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PURIFICATION AND PROPERTIES OF β-MANNOSIDASE FROM MALTED BARLEY

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SUMMARY

β-Mannosidase (β-mannoside mannohydrolase, EC 3.2.1.25) was extracted and purified about 100-fold from malted barley. The purified preparation was free of α-mannosidase, β-*N*-acetylhexosaminidase, α-galactosidase and β-glucosidase. The purified enzyme was active between pH 3 and 6 and exhibited a K_m value of $3.2 \cdot 10^{-4}$ M using the *p*-nitrophenyl-β-D-mannopyranoside as the substrate. 2-Amino-2-deoxy-D-mannose is a competitive inhibitor ($K_i = 1.18 \cdot 10^{-4}$ M). *p*-Nitrophenyl-α-D-mannopyranoside activated the enzyme at low concentration but competitively inhibits at higher concentrations. β-Mannosidase had maximum activity at 55 °C but this dropped significantly at 70 °C. Specific cleavage of β-mannoside linkages by the purified β-mannosidase was demonstrated using a β-(1-4)mannosylmannose as substrate. The enzyme showed the molecular weight is about 88 000 as determined by acrylamide gel electrophoresis.

INTRODUCTION

Previously [1] we have reported that malted barley extract contained α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-N-acetylhexosaminidase and α-mannosidase. β-Mannosidase (β-D-mannosidase mannohydrolase, EC 3.2.1.25) has been partially purified from viscera of snails, synovial fluid of humans [2, 3] from rat epididymis and from hen oviduct [4]. The purpose of the present study is to separate β-mannosidase enzymes from other carbohydrate-hydrolyzing enzymes and to study its activity in malted barley.

MATERIALS AND METHODS

Enzyme source. Malted barley was the gift of Kurth Malting Co., Milw. All enzyme purifications were carried out at 0–5 °C.

Enzyme substrates. The *p*-nitrophenylglycosides were purchased from Sigma Chemical, St. Louis, and the β-(1-4)dimannoside and β-(1-4)trimannoside were the gift of Professor G. O. Aspinall of Trent University, Ontario, Canada.

Enzyme assays. β-Mannosidase was assayed at pH 5.5 in a 2 ml vol. of 10 mM sodium acetate buffer solution containing 1.5 μmole of substrate and the properly diluted enzyme. The mixture was incubated for 15 min at 30 °C and the reaction was terminated by adding 3 ml of 5% Na_2CO_3 solution. The liberated *p*-nitrophenol was determined spectrophotometrically at 420 nm ($\varepsilon = 2.74 \cdot 10^{-4}$ $M^{-1} \cdot cm^{-1}$). 1 unit is defined as the amount of enzyme that releases 1 μmole of *p*-nitrophenol/min and specific activity is expressed as the number of units of enzyme per mg of protein. Protein was determined by the method of Lowry et al. [5].

Analytical methods. Degradation of the di- and trimannoside with β-mannosidase was followed by determining the reducing power using mannose as the standard [6].

Polyacrylamide gel electrophoresis was conducted in the system described by Davis [7] and sodium dodecylsulfate–polyacrylamide gel electrophoresis molecular weight determination as described by Weber and Osborn [8].

RESULTS

Purification of β-mannosidase

$(NH_4)_2SO_4$ precipitation. Malted barley was ground in a Wiley mill through a 30 mesh screen. A 700 g quantity was extracted with 3 l of 10 mM sodium acetate buffer (pH 5.51) for 8 h. The mixture was filtered through three layers of cheese-cloth and centrifuged at 14 000 × *g* for 15 min. This crude extract was fractionated with $(NH_4)_2SO_4$, the precipitates collected by centrifugation and suspended in 10 mM sodium acetate buffer, (pH 5.5). The solutions were dialyzed against the same buffer and assayed for the various glycosidase activities. For β-mannosidase preparation a 20–70% $(NH_4)_2SO_4$ saturation fraction (114–472 g/l) was utilized.

Biogel P-100 chromatography. The material obtained from the $(NH_4)_2SO_4$ saturation was applied to a Biogel P-100 column (5 cm × 50 cm) equilibrated with 10 mM acetate buffer (pH 5.5) and eluted with the same buffer as shown in Fig. 1. Fractions of 10 ml were collected and Fractions 40–74 containing β-mannosidase were pooled and lyophilized.

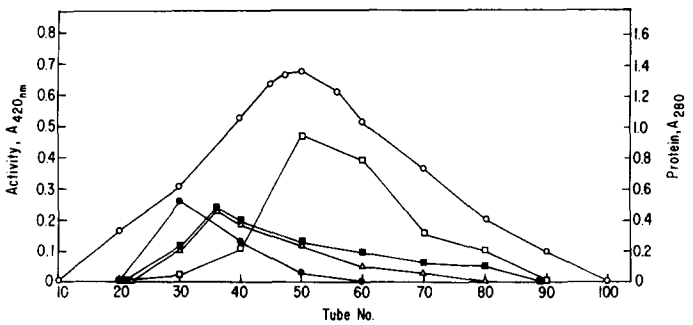

Fig. 1. Chromatography of the crude extract on a Biogel P-100 column (5.0 cm × 50 cm) equilibrated and eluted with 10 mM sodium acetate buffer (pH 5.5); 9-ml fractions were collected and Fractions 40–65 were pooled for further purification. ○—○, protein; □—□, β-mannosidase activity; △—△, β-*N*-acetylhexosaminidase activity; ●—●, α-mannosidase activity; ■—■, α-galactosidase activity.

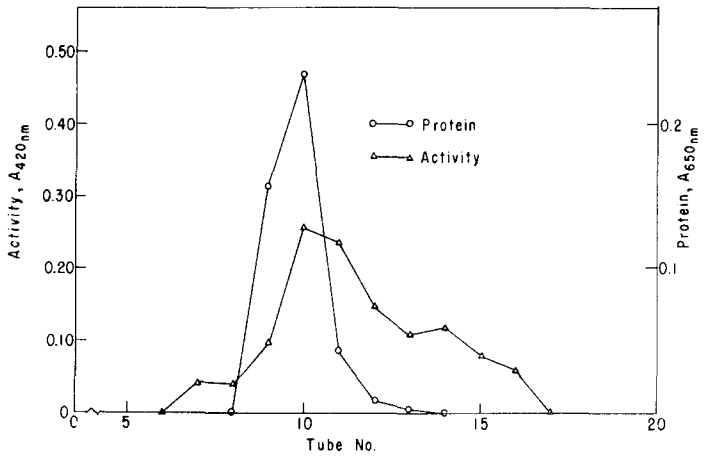

Fig. 2. DEAE-cellulose chromatography. A 2.5 cm × 42 cm column equilibrated 10 mM Tris buffer (pH 7.2) and a NaCl gradient of 0.01–0.1 M. Each reservoir contained 250 ml and 5-ml fractions were collected. ●—●, protein; ○—○, β-mannosidase activity.

DEAE-cellulose and CM-cellulose chromatography. The partially purified β-mannosidase was applied to a DEAE-cellulose column (2.5 cm × 42 cm) and eluted with 10 mM Tris buffer (pH 7.2) with increasing ionic strength of NaCl (0.01–0.1 M) as shown in Fig. 2. Each reservoir contained 250 ml. 5-ml fractions were collected and Fractions 9 and 10 were pooled. Fig. 3 shows the elution pattern from a carboxymethylcellulose column (1.5 cm × 25 cm) equilibrated with sodium acetate buffer (pH 5.5) and eluted with the same buffer. 3-ml fractions were collected and Fractions 7–10 were pooled. This preparation was free of α-mannosidase, α- and β-glucosidase,

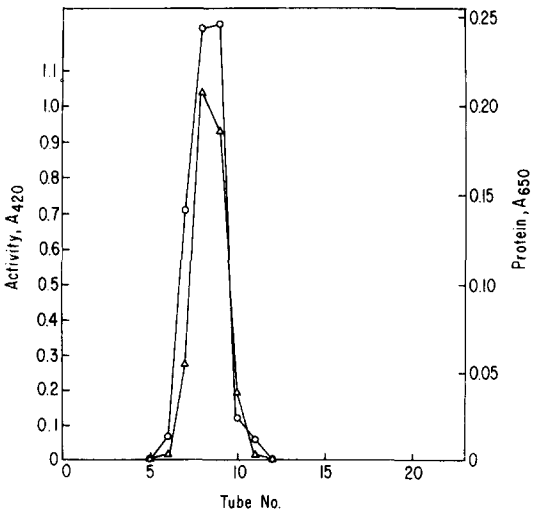

Fig. 3. Carboxymethylcellulose chromatography. A 1.5 cm × 25 cm column equilibrated and eluted with 10 mM sodium acetate buffer (pH 5.5). 3-ml fractions were collected. ○—○, protein; △—△, β-mannosidase activity.

β-*N*-acetylhexosaminidase and α-galactosidase activities. The specific activity of the purified enzyme was 0.505 units/mg under our assay conditions but 0.611 units/mg was calculated from a Lineweaver–Burk plot. The purification process is summarized in Table I.

TABLE I

PURIFICATION OF β–MANNOSIDASE FROM CRUDE EXTRACT OF BARLEY

Fraction	Volume (ml)	Concentration (units/ml)	Total activity	Protein (g)	Specific activity (units/mg)	Yield %	Purification
Crude Extract	3,000	0.164	492	40.4	0.012	100	1
20-70% Ammonium Sulfate	400	0.901	360	24.5	0.015	76	1.22
Bio–Gel P–100 Column	507	0.490	248	4.9	0.100	55	8.25
DEAE Cellulose Column	98	0.535	52	0.22	0.244	17	20.04
CM–32 Column	43	0.975	42	0.08	0.505	11	41.42

The purified β-mannosidase obtained from the CM-cellulose column contained a major and a minor protein band when analyzed by disc gel electrophoresis. The major band had β-mannosidase activity. Sodium dodecylsulfate–polyacrylamide gel electrophoresis for molecular weight determination exhibited a major band which gave an apparent molecular weight of 88 000 (Fig. 4). Carbohydrate analysis of the purified protein as done by Kesler [9] revealed less than 0.01 % carbohydrate covalently bound to the partially purified β-mannosidase enzyme.

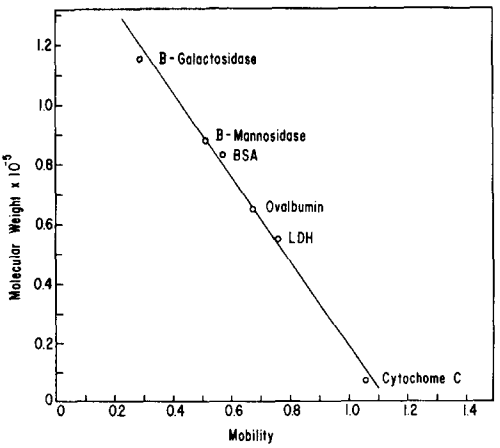

Fig. 4. Polyacrylamide gel electrophoresis of β-mannosidase and standard proteins in sodium dode cylsulfate.

Properties of β-mannosidase

The pH–activity profile was determined using acetate–citrate buffering systems. The optimum pH for this enzyme was 5.5 whereas the purified snail enzyme [2] has a optimum at pH 4.5.

The purified enzyme was stable in aqueous solutions at 4 °C for several months; however, because of possible contamination for long storage time, we stored the lyophilized enzyme frozen at −10 °C for over a year without any loss of activity. Freezing and thawing the enzyme solution did not show loss of activity. To determine heat stability the enzyme was assayed at various temperatures at the optimum pH (pH 5.5). Maximum activity of the enzyme was obtained at 55 °C with a very sharp loss of activity at 70 °C.

A dimannoside and trimannoside (β-1,4 linkages) were used to determine the specificity of the purified β-mannosidase. Both the dimannoside and trimannoside were substrates since they showed increase reducing power [6] when incubated with β-mannosidase at pH 5.5 at 37 °C for 2 h. Mannose release was qualitatively determined [1] by paper chromatography.

A Lineweaver–Burk plot and a substrate saturation curve is shown in Fig. 5

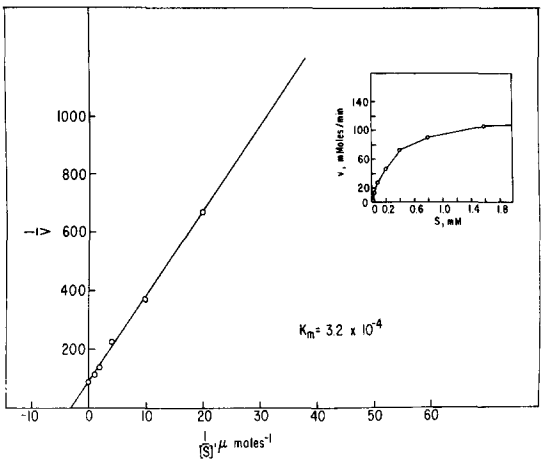

Fig. 5. Lineweaver–Burk plot for β-mannosidase (0.007 unit) with *p*-nitrophenyl-β-mannopyranoside as substrate. The insert presents the substrate saturation curve.

for β-mannosidase activity using the *p*-nitrophenyl-β-D-mannopyranoside as the substrate. The K_m value was about $3.2 \cdot 10^{-4}$ M. *p*-Nitrophenyl-α-D-mannopyranoside at 0.1–1.0 mM increased the V and acted as an inhibitor at 2–3 mM. Fig. 6 shows the results of a Lineweaver–Burk plot using various concentrations of the *p*-nitrophenyl-α-mannopyranoside substrate. The 2-amino-2-deoxy-D-mannose derivative showed competitive inhibition kinetics which is exhibited in Fig. 7. The K_i value was $1.8 \cdot 10^{-4}$ M.

DISCUSSION

The β-mannosidase-susceptible linkages are present in the core region of the neutral and acidic glycopeptides from a variety of sources and several investiga-

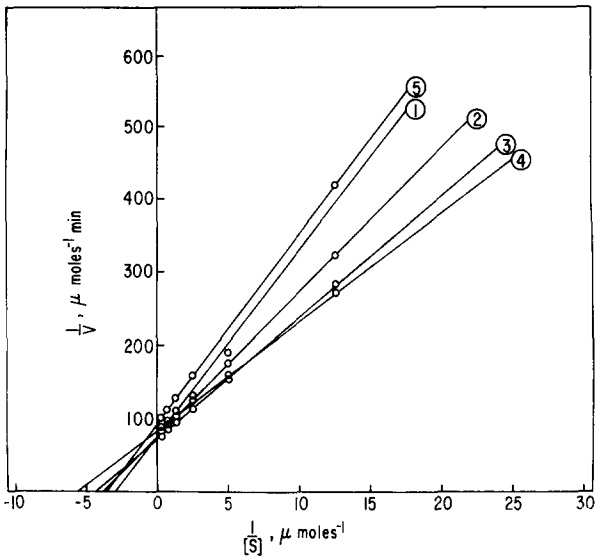

Fig. 6. Lineweaver–Burk plot of β-mannosidase (0.007 unit) with *p*-nitrophenyl-β-D-mannopyranoside as substrate and *p*-nitrophenyl-α-D-mannopyranoside as inhibitor. (1) No inhibitor; (2) 0.1 mM inhibitor; (3) 1.0 mM inhibitor; (4) 2.0 mM inhibitor and (5) 3.0 mM inhibitor.

tors [2, 3, 10] and have used β-mannosidase for glycoprotein carbohydrate studies. β-Mannosidase activities have been studied from various sources including mollusks [2, 3,11] hen oviduct [4] and seminal fluid [12]; however, we are reporting the first partial purification of this enzyme from a plant source. We have reported the partial purification of α-mannosidase from malted barley [1] and found higher activity for β-mannosidase in the crude extract than for α-mannosidase activity. The present study suggests a higher pH optimum than that for the enzyme from mollusks. The K_m of barley β-mannosidase with *p*-nitrophenylmannopyranoside as substrate is lower than the value obtained with other purified β-mannosidase enzyme. Heat

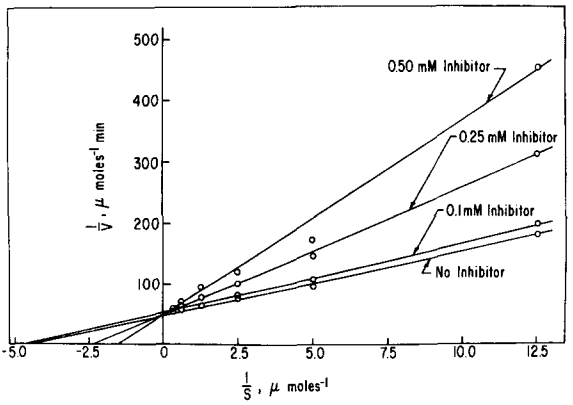

Fig. 7. Lineweaver–Burk plot of β-mannosidase (0.007 unit) with *p*-nitrophenyl-β-D-mannopyranoside as substrate and different concentrations of 2-amino-2-deoxy-D-mannose as an inhibitor.

stability studies are similar to those reported previously. Of particular interest is the effect of the *p*-nitrophenyl-α-D-mannopyranoside on the activity of β-mannosidase using the β-mannoside substrate. Apparently at low concentrations approaching the K_m of the substrate, the effector increases K_m without affecting V and at higher concentration (> 2 mM effector) there is a decreased V and lower K_m for the substrate. The *p*-nitrophenyl-α-D-mannoside is not a substrate for the enzyme.

On the other hand the 2-amino-2-deoxy-D-mannose derivative exhibited strictly competitive inhibition kinetics and showed no mixed inhibition. This information is useful in as much as we are interested in using affinity chromatography for purification of the mannosidase enzymes.

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