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Purification and Properties of a Thermophilic Amyloglucosidase from *Aspergillus niger*

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Summary. A thermophilic amyloglucosidase (EC 3.2.1.3) from a strain of *Aspergillus niger* was purified and separated from contaminating α-amylase (EC 3.2.1.1) and transglucosidase (EC 2.4.1.24) by ammonium sulphate fractionation, acetone precipitation and CM-BIO-GEL A chromatography. A 61-fold purification was achieved. The enzyme had highest affinity for starch (100), maltotriose (68) and maltose (31) and K_m values of 0.025% and 1.42 mM with starch and maltose, respectively. It had a molecular weight of 63,000. The enzyme operated most efficiently on starch and maltose at pH 4.5 and surprisingly at the high temperature of 70° C. It possessed considerable pH stability, with 79% and 50% activity retained at pH 2.0 and pH 11.0, respectively, after 30 min at 40° C. The enzyme was 100% stable up to 50° C and 90% stable at 60° C for 30 min; above this latter temperature activity was rapidly destroyed. The presence of starch or glycerol improved the thermal stability of the enzyme. Polyvalent anions stimulated activity while the cations Cu^{2+} and Ag^{+} and to a lesser extent Ni^{2+} and Co^{2+} caused notable inhibitory effects. When incubated with high concentrations of glucose the enzyme formed small amounts of isomaltose as a reversion product.

Introduction

Starch-degrading enzymes of microbial origin have a number of industrial applications (Fogarty and Kelly 1979, 1980). The mould extracellular enzyme, amyloglucosidase, is of major importance in the starch industry in the commercial production of D-glucose from corn syrups (Aunstrup 1979; Fogarty 1983). Consequently the enzyme and the many fungi producing it have received considerable attention (Fleming 1968; Fogarty 1983; Fogarty and Kelly 1980). Of prime importance in this context are the fungi belonging to the *Aspergillus niger* group which have properties highly suited to the industrial scale saccharification of starch (Underkofler 1968).

The current study presents a comparitively simple but effective method of purifying the amyloglucosidase of *A. niger* and separating it from contaminating α-amylase and transglucosidase. This latter enzyme is produced simultaneously with amyloglucosidase and is a highly undesirable contaminant since it lowers the efficiency of the saccharification process by forming unfermentable sugars (Maher 1968). Many of the properties of amyloglucosidases isolated from *A. niger* are still in dispute (Pazur et al. 1971; Lineback et al. 1972; Freedberg et al. 1975; Alazard and Raimbault 1981) and so we undertook an examination of the properties of the purified preparation obtained in this study.

Materials and Methods

Microorganism and Cultivation. The organism used was a strain of *Aspergillus niger*, IMDCC No. 1203. The enzyme was obtained by growing the mould in batch culture in 2.5% (w/v) corn steep liquor and 15% (w/v) barley syrup (pH 5.9 before autoclaving). This medium also served as the inoculating medium. A 2-day old inoculum (50 ml) – produced from a spore suspension – was added to the medium (450 ml) in 2-l Erlenmeyer flasks and incubated at 120–150 revs/min at 34° C for 4 days in a New Brunswick orbital incubator. The culture was then filtered through muslin gauze and centrifuged at 10,000 *g* for 10 min to obtain a mycelial-free supernatant. The organism was maintained on Czapex Dox agar slopes at 4° C, following cultivation at 30° C for 5 days.

Enzyme Assays. α-Amylase activity was detected by studying the action pattern on 1% (w/v) starch. An additional method involved assaying the enzyme solution on 1% (w/v) starch and comparing the production of glucose (GOPOD method – Hugget and Nixon 1957) with that of reducing sugars (DNS method – Bernfeld 1955).

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A higher level of reducing sugars to that obtained for glucose was taken to indicate the presence of α-amylase.

The amyloglucosidase (AG) was assayed by incubating the enzyme solution (0.5 ml) with 1% (w/v) soluble starch (0.5 ml) in 0.1 M acetate buffer, pH 4.5 at 40° C. After 30 min 3 ml of glucose oxidase-peroxidase (GOPOD – Hugget and Nixon 1957) reagent was added and the intensity of the orange-red colour measured at 420 nm after a further 30-min incubation. A unit of activity is defined as that amount of enzyme which releases 1 mg of glucose from starch per milliliter of enzyme solution under assay conditions.

The transglucosidase activity was measured initially by incubating enzyme solution (1 ml) with 6% (w/v) maltose (1 ml) in 0.1 M acetate buffer, pH 4.5, for 60 min and then detecting panose and isomaltose – the main transfer products – by thin-layer chromatography (Hansen 1975). The method was replaced subsequently by that described here (Benson and Fogarty 1979; Benson et al. 1982). Enzyme solution (0.5 ml) was incubated with 2% (w/v) α-methyl-D-glucoside (α-MG, 0.5 ml) for 60 min and glucose released was then measured as described above for amyloglucosidase. A unit of activity is defined as that amount of enzyme which releases 1 mg of glucose from α-MG per milliliter of enzyme at 40° C in 60 min.

Analytical Isoelectric Focusing. Analytical isoelectric focusing in polyacrylamide gel was carried out using an LKB Multiphor system. The ampholine carrier ampholytes used had a pH range of 3.5–9.5. Enzyme solutions were prepared with a protein concentration of 1–2 mg/ml. LKB procedures for precipitating, fixing, staining, and destaining the focused proteins were followed as detailed in the practical information leaflet entitled "LKB ampholine PAG plates for analytical electrofocusing on polyacrylamide gels" (LKB-Produkter AB, Sweden).

Thin-Layer Chromatography (TLC). The action of the enzymes on various substrates and the detection of end-products was determined by TLC (Hansen 1975).

Stability to pH. Enzyme samples were added to 0.01 M Britton and Robinson's universal buffer (Campbell 1955) in the range pH 2.0–11.5 and incubated at 40° C. After 30 min the contents of the tubes were brought to the pH optimum of the enzyme and the residual activity determined.

Stability to Temperature. Enzyme samples were incubated in 0.1 M universal buffer (Campbell 1955) at pH 4.5 over a temperature range of 20–80° C. After 30 min the samples were assayed at 40° C for residual activity.

Effect of Metal Ions, Anions and Inhibitors on Enzyme Activity. The enzyme and the individual reagents being examined were preincubated at pH 4.5 and 40° C for 30 min and then assayed for activity. Results are expressed as percentage residual activity compared to a control containing substrate only.

Molecular Weight Determination. The molecular weight of the enzyme was estimated using Sephadex G-150 (Andrews 1964) in a Pharmacia K40 column (2.5 × 40 cm). Cytochrome C, α-chymotrypsinogen A, ovalbumin and human immunoglobulin G served as the reference proteins. Elution was performed using 0.01 M universal buffer, pH 4.5 (Campbell 1955).

Results and Discussion

Purification of Amyloglucosidase

Step 1: Fractionation with Ammonium Sulphate. In this the initial purification step, the cell-free supernatant (CFS) was treated with solid ammonium sulphate, with continuous stirring at 4° C and separated into the following saturation ranges: 0–30%, 30–50%, 50–70%, and 70–100% (Green and Hughes 1955). Precipitates were collected after 30 min by centrifugation at 10,000 *g* for 10 min in a Sorvall RC2-B centrifuge. They were then dissolved in a minimum of sodium acetate buffer (0.01 M, pH 5.0) and dialysed overnight against tap water. Transglucosidase was detected only in the 70–100% fraction while amyloglucosidase was located primarily in this fraction and to a small extent in the 50–70% fraction.

Chromatographic analysis of all the fractions, including the CFS and the dialysed supernatant (after 100% saturation) following incubation with 1% (w/v) soluble starch (pH 4.5), showed that small amounts of α-amylase were also produced. The presence of this enzyme, however, was not revealed in the CFS, perhaps because it was present in very small amounts or because the amyloglucosidase was so active that it hydrolysed the α-amylase products too rapidly to be detected by TLC.

Step 2: Fractionation with Acetone. To the redissolved 70–100% fraction, one volume of chilled acetone (−16° C) was added slowly with constant stirring. After standing overnight at 4° C the solution was centrifuged at 10,000 *g* for 30 min. The resultant precipitate was then dissolved in a small amount of acetate buffer (0.01 M, pH 5.0) and dialysed overnight against tap water. When compared with Step 1, 85% of amyloglucosidase and 98% of transglucosidase were recovered at this stage with an overall purification of 10.3 and 12.4, respectively. This fraction, however, failed to show the presence of α-amylase using TLC and in addition gave a glucose to reducing sugar ratio of 1 : 1.007 after 30 min hydrolysis of starch (1.0% w/v) at 40° C. It was thus judged to be free of α-amylase.

Step 3: CM-BIO-GEL A Chromatography. Using CM-BIO-GEL A equilibrated with 0.02 M acetate buffer pH 4.5 (Kobrehel 1979) the two remaining activities – from the combined treatments described above – were successfully separated (Fig. 1). Amyloglucosidase was eluted first by running equilibrating buffer through the column (0.9 × 15 cm). The combined fractions of the amyloglucosidase peak contained a total of 2,654 U of activity and 5.07 mg of protein. Elution of the bound transglucosidase was achieved only after the addition of buffer containing 0.2 M sodium chloride. Chromatographic analysis (Table 1) confirmed that the first peak was indeed amyloglucosidase (AG) as it characteristically

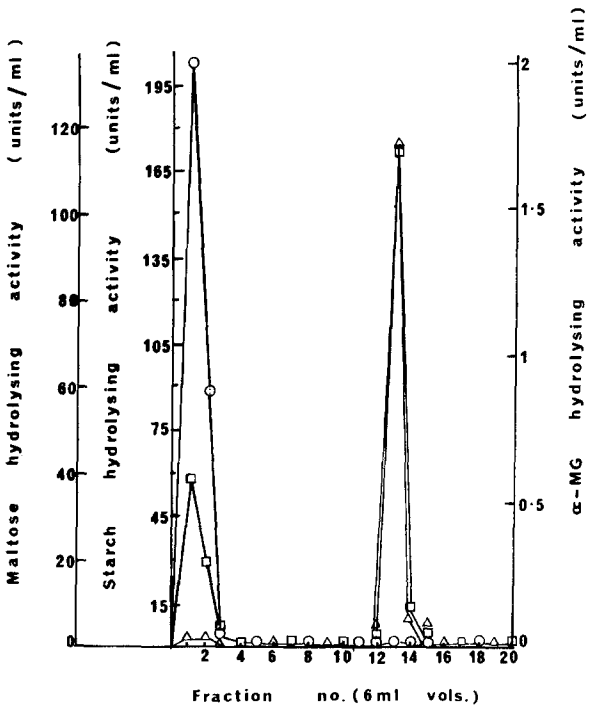

Fig. 1. Chromatographic separation of amyloglucosidase and transglucosidase using a column (0.9 × 15 cm) of CM-BIO-GEL A. (○) starch hydrolysing activity; (□) maltose hydrolysing activity; (△) α-MG hydrolysing activity. The column was eluted with 0.02 M acetate buffer, pH 4.5 (25 ml) and then with 0.02 M acetate buffer, pH 4.5 containing 0.2 M NaCl

degraded starch rapidly to glucose only. Also its action on 6% (w/v) maltose showed no product other than glucose. These findings were consistent with the assay result obtained with α-MG as substrate which also showed transglucosidase (TG) was absent since no hydrolysis of the TG-specific substrate took place. The second peak was identified as transglucosidase as it not only hydrolysed α-MG but showed the characteristic transfer products, panose and isomaltose, on incubation with 6% (w/v) maltose. A summary of the amyloglucosidase purification scheme using 400 ml of CFS is presented in Table 2.

Analytical Isoelectric Focusing. This technique confirmed the separation of amyloglucosidase and transglucosidase after CM-BIO-Gel A chromatography. Amyloglucosidase from Step 3, which had an overall purification of 60.9, consisted of only two protein bands. These bands had very similar isoelectric points in the low acidic region (pH 4.0) and though not precisely determined were consistent with previously reported values (Pazur and Ando 1959; Lineback et al. 1969). Earlier studies (Pazur and Ando 1959; Lineback et al. 1969; Freedberg et al. 1975) have reported the production of two AG isoenzymes by *A. niger* and the observations made here are similar.

Table 1. Data from the chromatographic analysis of the action of purified amyloglucosidase, AG (1st Peak) and transglucosidase, TG (2nd Peak) on maltose (6%, w/v) and starch (4%, w/v). Symbols: + = present; + + = present to considerable extent; + + + = present in excess as major product; − = absent; ± = trace amount

Product	Maltose (6% w/v)				Starch (4% w/v)			
	1st peak (AG)		2nd peak (TG)		1st peak (AG)		2nd peak (TG)	
	2 h	4 h	2 h	4 h	2 h	4 h	2 h	4 h
Glucose	+	+ +	+	+ +	+ +	+ + +	±	+
Isomaltose	−	−	+	+	−	−	−	−
Panose	−	−	+	+ +	−	−	−	−

Table 2. Purification of *Aspergillus niger* amyloglucosidase from cell-free supernatant (400 ml)

	Total protein (mg)	Activity (units)	Yield %	Specific activity (units/mg protein)	Purification (x-fold)
CFS	3,920	33,720	100	8.6	1
$(NH_4)_2SO_4$ Fractionation (70–100%)	403	12,038	36	29.87	3.5
Acetone precipitation (Vol. 1)	64.4	4,388	13	88.9	10.3
CM-Bio-Gel A chromatography	5.07	2,654	8	523.5	60.9

Table 3. Comparative hydrolysis of various substrates by amyloglucosidase in 0.1 M acetate buffer, pH 4.5, at 40° C for 30 min

Substrate (1%, w/v)	Linkage/Substituted group	% Hydrolysis
Starch	α-1,4 and α-1,6	100
Maltotriose	α-1,4	68
Maltose	α-1,4	31
Panose	α-1,4 : α-1,6	14.0
Sucrose	Fructose-α-1,2-glucose	3.3
Pullulan	α-1,4 and α-1,6	1.46
p-nitrophenyl-α-D-glucoside	α-nitrophenyl	1.0
Glucose-1-phosphate	α-1-phosphate	0.8
Maltitol	OH	0.75
Laminarin	β-1,3	0.46
Gentiobiose	β-1,6	0.33
Isomaltose	α-1,6	0.3
Melibiose	Galactose-α-1,6-glucose	0.12
* β-naphtyl-α-D-glucoside	β-naphthyl	0.1
Trehalose	α-1,1	0.1
Cellobiose	β-1,4	0.1
Sophorose	β-1,2	0.07
α-CHA	α-cyclohexaamylose	0.06
* Nigeran	α-1,3	0.05
α-MG	α-methyl	0.05

* 0.1% (w/v)

Properties of Amyloglucosidase

Hydrolysis of various compounds (1% w/v, in 0.1 M acetate buffer, pH 4.5) by amyloglucosidase showed (Table 3) that the enzyme possesses a greater ability to hydrolyse large molecular weight compounds as illustrated by the relative values for starch (100), maltotriose (68), and maltose (31). The influence of an α-1,6 linkage on amyloglucosidase activity is also clear by comparing the hydrolysis of maltotriose (68) and maltose (31) with related α-1,6 sugars, e.g., panose (14) and isomaltose (0.3). The apparent hydrolysis however of the β-linkage, e.g., in gentiobiose, is attributed to the breakdown of contaminating α-linked sugars rather than to intrinsic or even contaminating α-glucosidase activity.

The hydrolysis of sucrose (fructose-α-1,2-glucose) was approximately 10 times slower than maltose. The enzyme also effected the hydrolysis of a variety of other glucosides (Table 3) but there were large differences in the rates of hydrolysis of these compounds. This broad specificity was referred to earlier by Pazur and Kleppe (1962). Kinetic analysis of amyloglucosidase action on starch and maltose, yielded Km values of 0.023% and 1.42 mM, respectively.

The optimum pH and temperature values for the amyloglucosidase with maltose and starch as substrates were pH 4.5 and surprisingly, 70° C, with both substrates. Amyloglucosidases produced by Aspergilli generally have optima within the range pH 4.5–5.0 while most temperature optima lie within the range 40–60° C (Banks et al. 1967; Fogarty and Kelly

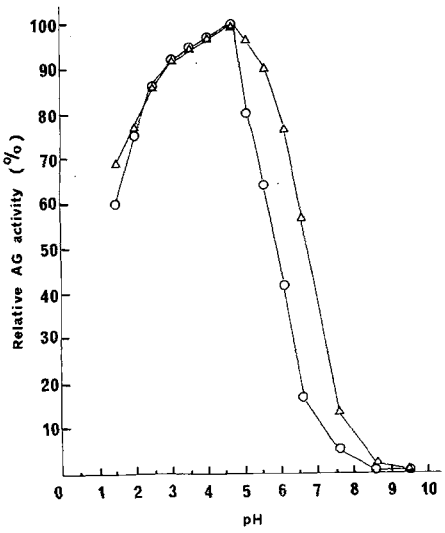

Fig. 2. Determination of the pH activity profile of AG in universal buffer (0.1 M). (△) 1% (W/V) starch as substrate; (○) 1% (W/V) maltose as substrate

1980). While the pH activity profiles in this instance were very similar on both substrates up to pH 4.5, the enzyme had much less activity at higher pH values on maltose than on starch. Thus, at pH 6.5 the activity on starch was 56% (100% at pH optimum, i.e., 4.5) and only 16% on maltose (Fig. 2). The amyloglucosidase from the thermophilic fungus *Thermomyces lanuginosus* also possesses an optimum value of 70° C (Rao et al. 1979). High operating temperature is of considerable significance in an industrial context since it offers the advantages of increased reaction

Table 4. Stability of amyloglucosidase over the pH range 2.0–11.5. The stability of the enzyme after 30 min at 40° C in universal buffer 0.01 M was determined at the pH values indicated

pH	Residual AG activity (%)
2.0	82
3.0	96
4.0	100
5.0	94
6.0	92
7.0	91
8.0	89
9.0	85
10.0	60
11.0	50
11.5	18

rates, decreased viscosity, reduced microbial contamination and better storage stability.

The enzyme showed considerable stability (Table 4) to pH over the range pH 2.0–11.5; after 30 min in 0.01 M universal buffer at 40° C–79% and 82% activity was retained at pH 2.0 and pH 9.5, respectively, with 50% activity still remaining at pH 11.0 but dropping quickly to 18% at pH 11.5. Such stability to pH is unusual for amyloglucosidase since it is generally regarded as unstable above pH 7.0 (Krzechowska and Urbanek 1975; Okada 1977). The amyloglucosidase from *Humicola lanuginosa* (Taylor et al. 1978) and from *Corticium rolfsii* (Kaji et al. 1976) are also exceptions in this context as they possess considerable stability above pH 7.0.

With starch (1%, w/v) or maltose (1%, w/v) as substrate in 0.1 M acetate buffer, pH 4.5, the amyloglucosidase was maximally stable up to 50° C for 30 min. At 60° C a loss of 10% was recorded while at 70° C about 90% of the activity was destroyed (Fig. 3). The thermal stability, however, was improved in the presence of higher levels of substrate (starch) or glycerol when incubated at 75° C for 10 min. Under these conditions the thermal stability was observed to be linearly related to the concentration of glycerol up to 30% (Fig. 4).

Alazard and Raimbault (1981) reported that the amyloglucosidase of *Aspergillus niger* synthesised in solid culture was significantly more thermostable than that from liquid culture and had optimal activity between 65° C and 70° C compared to 50° C for the enzyme from liquid culture. The information obtained in this study shows that the temperature optimum obtained here in liquid culture was 70° C. Furthermore Alazard and Raimbault (1981) claim that the enzyme from solid culture is more stable than that from liquid culture. The enzyme from solid state culture was stable at pH 7.0 for 30 min between 45° C and 65° C and at higher temperatures activity

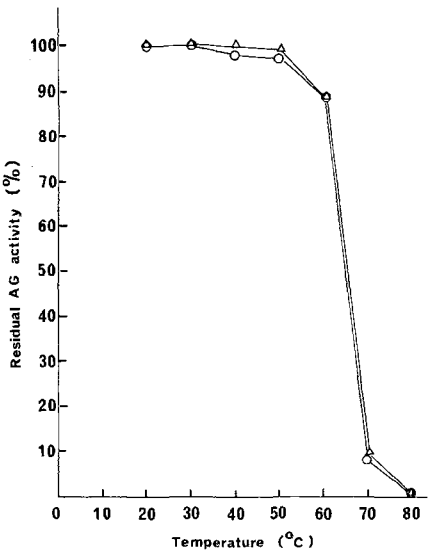

Fig. 3. Effect of temperature on the stability of AG. (○) 1% (W/V) starch substrate, pH 4.5; (△) 1% (W/V) maltose substrate, pH 4.5

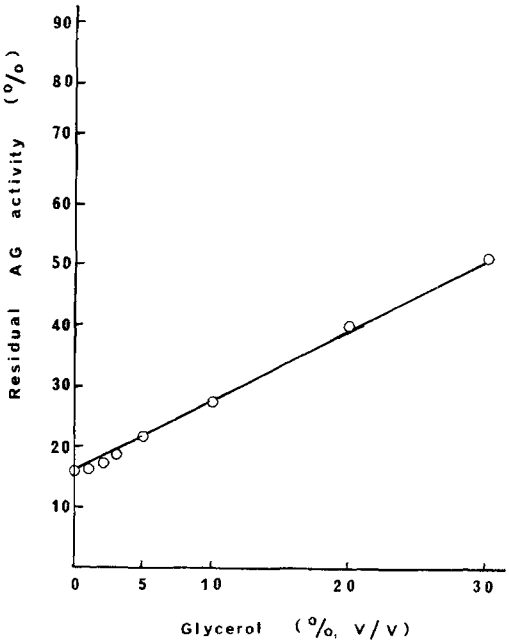

Fig. 4. Effect of glycerol on thermal stability of amyloglucosidase. The enzyme was mixed with glycerol and incubated at 75° C for 10 min and the residual activity determined on starch (1% w/v) at pH 4.5 and 40° C

decreased sharply and a complete inactivation was noted after 15 min at 75° C. From liquid culture, inactivation occurred at 55° C and the activity was totally lost in 15 min at 65° C. The enzyme investigated here was 90% stable at 60° C for 30 min and was considerably more stable than that obtained above in liquid culture.

All the metal ions examined (chloride salts) caused some loss in activity at a 10-mM level. Cu^{2+}

and Ag^+ reacted with the GOPOD reagent at this concentration but were shown at a 1-mM level to cause a 30% and 24% loss, respectively, while Co^{2+} inhibited activity by 12% (control = 100%). Other metals such as Zn^{2+} (114%), Sn^{2+} (107%), Ba^{2+} (105%), Pb^{2+} (104%), and Hg^{2+} (103%) caused some stimulation at a 1-mM level. Hg^{2+} very strongly inhibits amyloglucosidase of a number of other fungi (Okada 1977; Yamasaki et al. 1977). The thermophilic amyloglucosidase of *Cephalosporium eichhorniae* (Day 1978) did not show a requirement for monovalent (Na^+, K^+) or divalent (Mg^{2+}, Ca^{2+}) cations for either activity or stability. However, the enzyme showed a three-fold increase in activity in the presence of phosphate anion.

Polyvalent anions were found to be beneficial for amyloglucosidase activity (Table 5) as they all stimulated activity at 10 mM, 1 mM, and 0.1 mM concentrations, especially pyrophosphate.

Amyloglucosidase was also stimulated by a number of chelating agents at concentrations of 10 mM and 1 mM, in particular, α,α^1-dipyridyl, which gave 121% and 109% activity, respectively, relative to a control. The oxidising agent iodoacetamide also caused some stimulation while sulphydryl agents, especially p-hydroxymercuribenzoate (p-HMB), inhibited activity. In the presence of 10 mM, 1 mM, and 0.1 mM p-HMB, activity was 26%, 46%, and 80%, respectively. A comparison of the effect of mercuric chloride, mercuric and mercurous acetate on amyloglucosidase activity showed (Table 6) that organic salt is a more potent inhibitor than inorganic salt and furthermore that organic mercury as the mercuric ion is more potent than as the mercurous ion. No inhibitory effects by the acetate ion were detected under the assay conditions described.

α-Methyl-D-glucoside was found to inhibit amyloglucosidase acting on maltose and starch. The inhibition was found to be competitive with maltose as substrate and non-competitive in the case of starch. With Schardinger dextrin (α-CHA) mixed inhibition was observed with starch as substrate while slight stimulation of activity was recorded in the case of maltose. Previous work (Cramer and Kampe 1962) indicated that α-CHA may in fact accelerate certain reactions and this could well account for the above phenomenon. Since maltose is a smaller molecule than starch it could be that α-CHA – due to its clathrating ability – imposes some bond strain on the maltose, thus making it somewhat more vulnerable to hydrolysis. Maltitol and gentiobiose at a 20-mM level caused non-competitive and uncompetitive inhibition, respectively, with starch as substrate.

The recombination of D-glucose to yield oligosaccharides (reversion reactions) when high concentrations of glucose are incubated with crude or highly purified preparations of amyloglucosidase has been reported (Underkofler et al. 1965; Pazur and Okada 1967). In this study incubation of amyloglucosidase with concentrated solutions of D-glucose (10%, w/v and 30%, w/v) at 60° C over 22 h was shown by TLC (Hansen 1975) to produce small amounts of isomaltose as the sole reversion product (Table 7). Somewhat more isomaltose was present in the 30% (w/v) glucose digest.

The molecular weight of amyloglucosidase from different sources varies considerably from 48,000 (Miah and Ueda 1977; Tsuboi et al. 1974) up to

Table 5. Effect of polyvalent anions on amyloglucosidase activity. Anions (sodium salts) were preincubated with enzyme for 30 min at 40° C. A control (100%) was also incubated

Anion	Anion concentration		
	10 mM	1 mM	0.1 mM
	% Activity of control		
Borate	110	110	116
Arsenate	114	110	110
Pyrophosphate	121	120	112
Orthophosphate	114	111	100
Carbonate	119	109	106

Table 6. Inhibition of amyloglucosidase by some mercury compounds. Control = 100

Inhibitor	Concentration		
	10 mM	1.0 mM	0.1 mM
	Enzyme activity – % control		
Mercuric chloride	92	97	103
Mercuric acetate	0.0	0.0	32
Mercurous acetate	12.5	31	67

Table 7. Action of amyloglucosidase on 10% (w/v) and 30% (w/v) glucose at 60° C. Symbols: + = present in trace amount; ++ = present; +++ = present in small amount; ++++ = present in considerable amount; – = absent

Reversion products	10% Glucose					30% Glucose				
	0 h	1 h	2 h	4 h	22 h	0 h	1 h	2 h	4 h	22 h
Maltose	–	–	–	–	–	–	–	–	–	–
Isomaltose	–	–	+	++	+++	–	–	++	+++	++++
Maltotriose	–	–	–	–	–	–	–	–	–	–
Panose	–	–	–	–	–	–	–	–	–	–

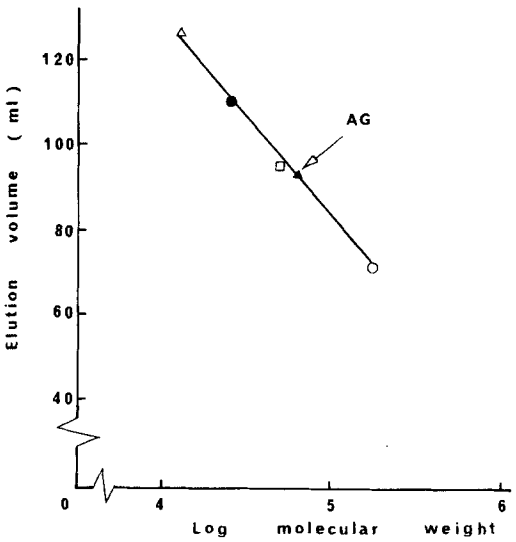

Fig. 5. Determination of molecular weight of amyloglucosidase using Sephadex G-150. (○) cytochrome C; (▲) amyloglucosidase; (□) ovalbumin; (●) chymotrypsinogen A; (△) human immunoglobulin G

112,000 and greater (Lineback and Baumann 1970; Pazur et al. 1971; Freedberg et al. 1975). Lineback et al. (1972) found two forms of amyloglucosidase in *A. niger* having molecular weights of 74,900 and 54,300 and Pazur et al. (1971) recorded molecular weights of 99,000 and 112,000 for the two iso-enzymes. The value of 63,000 obtained in this study by the gel-filtration technique (Fig. 5) is in good agreement with the findings of Freedberg et al. (1975) who recorded molecular weights of 63,000 and 57,500 for the two major components present. Ramasesh et al. (1982) isolated two iso-enzymes of amyloglucosidase from *A. niger* which had molecular weights of 69,810 and 89,130. Both were glycoproteins and differed in their carbohydrate contents, pH and temperature stabilities and optima for activity. Two forms of the enzyme with different properties have also been recorded by Pazur and Ando (1959), Fleming and Stone (1965) and Smiley et al. (1971). Alazard and Baldensperger (1982) detected two forms of amyloglucosidase in *A. hennebergi* (*A. niger* group) with molecular weights of 60,000 and 72,000. Isoelectric focusing of each preparation yielded a single protein band at pH 3.9. However only one peak was detected here in determining molecular weight (Fig. 5) although two closely related bands were observed in isoelectric focusing at pH 4.2.

Most notable among the properties of the enzyme from a biotechnological viewpoint, could be the stability to pH and not least its suitability to high operating temperatures – a feature that distinguishes it from many similar enzymes reported to-date.

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