in Chapter 2, namely, the PAHBAH assay and the micro-haze method (using purified commercial dextran as the substrate).

6.2.4 Determination of Protein Concentration

(a) U.V. Absorption

The concentration of protein was determined by measuring the absorbance of the optically clear solution at 280 nm. This method was used for monitoring protein in the eluent from column chromatography.

(b) Biorad Standard Assay

Protein levels in the dextranase solutions were determined by the Biorad standard assay (Bradford, 1976). This method was utilised for determination of protein levels required for calculation of specific activity.

6.2.5 Detection of Other Catalytic Activity

The dextranase from RT364 isolate was checked for contaminating activities by estimation of the reducing sugars (PAHBAH assay) released from the either potato starch or pullulan.

6.2.6 Measurement of Molecular Weight on Sepharose CL-6B.

The apparent molecular weight of the dextranase was estimated by gel filtration chromatography, at 5°C, using the following markers: yeast alcohol dehydrogenase (MW 141 000), bovine serum albumin (MW 68 000), ovalbumin (MW 43 500) and chymotrypsin (MW 25 700). Sepharose CL-6B was washed with distilled water to remove fines and equilibrated at 5°C with 0.05 M Tris buffer, pH 7.5, containing 0.15 M KCl. The column (2.5 x 35 cm) was packed under gravity. Between samples, the column was washed exhaustively with the buffer until the eluent and feed buffer had the same absorbance at 280 nm.

The enzyme solution was dialysed against the column buffer prior to the application of 2.0 mL of the sample (containing a total A_{280} = 3.0) to the column. Fractions of approximately 2 mL were collected and weighed: the density of the buffer was taken as 1.00 in calculating elution volumes. The detection of proteins in the eluent was carried out by absorbance measurements at 280 nm. Elution volumes (V_e) were measured with respect to 0.2 % (w/v) Blue Dextran 2000 which gave a measure of the void volume (V_o).

6.2.7 Purification of Dextranase

(i) Affinity Absorption

Ammonium sulphate to 30 % saturation was added to a 5-mL aliquot of the concentrated crude dextranase and the pH then adjusted to 7.5. Sephadex G-100 (4 % w/v) was added to form a slurry and the mixture stirred for 1 hour (5°C). The

Sephadex-dextranase complex was isolated as a solid on a fine sinter (the filtrate containing unbound enzyme was retained for analysis). The complex was resuspended in buffer (10 mL, 0.05 M citrate, pH 5.0) and incubated at 60°C for 4 hour to achieve disociation of the complex. The free enzyme and Sephadex were separated by filtration. The protein present in the filtrate was concentrated with ammonium sulphate (80% saturation) and centrifugation (5°C, 30 min, 20 000*g*).

The precipitated enzyme was redissolved in buffer (~1 mL, 0.05 M citrate, pH 5.0) and dialysed against the same buffer (5°C). The dialysate was centrifuged to obtain a clean solution prior to activity and protein assays.

(ii) Ammonium Sulphate Fractionation

The ammonium sulphate fractionation and subsequent centrifugation and dialysis were carried out at 5°C. A gently stirred extract of the RT364 dextranase was brought slowly to the required level of saturation (10, 20, 30, 40, 50 or 80 %) by the addition of solid ammonium sulphate. Once all the salt had dissolved, the solution was allowed to stand for 1 hour at 5°C prior to centrifugation (20 000*g* for 30 min). The precipitate was redissolved in a small amount of buffer (0.05 M citrate, pH 5.0) and dialysed against the same buffer. After dialysis, any turbidity was removed by centrifugation.

(iii) Chromatofocusing (Pharmacia, 1982)

Polybuffer exchanger PBE 94 was equilibrated with the start buffer (0.025M histidine-HCl, pH 6.2) and the column (1.0 x 40 cm) packed under gravity. The

uniformity of the packing was confirmed using cytochrome c, a coloured and strongly basic (pI = 10.5) marker protein. A clear solution of the partially purified RT364 dextranase (2 mL 10-30% ammonium sulphate fraction) was loaded onto the chromatofocusing column. The proteins were eluted with polybuffer 74-HCl (pH 3.8) at 1 mL min^{-1}. Once the pH of the eluent reached 3-8 any residual protein was washed off the column with dilute HCl (0.1M).

Fractions of approximately 2 mL were collected and weighed: the density of the buffer was taken as 1.00 in calculating elution volumes. The detection of protein in the eluent was carried out by absorbance measurements at 280 nm. Enzyme activity and the pH of the aliquots were also measured. The results from the chromatofocusing run were utilised to determine the isoelectric point (pI) of the RT364 dextranase following the method described by Sluyterman and Elgersma (1978) and Sluyterman and Wijdenes (1978).

6.2.8 Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was carried out in 1-mm-thick polyacrylamide slab gels using the Gradipore Micrograd vertical electrophoresis system at a constant voltage.

(i) Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS/PAGE)

The following procedure was based on the method of Inkerman *et al.* (1975a) which was a modification of the original method of Weber *et al.* (1972). Individual proteins standards and a mixture of the molecular weight markers for (SDS) gel electrophoresis were used for standardisation. The standard proteins

used were: myosin (rabbit muscle, MW 205 000), β-galactosidase (*Escherichia coli*, MW 116 000), phosphorylase b (rabbit muscle, MW 97 400), albumin (bovine serum, MW 66 000), albumin (egg, MW 45 000) and carbonic anhydrase (bovine erythrocytes, MW 29 000).

An appropriate amount of sample buffer (Tris-Tricine-SDS buffer, pH 7.4; SDS 0.4 %; bromophenol blue 0.005%; sucrose 20 %) was added to each vial of standard protein or each sample protein to give a concentration of about 10 mg mL^{-1}. The samples were heated to 100°C for 5 min to solubilise the protein. Centrifugation (5 min, room temperature, 12 000*g*) was employed to clarify the solutions. Less than 5 μg of protein per well was loaded onto the SDS 10-20 % crosslinked gels. Electrophoresis was carried out at a constant voltage (200 V), using the Micrograd with Tris-Tricine-SDS (pH 7.5) as the buffer, until the dye front moved the length of the gel. Gels were fixed with TCA (10 %, 10 min) prior to staining with Gradipure (10 mL per gel) overnight. Contrast was improved after destaining in acetic acid (10%, overnight).

(ii) Gradient Polyacrylamide Gel Electrophoresis (Native PAGE)

Electrophoretic analysis of the native enzyme was performed by modification of the method of Inkerman *et al.* (1975b). Samples (200 μL) were prepared for electrophoresis by addition of sucrose [20 % (w/v)] and bromophenol blue [20 μL, 0.025 % (w/v)]. An aliquot containing about 5 μg protein per well was loaded (HYLINX gel). Electrophoresis was carried out at a constant voltage (200 V), using the Micrograd with Tris-EDTA-Borate buffer (pH 8.3; Tris, 10 % w/v; boric acid, 5% w/v; EDTA, 0.5 % w/v) until the dye front migrated the length of the gel (about

20 min). Proteins were visualised using the Gradipure staining technique outlined above. Detection of dextranase activity was attempted using a method described by Dr. C. Wynter (personal communication). This procedure involved rinsing the HYLINX gel with buffer (0.05 M citrate, pH 5.0) immediately after electrophoresis was completed. The gel was then overlayed on a BDA plate and incubated at 65°C until clearing zones became visible. The gel was removed from the plate and the reaction stopped by flooding with alcohol. Dextranase activity could be seen on the BDA plates as colorless (or clearer) bands on a blue background.

6.2.9 U.V. Absorption Spectroscopy

The absorption of the solution was measured over the range 350 nm to 240 nm using a Cary 118C recording spectrophotometer.

6.3 RESULTS AND DISCUSSION

6.3.1 Large Scale production of RT364 Dextranase

A large volume (about 37 L) of the cell-free culture extract of RT364 (prepared by Miss L.M. Cox and Dr. M. Dawson, Sugar Research Institute) was obtained from 9 separate fermentations. Individual cell-free culture broths were assessed for dextranase activity (PAHBAH assay) and protein content. The results are given in Table 6.1.

The extracts were concentrated as described in the experimental section using ammonium sulphate precipitation (80% saturation). The respective concentrates were reassayed for dextranase activity (PAHBAH assay; Table 6.2;

TABLE 6.1 ACTIVITY AND PROTEIN LEVELS IN CELL-FREE CULTURE BROTH FROM SEPARATE FERMENTATIONS OF RT364 DEXTRANASE.

Batch Number	Volume (mL)	Activity[a] (nanomoles min^{-1} ml^{-1})	Protein Conc[b] (mg mL^{-1})	Total Activity (µmole min^{-1})	Specific Activity (µmole min^{-1} mg^{-1})	Total Protein (mg)
1 (Fermenter)	1 508					
2 (Fermenter)		>AS ppt	sent to	University of	Queensland	
3 (Bottles)	4 483	28.5	0.02	128	1.43	90
4 (Bottles)	8 446	64.8	0.022	547	1.03	186
5 (Bottles)	7 699	51.9	0.014	399	2.95	108
6 (Fermenter)	1 904	129.6	0.011	247	3.7	209
7 (Fermenter)	1 546	4.2	0.05	6.4	0.08	77
8 (Bottles)	11 593	10.4	0.045	121	0.23	522
9 (Fermenter)	1 575	17.6	0.052	27.7	0.34	82
TOTAL	37 246					

[a]Activity determined using the PAHBAH assay (30 min, pH 5.0, 75°C). [b]Protein concentration determined by the Biorad protein assay. AS:ammonium sulphate.

TABLE 6.2 ACTIVITY AND PROTEIN LEVELS IN CONCENTRATED EXTRACTS FROM SEPARATE FERMENTATIONS OF RT364 DEXTRANASE (PAHBAH ASSAY).

Batch Number	Volume (mL)	Activity[a] (nanomoles min^{-1} mL^{-1})	Protein[b] conc (mg mL^{-1})	Total Activity (µmole min^{-1})	Specific Activity (µmole min^{-1} mg^{-1})	Total Protein (mg)	Recovery %	
							Activity	Protein
1 (Fermenter)	4.4	2 150	2.35	9.46	0.92	10.34		
2 (Fermenter)		>As ppt	sent to	University	of	Queensland		
3 (Bottles)	1 500 →19.9	648	0.618	12.9	1.05	12.3	30.1%	40.9%
	2 983 →79.1	2282	1.78	180.4	1.28	140.7		
4 (Bottles)	~ 25%							
	~75%	>AS ppt	sent to	University	of	Queensland		
5 (Bottles)	79.8	1 815	1.34	144.8	1.35	106.9	36.3%	98.8%
6 (Fermenter)	49.06	1 763	1.95	86.5	0.904	95.7	35.0	45.8%
7[c] (Fermenter)	35.1	176.3	0.905	6.2	0.195	31.8	~100% but very low	41.3%
8 (Bottles)	320.4	329	0.74	105.4	0.445	237	87.1	45.4%
9 (Fermenter)	46.2	648	0.845	29.96	0.77	39.06	~100%	47.6%
Pooled[d]	576.0	777.8	1.05	448	0.74	604.8		

[a]Activity determined using the PAHBAH assay (30 min, pH 5.0, 75°C). [b]Protein concentration determined by the Biorad protein assay. [c]Not included in pooled sample because of low activity and low specific activity. [d]Half of pooled sample sent to University of Queensland for analysis, purification and characterisation. AS:ammonium sulphate

micro-haze test; Table 6.3) and protein content. Recovery of activity and protein were determined and the specific activity calculated for each concentrated extract (Table 6.2). Concentrated extracts from separate batches were pooled with the exception of the concentrate from Batch 7 which exhibited a relatively low specific activity. The pooled concentrate (570 mL) was estimated to contain 1.05 mg protein mL^{-1} and exhibit a specific activity of 0.74 μmole glucose min^{-1} mL^{-1}*. This concentrate was used as the starting material in development of a purification protocol for RT364 dextranase.

6.3.2 Gel Filtration Chromatography

Preliminary investigations to develop a purification procedure for the RT364 dextranase were carried out using gel filtration chromatography. No separation of protein components in the crude extract was achieved as the sample eluted from the Sepharose CL-6B column as a single Gaussian peak (Figure 6.1). However, the dextranase activity exhibited by the fractions indicated that the enzyme eluted prior to the fraction displaying maximal absorbance at 280 nm. Fractions corresponding to the activity peak (100 to 170 mL) were pooled and concentrated. Further study on this sample was not carried out as a significant increase in purity was not achieved. Hence, alternative methods for the purification of the RT364 dextranase were sought.

**Half the sample volume was retained at SRI for protein purification and characterisation while the other half was forwarded to co-workers at University of Queensland for experimentation.*

TABLE 6.3 ACTIVITY AND PROTEIN LEVELS IN CONCENTRATED EXTRACTS FROM SEPARATE FERMENTATIONS OF RT364 DEXTRANASE (MICRO-HAZE TEST).

Batch Number	Volume (mL)	Activity[a] (ppm dextran hydrolysed min^{-1} mL^{-1})	Protein Conc (mg mL^{-1})	Total Activity (ppm dextran hydrolysed min^{-1})	Specific Activity (ppm dextran hydrolysed min^{-1} mg^{-1})	Total Protein (mg)
1 (Fermenter)	4.4	38 360	2.35	1.69×10^5	1.63×10^4	10.34
2 (Fermenter)		>AS ppt	sent to	University of	Queensland	
3 (Bottles)	1 500 → 19.9	10 560	0.618	2.1×10^5	1.71×10^4	12.3
	2 983 →79.1	32 247	1.78	2.55×10^6	1.81×10^4	140.7
4 (Bottles)	~25%					
	~75%	>AS ppt	sent to	University of	Queensland	
5 (Bottles)	79.8	22 320	1.34	1.78×10^6	1.67×10^4	106.9
6 (Fermenter)	49.06	30 513	1.95	1.50×10^6	1.56×10^4	95.7
7 (Fermenter)	35.1	2 363	0.905	8.3×10^4	2.61×10^3	31.8
8 (Bottles)	320.4	6 762	0.74	2.17×10^6	9.14×10^3	237
9 (Fermenter)	46.2	11 929	0.845	5.51×10^5	1.41×10^4	39.06
Pool	576	16 792	1.05	9.67×10^6	1.60×10^4	605

[a]Activity determined using the micro-haze test (30 min), pH 5.0, 65°C). [b]Protein concentration determined by the Biorad protein assay. AS:ammonium sulphate.

In addition to protein separation, gel filtration may be used for molecular weight estimation. The technique was therefore utilised for the determination of the apparent molecular weight of the activity in the crude RT364 dextranase preparation. Calibration of the column was carried out using four proteins of known molecular weight. The results are shown in Figure 6.2. Using the method described by Inkerman (1973), the apparent molecular weight for the active dextranase fraction was calculated to be 50 000 (Table 6.4).

TABLE 6.4 CALIBRATION OF THE SEPHAROSE CL-6B COLUMN FOR MOLECULAR WEIGHT ESTIMATION.

Sample	V_e (mL)	Log MW	Molecular Weight
Blue dextran	60.5 (V_o)	-	-
Alcohol dehydrogenase (Yeast)	127.0	5.149	141 000
Bovine Serum Albumin	135.0	4.821	68 200
Egg Albumin (ovalbumin)	144.0	4.638	43 500
Chymotrypsin	155.5	4.398	25 000
RT364 (Activity)	141	4.700	50 100

6.3.3 Affinity Adsorption

Successful application of highly branched substrates as affinity absorbents have been reported for enzyme purification (Saito, 1973; Rozie *et al.*, 1992). An experiment was devised to determine if Sephadex G-100 (a modified cross-linked dextran) could be used as an affinity absorbent for purification of the RT364 dextranase. Results obtained for the crude enzyme, the filtrate containing unbound

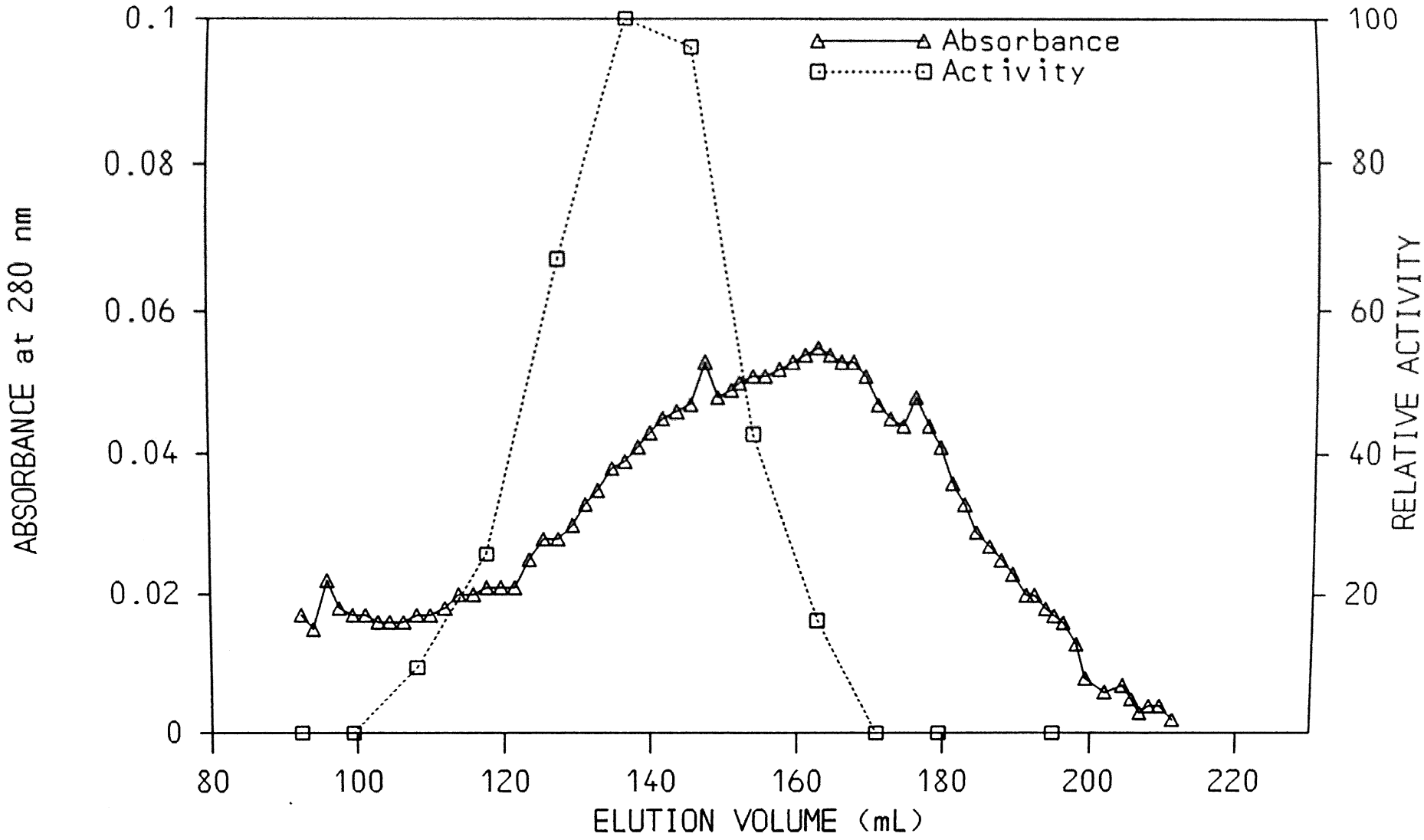


Figure 6.1 **Chromatography of RT364 dextranase using sepharose CL-6B gel filtration media. Activity determined by the PAHBAH assay (30 min, pH 5.0, 75°C)**

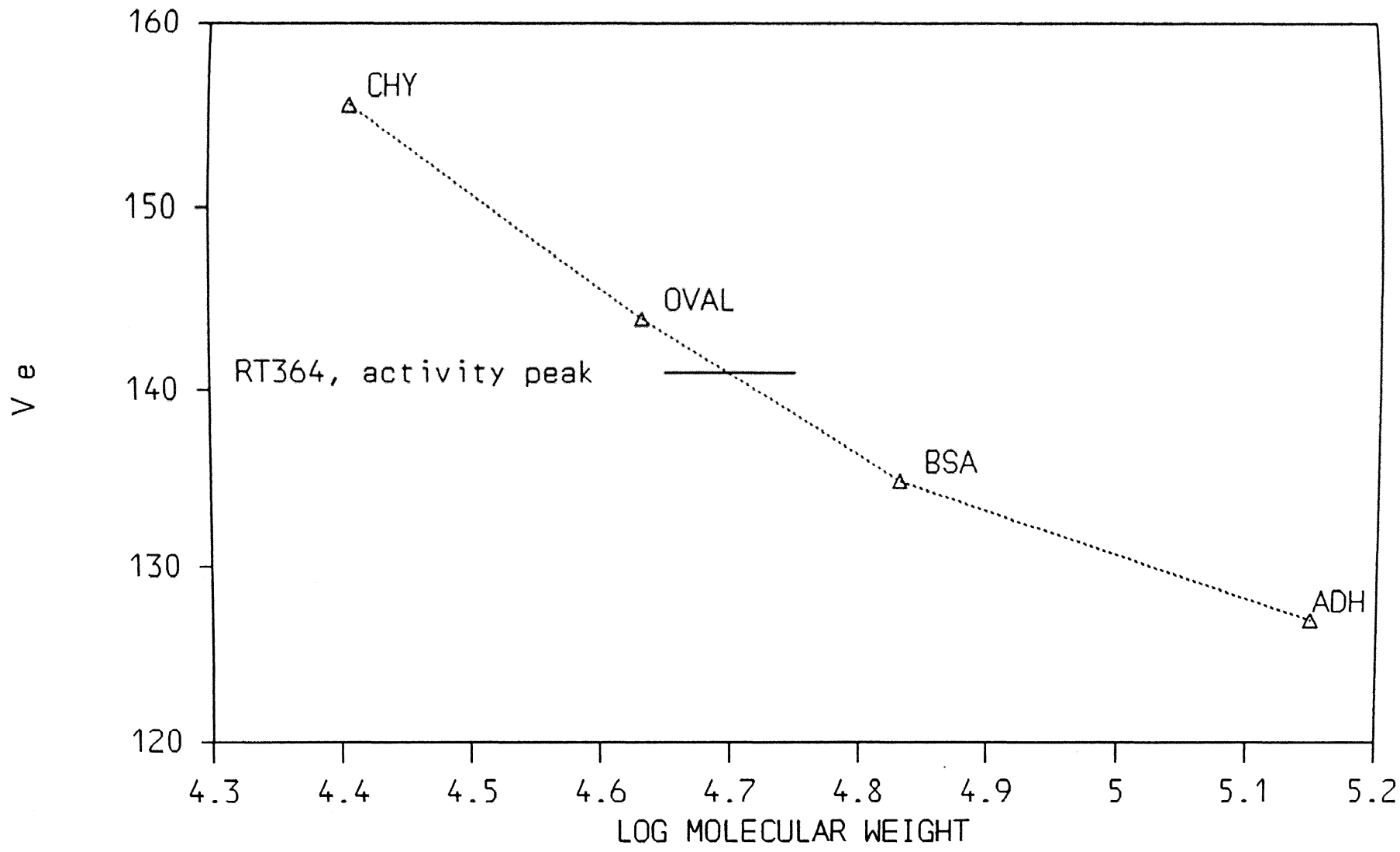


Figure 6.2 Calibration curve for sepharose CL-6B gel filtration chromatography. CHY:chymotrypsin; OVAL:ovalbumin' BSA:bovine serum albumin; ADH:alcohol dehydrogenase (yeast).

dextranase (first filtrate), the final filtrate and the concentrated final filtrate are given in Table 6.5. An apparent increase in purity was achieved with the final filtrate exhibiting about double the specific activity of the crude preparation. However, the recovery of activity was very low *e.g.* less than 20 % of the enzymic activity was recivered in the final filtration. Further loss of activity was apparent after concentration of the final filtrate with recovery of activity at a level of around 3 %.

TABLE 6.5 PROPERTIES OF AFFINITY ADSORPTION FRACTIONS.

Sample	Volume (mL)	Specific Activity (μmole min^{-1} mg^{-1})	Protein (mg mL^{-1})	Recovery of Activity (%)
Crude Enzyme	5.0	0.811	1.96	-
First Filtrate	6.5	0.586	1.65	79.1
Final Filtrate	4.8	1.8	0.18	18.4
Concn Final Filtrate	0.8	1.25	0.27	3.4

6.3.4 Ammonium Sulphate Fractionation

Fractional precipitation of protein extracts with salts such as ammonium sulphate (Scopes, 1982) have been widely used for both protein concentration and protein purification. Ammonium sulphate fractionation of the crude RT364 concentrated extract was investigated as shown in Table 6.6. The precipitate from each cut was dialysed against buffer (0.05 M, pH 5.0) prior to assay. Most enzyme activity was detected in the 20 - 30 % cut, however, significant activity was also

present in the 10-20 % cut. An approximate a two-fold increase in specific activity (determined by the PAHBAH assay) was achieved for both fractions.

TABLE 6.6. AMMONIUM SULPHATE FRACTIONATION OF RT364.

Sample	Volume (mL)	Specific Activity (µmole/min/mg)	Protein (mg/mL)	Recovery of Activity (%)
Crude Enzyme	271.6	0.72	1.29	-
Fraction 1 0-10 % AS	10.4	0.30	0.16	0.2
Fraction 2 10-20 % AS	10.2	1.32	3.2	17.1
Fraction 3 20-30 % AS	24.6	1.20	5.3	62.2
Fraction 4 30-40 % AS	23.8	0.13	2.26	2.74
Fraction 5 40-50 % AS	20.5	0.05	1.39	0.5
Fraction 6 50-80 % AS	20.0	0.008	0.63	0.04

Each of the fractions were also assayed for activity using the micro-haze test. The relative increase in specific activity was of the same order (about two-fold) as determined from the reducing sugar assay. A profile for the recovery of enzyme activity (determined using the PAHBAH assay and micro-haze test), as well as protein recovery is shown graphically in Figure 6.3.

Salt fractionation resulted in only a small increase in purity of the RT364 dextranase. As a significantly greater increase in purification of the RT364 dextranase was necessary prior to physico-chemical characterisation, other techniques were investigated.

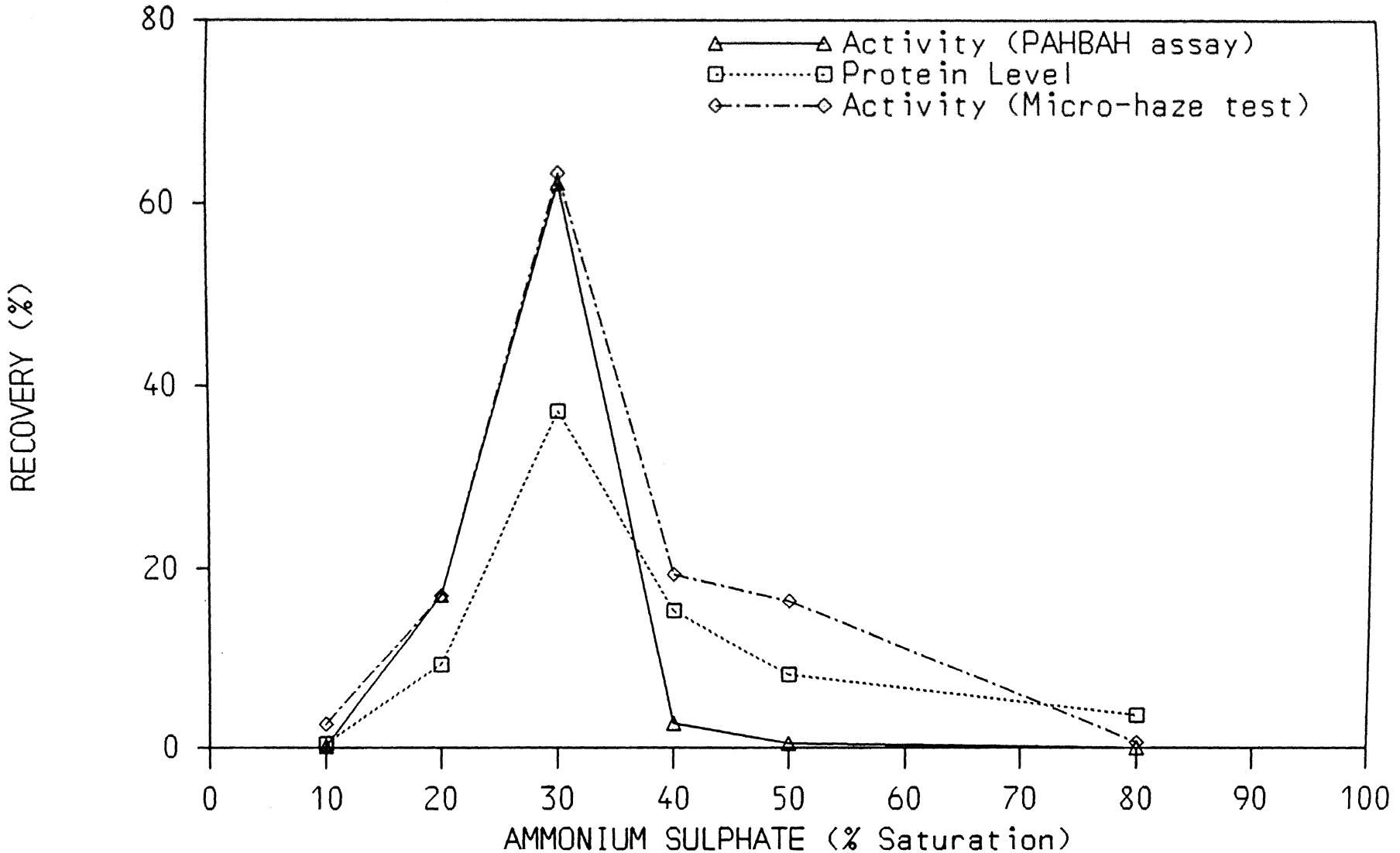


Figure 6.3 **Recovery of dextranase activity and protein during salt fractionation of the concentrated crude culture extract from RT364.**

6.3.5 Chromatofocusing of RT364 dextranase

The technique of chromatofocusing was investigated next for purification of the RT364 dextranase. The two ammonium sulphate fractions exhibiting the highest activity (fractions 2 and 3) were pooled and applied to a chromatofocusing column.

The proteins present in these fractions were separated into five separate regions by chromatofocusing (Figure 6.4). Column fractions were pooled, as indicated in Figure 6.4, prior to assay. Each pooled sample was concentrated using ammonium sulphate (80 % saturation) and then dialysed (0.05 M citrate buffer, pH 5.0) to remove the polybuffer. Dextranase activity was determined for the concentrated aliquots using the PAHBAH assay.

The majority of the activity was recovered in the region designated IV. Chromatofocusing led to a further eight-fold increase in purity resulting in a final specific activity of 11.2 μmole min^{-1} mg^{-1} (Table 6.7) at 75°C.

TABLE 6.7 PROPERTIES OF CHROMATOFOCUSING FRACTIONS.

Sample	Volume (mL)	Specific Activity (μmole min^{-1} mg^{-1})	Protein (mg mL^{-1})	Recovery of Activity (%)
Partially purified Ammonium sulphate 10-30 % cut	2.0	1.46	6.156	-
Region I	4.38	0.205	0.095	0.5
Region II	5.48	0.093	0.07	0.2
Region III	3.71	1.17	0.10	2.4
Region IV	7.96	11.2	0.185	62.1
Region V	7.12	0.17	0.46	3.1

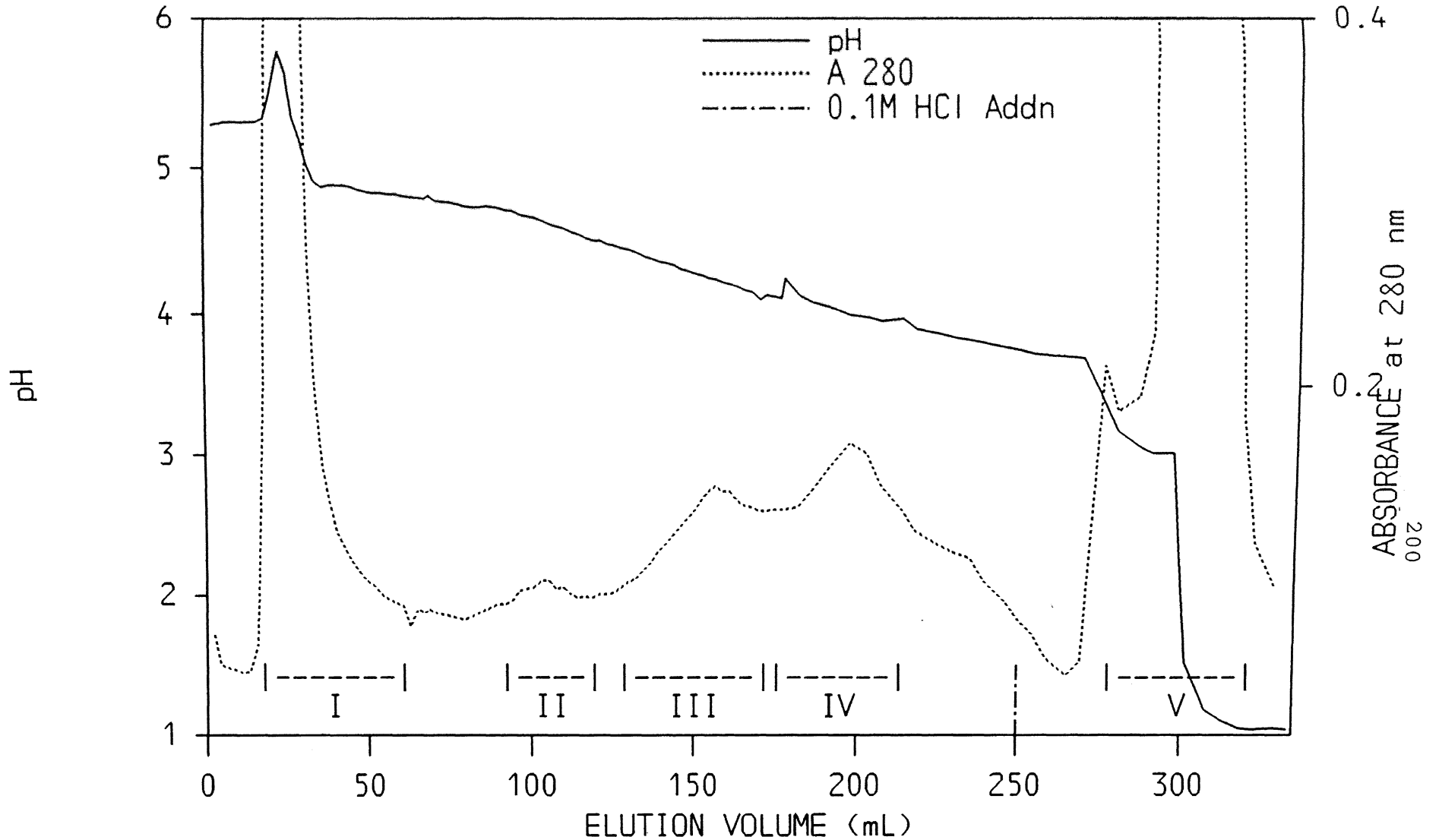


Figure 6.4 **Chromatography of a partially purified preparation of RT364 dextranase on PBE94, pH gradient 5.4 to 3.8 (polybuffer 74-HCl).**

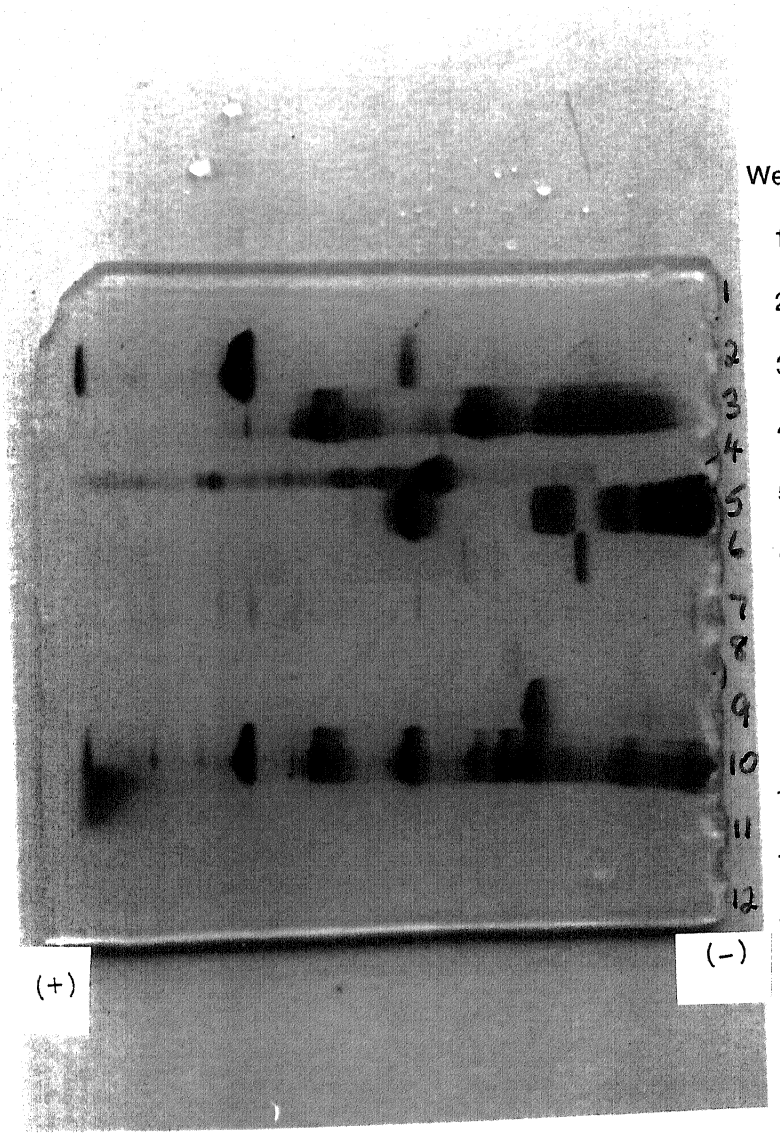
The pH profile over the chromatofocusing run was determined from measurement of the pH of the column fractions. The region containing the highest dextranase activity (Region IV) eluted at the lower pH range of the separation. This corresponded to a value of 4.0 for the isoelectric point of the RT364 dextranase.

To determine if further purification steps would increase the specific activity of the enzyme an estimate of the present state of purity was obtained using polyacrylamide gel electrophoresis (PAGE).

6.3.6 Polyacrylamide Gel Eelectrophoresis

(i) Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS/PAGE)

As shown in Figure 6.5, the RT364 dextranase (Chromatofocusing Region IV) was separated into three protein components using gradient polyacrylamide gel electrophoresis in the presence of SDS. Under these conditions, the enzyme should be fully denatured and dissociated into its constituent polypeptide chains with separation being achieved on the basis of molecular weight. Individual proteins of known molecular weight and a mixture of these molecular weight markers were used for standardisation. The molecular weight for each band in the dextranase preparation was estimated by comparison with the relative mobilities of the marker proteins. These data are presented in Figure 6.6 as a plot of the relative mobility against log MW for each of the reference proteins. The highest protein concentration was contained in the band of MW 152 000. The other two bands (MW 98 000 and 82 000) were composed of successively less amounts of protein. Subsequently, the 152 000 MW band was shown to contain the major portion of the



Well No.	Protein
1	-
2	Carbonic anhydrase
3	Albumin egg
4	Miles dextranase L4000
5	Albumin bovine plasma
6	Chromatofocusing region IV
7	Myosin
8	Phosphorylase
9	β-galctosidase
10	Mixed standards (Sigma)
11	AS 10-30% fraction
12	-

Figure 6.5 Polyacrylamide gel electrophoresis of the RT364 dextranase in the presence of sodium dodecyl sulphate.

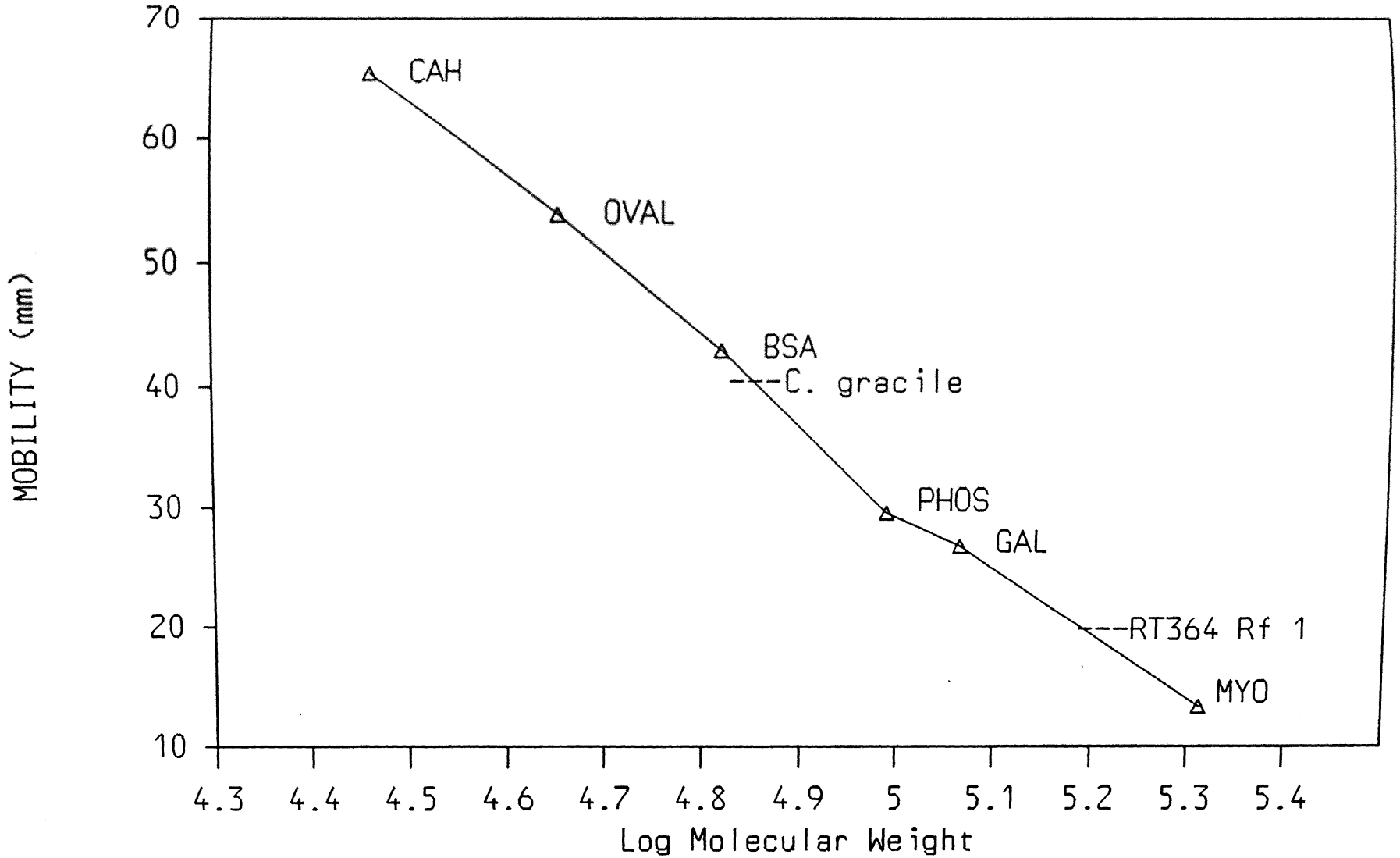


Figure 6.6 **Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of RT364 dextranase, *C. gracile* dextranase and proteins of known molecular weight (CAH:carbonic anhydrase; OVAL:egg albumin; BSA:bovine plasma albumin; PHOS:phosphorylase; GAL:β-galactosidase; MYO:myosin).**

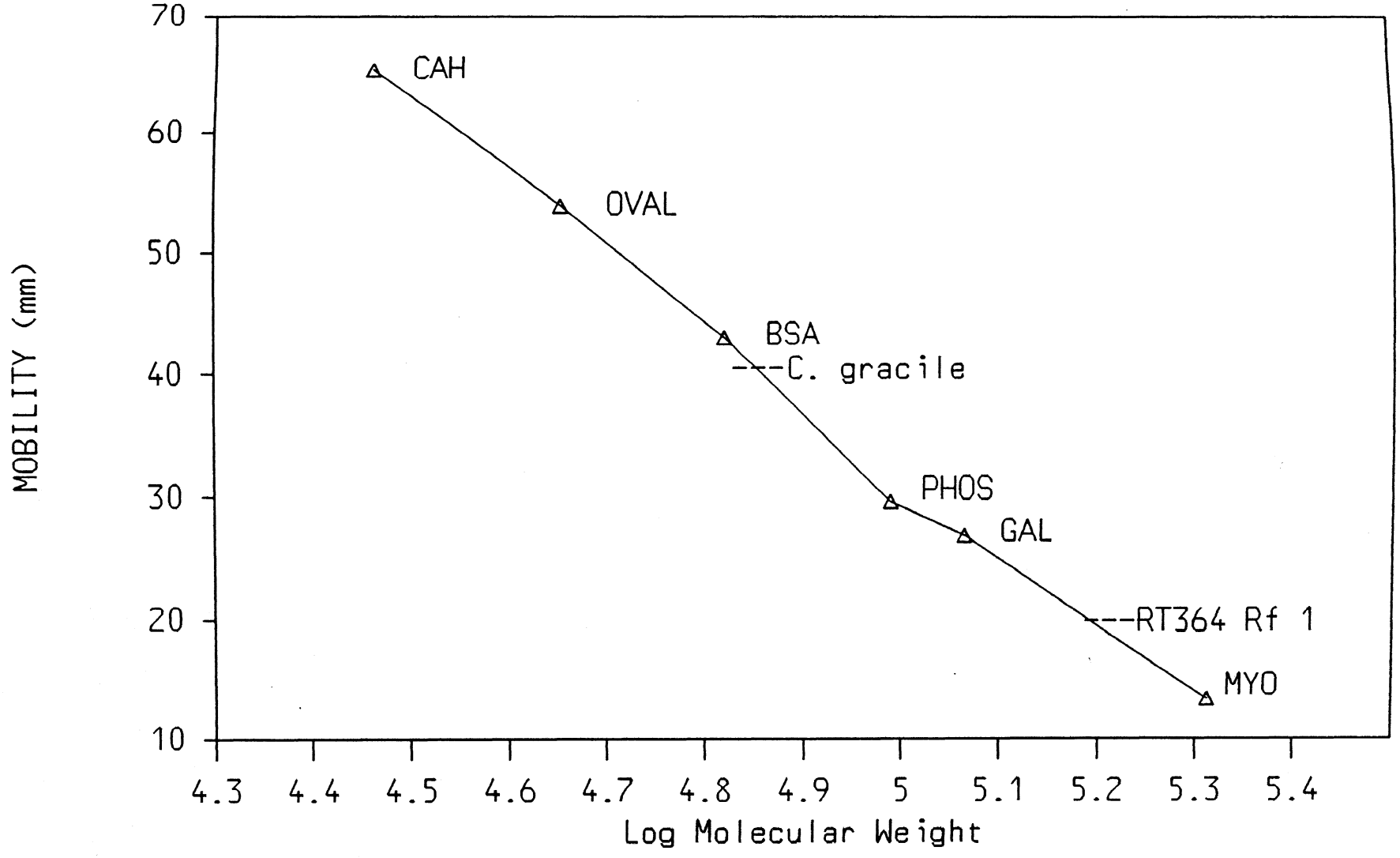


Figure 6.6 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of RT364 dextranase, *C. gracile* dextranase and proteins of known molecular weight (CAH:carbonic anhydrase; OVAL:egg albumin; BSA:bovine plasma albumin; PHOS:phosphorylase; GAL:β-galactosidase; MYO:myosin).

dextranase activity (this study; Dr. C. Wynter, private communication). The above results may be compared with the earlier finding of a MW of 50 000 for the RT364 dextranase activity obtained by gel filtration. These conflicting results indicated the possibility of either interaction of the dextranase with the gel filtration medium or the presence of non-protein material such as covalently bound carbohydrate. Some evidence for the latter is discussed under UV Absorption Spectra in this chapter.

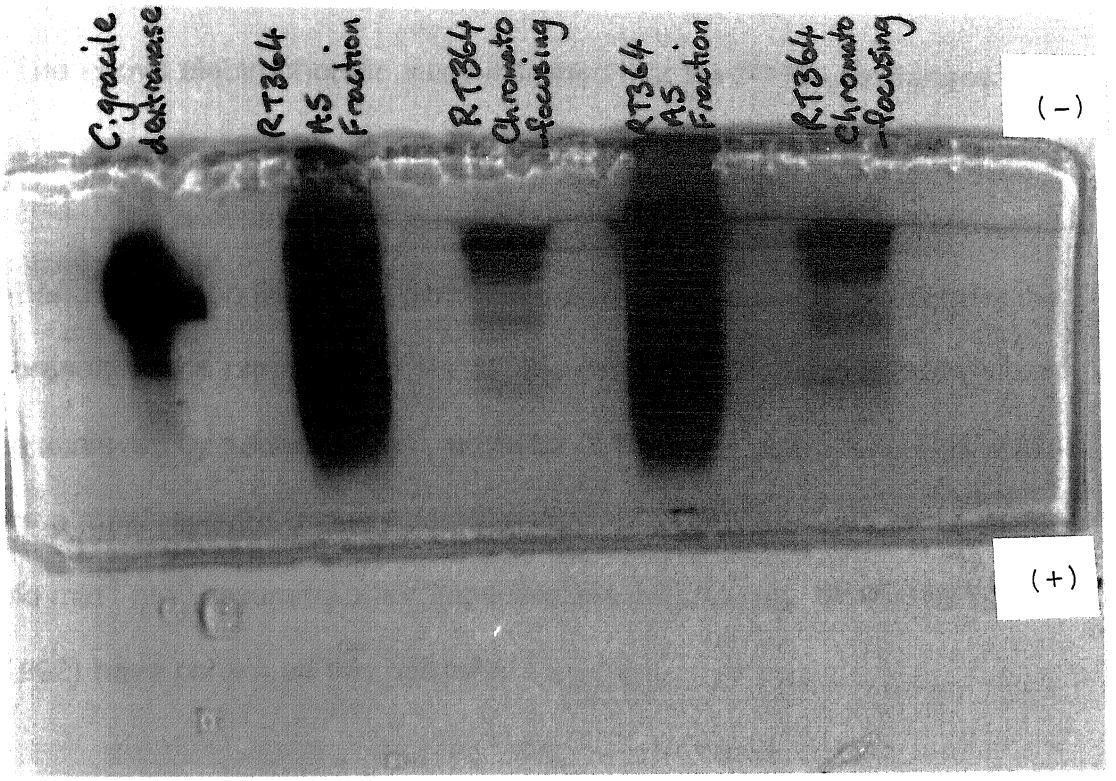
(ii) Gradient PAGE (Native PAGE)

Electrophoretic analysis of the native enzyme was performed on HYLINX gels (pH 8.3) for the ammonium sulphate fraction (AS 10-30 %) and the chromatofocusing fraction (Region IV). Proteins were visualised using the Gradipure stain. The electrophoretic pattern obtained for the native RT364 is given in Figure 6.7(a).

The AS 10-30 % fraction appeared as a broadish band after reaction with the protein stain. A number of intense and distinct bands were visible within the broad region. In comparison, the pattern obtained from the chromatofocusing Region IV preparation consisted of one intense band of low mobility, two light faster migrating bands and two very light diffuse areas.

Visualisation of the dextranase activity was attempted using the overlay technique on a BDA plate. The dextranase activity could be seen as clearing zones on the BDA plates in the area corresponding to the intense protein band described for Region IV (Figure 6.7 (b)), for both the AS 10-30 % fraction and the Region IV fraction. This corresponds to the 152 000 MW species obtained on SDS/PAGE. Unfortunately, the clearing zones were diffuse and proved difficult to photograph.

(a)



(b)

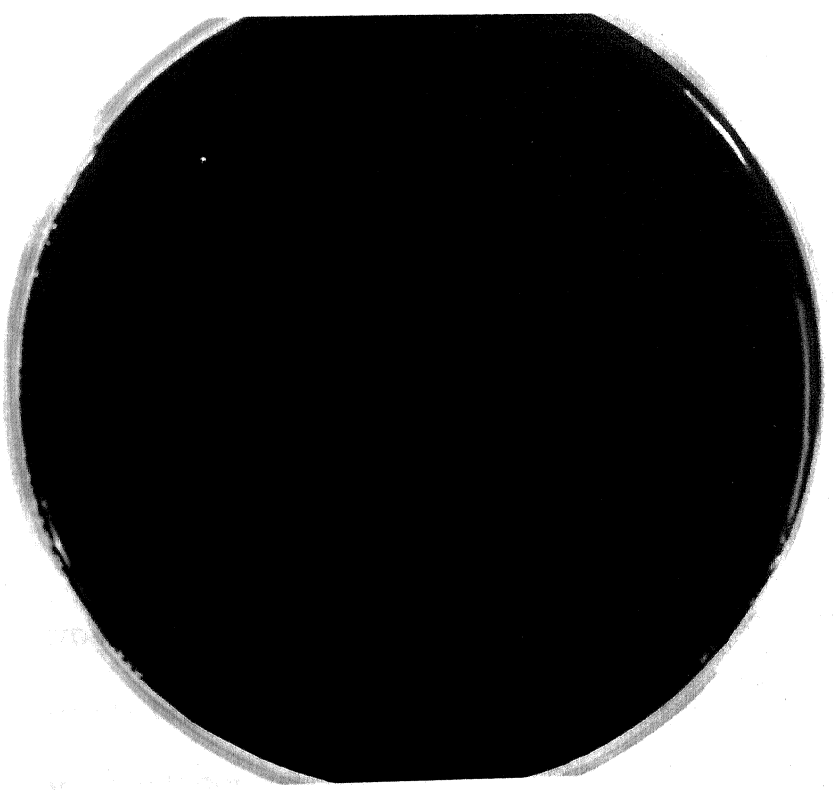


Figure 6.7 Electrophoretic separation (pH 8.3, Hylinx microgel) of the native RT364 enzyme from salt fractionation and chromatofocusing compared to Miles dextranase L4000

The above electrophoretic study indicated that the RT364 dextranase was not chromatographically pure. However, an estimate of its state of purity was possible using the relative intensities of the three major protein bands. It appeared that the band corresponding to the dextranase activity accounted for over half of the protein present in the preparation. Hence, the most likely increase in purity which could be achieved by additional work would be of the order of two-fold This would result in a final specific activity for the purified RT364 dextranase of about 20 μmole min^{-1} mg^{-1} assuming other 'impurities' are not proteins. Studies by Wynter et. al (1992) have confirmed this estimate.

6.3.7 Characterisation of Partially Purified RT364 Dextranases

(i) Temperature Optimum

The temperature-activity profile of the Region IV fraction was measured using the PAHBAH assay (Figure 6.8). The temperature optimum for exo-dextranase activity was approximately 83°C. About 80 % of the maximal activity was exhibited at 75°C.

The micro-haze test (a method specific for endo-dextranase activity) was used to measure the temperature-activity profile of the Region IV fraction under simulated factory conditions using deteriorated cane juice. The temperature optimum at which maximal endo-dextranase activity occurred was approximately 68°C (Figure 6.8). This variation indicated that the partially purified RT364 dextranase (Region IV) contained two or more dextranases with different

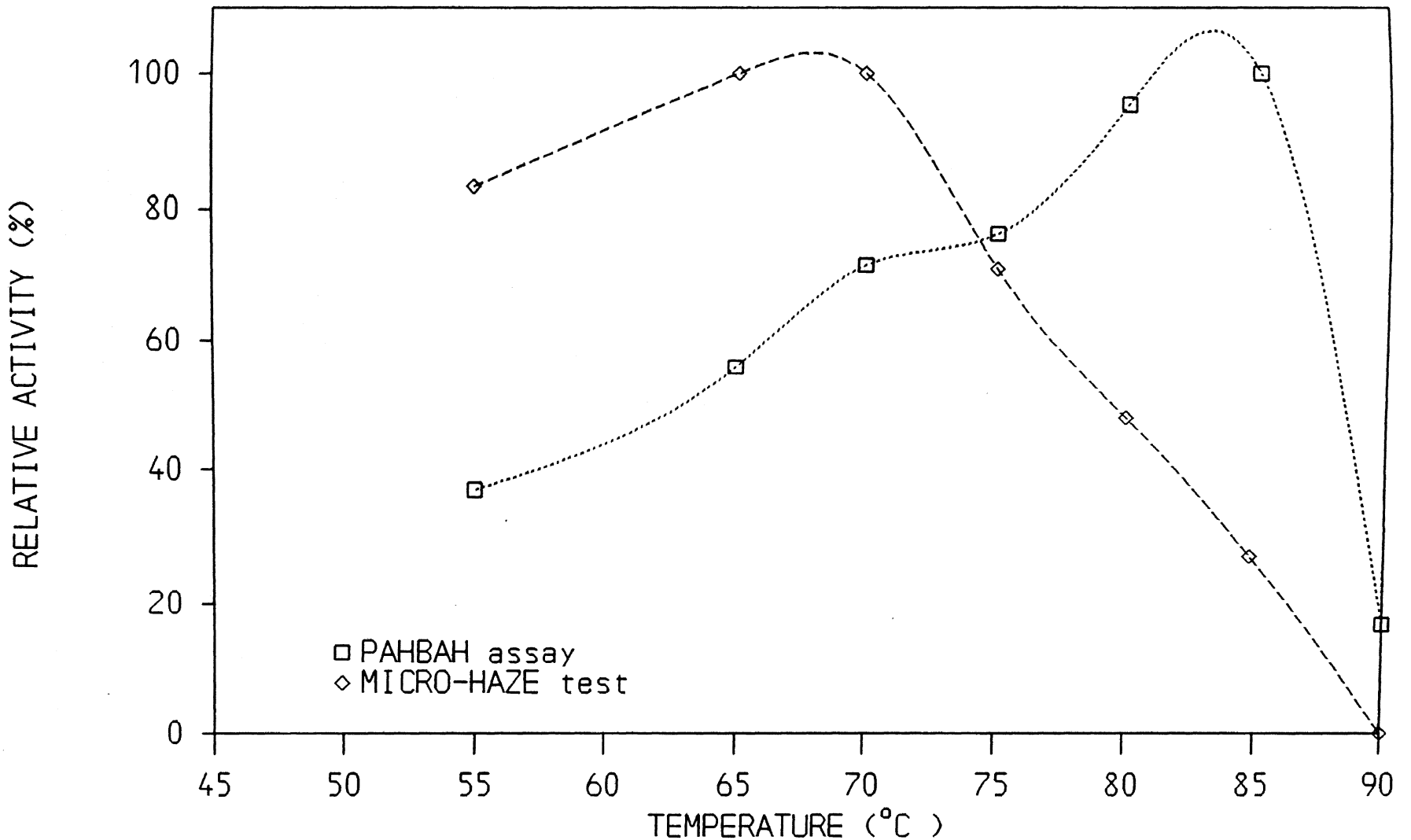


Figure 6.8 Temperature-activity profile of RT364 dextranase (Region IV from chromatofocusing) as determined by the PAHBAH assay (30 min, pH 5) and micro-haze test (30 min, pH 5.0).

thermostabilities. The most thermostable form(s) exhibited exo-dextranase activity while the endo-dextranase activity was significantly less thermostable.

(ii) U.V. Absorption Spectra

The absorption spectra for the AS 10-30 % fraction and the chromatofocusing Region IV fraction are given in Figure 6.9 and 6.10, respectively. The ratio (A_{280}/A_{260}) was taken as a measure of purity of the protein (Warburg and Christian, 1941). The statistical average for this ratio for pure proteins is 1.75-1.80 (see spectrum of *C. gracile;* Figure 3.11). A value approaching this figure would demonstrate that the preparation consisted largely of protein. The A_{280}/A_{260} ratio for the AS 10-30 % fraction was 1.05 (after appropriate correction was made for scattered light). In comparison, the chromatofocusing Region IV fraction exhibited a corrected ratio of 1.44. The relatively low values obtained for the A_{280}/A_{260} ratios indicated that impurities other than proteins were also present in these purified preparations.

(iii) Other Catalytic Activity

The concentrated extracts from the two partially purified fractions were checked for the presence of other enzymic activities. The activities of greatest relevance for application within the sugar factory are amylase and pullulanase activities. The relative amounts of these activities are listed Table 6.8, together with the corresponding results for the commercial *C. gracile* dextranase. Significant levels of amylase activity were present in the partially purified extracts.

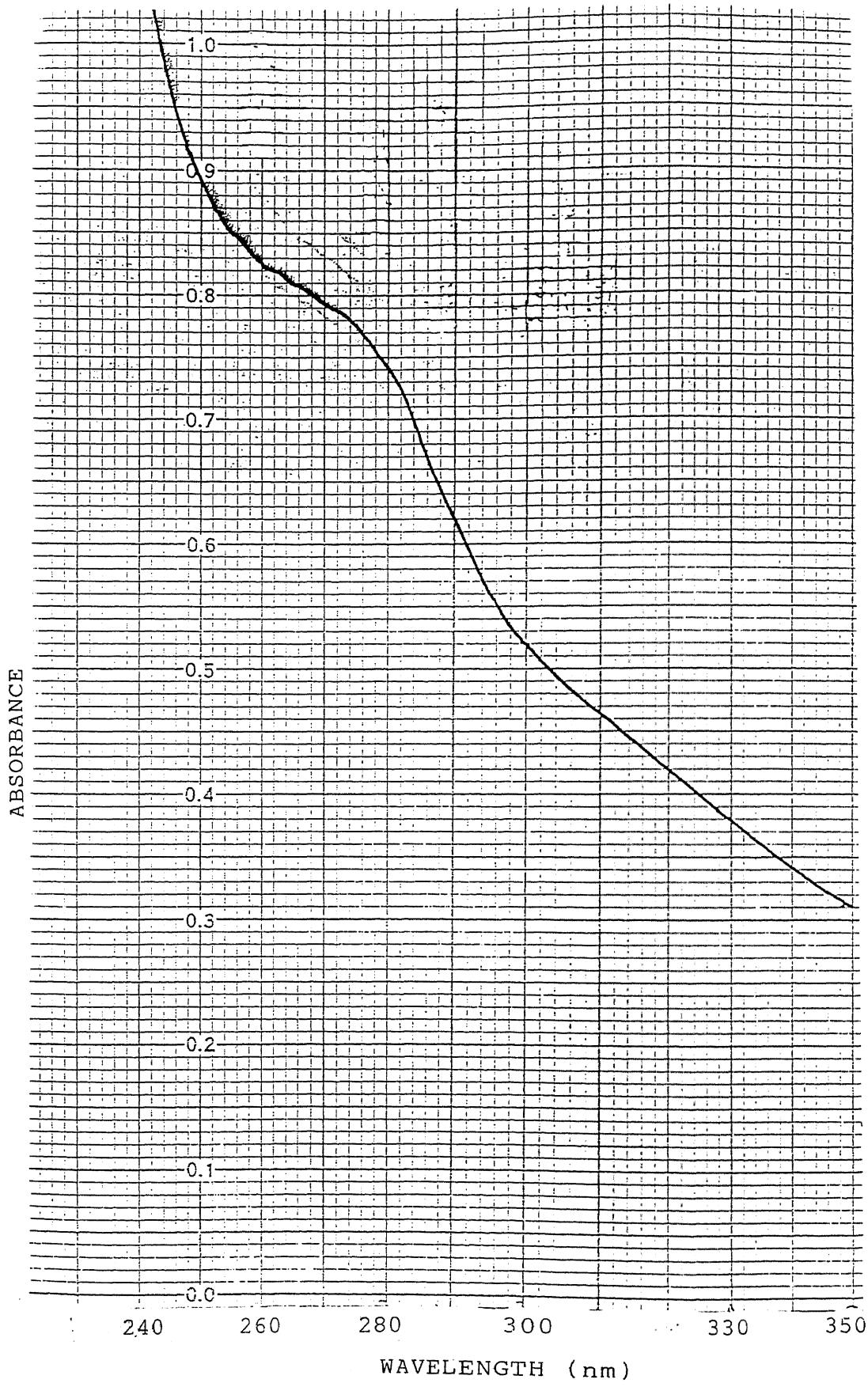


Figure 6.9 UV Spectrum of from 10-30% AS fraction of RT364 dextranase in 0.05M citrate buffer, pH 5.0.

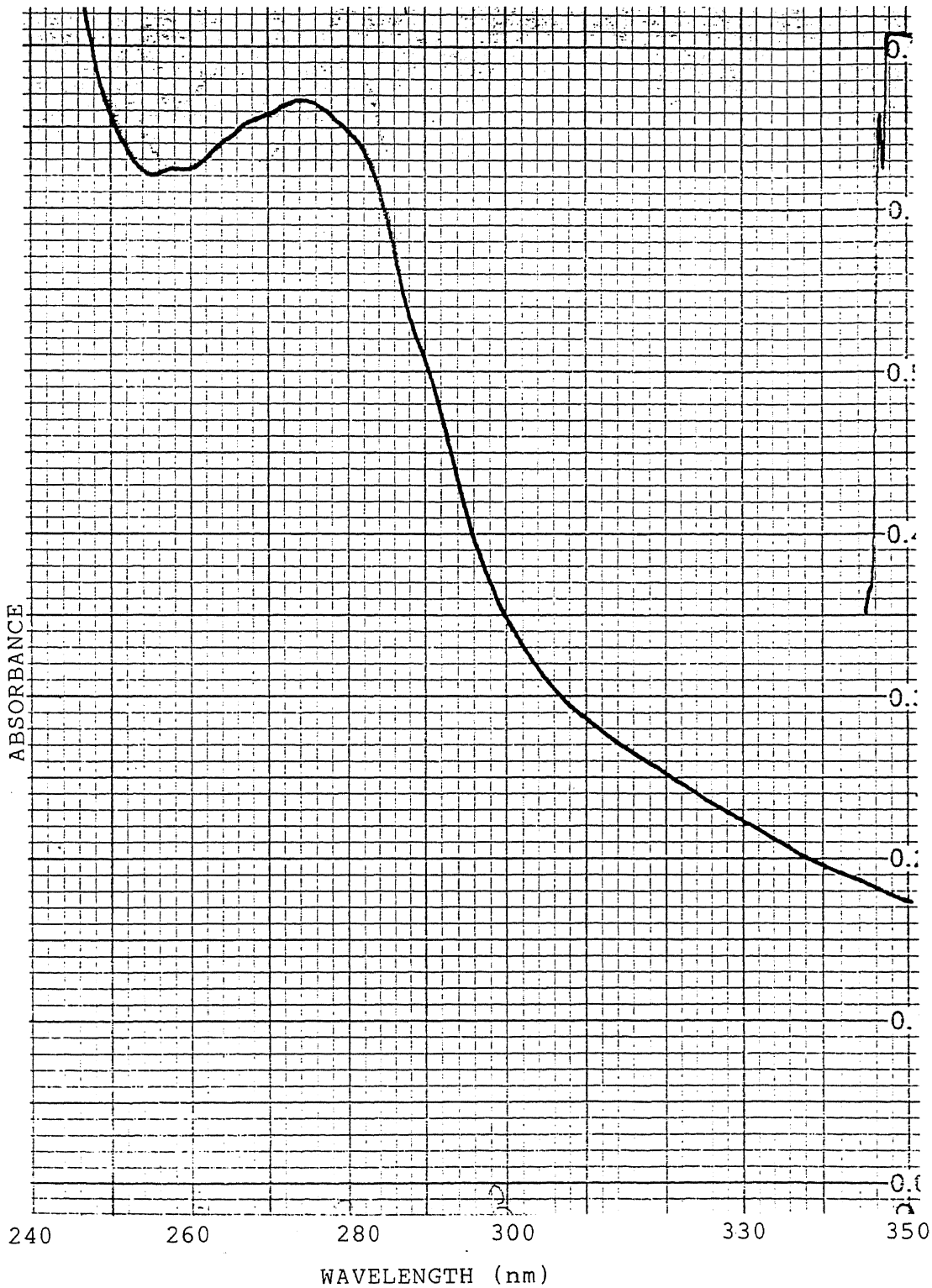


Figure 6.10. UV Spectrum of Region IV from chromatofocusing of RT364 dextranase in 0.05M citrate buffer, pH 5.0.

In comparison, most of the pullulanase activity present in the partially purified RT364 preparation had been removed.

TABLE 6.8 RELATIVE ACTIVITY AGAINST DEXTRAN, STARCH AND PULLULAN FOR PARTIALLY PURIFIED RT364 PREPARATIONS[a].

Dextranase Source	Dextran	Starch	Pullulan
C. gracile	100	1.45	0
10-30 % AS fraction	100	34.0	0.8
Region IV Chromatofocusing	100	23.9	0.3

[a]Activity determined using the PAHBAH Assay; 65°C, pH 5.0; 30 min. Dextran T2000 (0.4%); Starch (potato, 0.1%); and Pullulan (0.4%).

6.3.8 Commercial Potential of a Thermostable Dextranase

Several strains of thermophilic bacteria were selected which produce heat stable dextranases. The extracellular dextranases produced were characterised in terms of temperature optima and stability at 75°C and pH 5.0. The bacterial dextranase exhibiting improved stability compared to the currently used dextranase were selected for further study. The most important comparisons were related to thermal stability and degradation of cane dextran under factory conditions (determined using the micro-haze test developed during the course of this thesis) and specific activity.

Cloning and expression of the gene for the selected dextranase activity was deferred at this time. Instead, research was directed toward purification and

characterisation of the native dextranase from the most promising isolate. The dextranase selected exhibited enhanced thermal stability at 75°C under simulated factory conditions (Figure 6.11).

Purification prior to cloning was essential due to the very low specific activity exhibited by the crude preparation of the thermostable enzyme. Enhanced success of the gene cloning step would be expected if a purified enzyme exhibiting a high specific activity was available as the starting material.

A number of purification procedures were investigated and a final specific activity for the best thermostable dextranase (RT364 dextranase) was determined to be about 20 μmole min^{-1} mg^{-1} at 75°C. In comparison, the specific activities of the *Chaetomium gracile* dextranase currently used by mills are 2750 and 110 μmole min^{-1} mg^{-1} at 55°C and 75°C, respectively, measured by the same method.

Thus, despite exhibiting the required temperature and pH optima, the commercial potential of the RT364 dextranase is limited by the low specific activity. A comparison of specific activities indicated that approximately 150 times more thermostable enzyme would be required for use at 75°C relative to the amount of commercial enzyme currently used at 60°C. Such levels are impractical and uneconomic. The low productivity of the bacteria further reduces the economic viability of the thermostable enzyme.

However, this factor would be less significant if cloning of the dextranase gene into an aerobe such as *B. subtilis* could be achieved. This bacterium is relatively easy to culture and would express high levels of the thermostable enzyme.

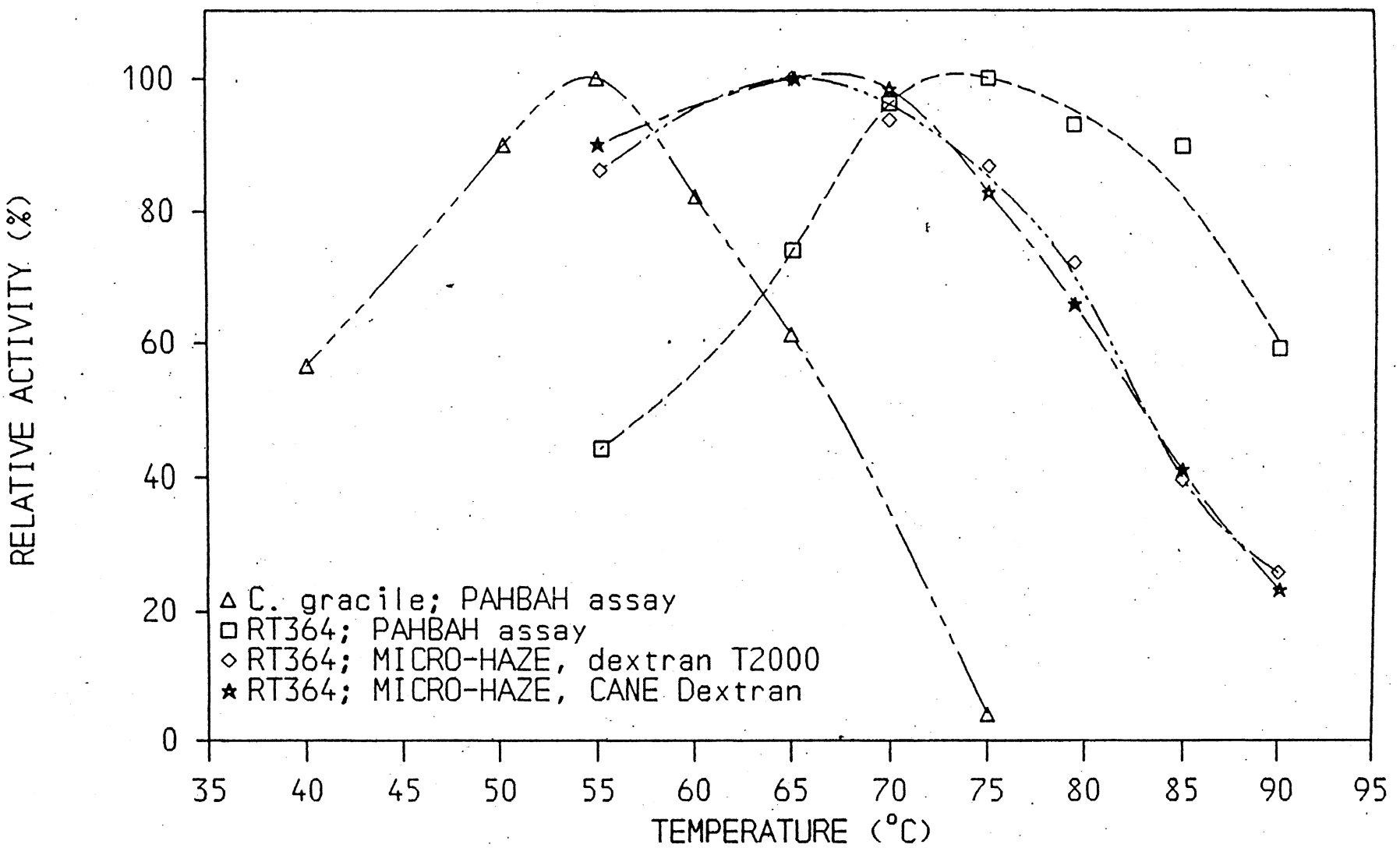


Figure 6.11. Temperature-activity profiles for *C. gracile* dextranase and crude RT364 dextranase (determined by the PAHBAH assay and micro-haze test).

Estimated cost of thermostable dextranase production (based on research scale work) would be of the order of $100 000 per 5 g (the amount of *C. gracile* dextranase per L of commercial preparation currently marketed for $100 - $130 L^{-1}).

Analysis of the current process for dextranase treatment resulted in a cost-benefit of a factor of 10 (L. Riddell and Dr. P.A. Inkerman, private communication). The thermostable enzyme would cost about 150 000 times more than the current commercial dextranase to obtain additional economic gains (*i.e.* immediate enzyme addition with no disruption to factory process, no extra sucrose loss, no additional commercial α-amylase. no effects on clarification). Therefore, no attempt was made to produce sufficient enzyme to carry out a limited factory trial.

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