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α-D-MANNOSIDASE FROM *CAPSICUM ANNUUM*

K. M. PRIYA SETHU and T. N. PRABHA*

Department of Plant Cell Biotechnology, Central Food Technological Research Institute, Mysore 570 013, India

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Key Word Index—*Capsicum annuum*; Solanaceae; bell-pepper; enzyme purification; characterization; glycosidase; α-mannosidase.

Abstract—α-D-Mannosidase (EC 3.2.1.24), a glycosidase showing a consistent increase in activity with the progress in softening in *Capsicum annuum*, was purified to electrophoretic homogeneity by fractionation with ammonium sulphate followed by gel filtration on Sephadex G-100, ion-exchange chromatography on DEAE Sephadex A-50 and HPLC gel filtration on GF 250. The purified enzyme had a native and a SDS M_r of 43 000 and 23 000, respectively. The optimal pH was 5.7 and the optimal temperature was 50°. The enzyme was thermally stable up to 60° for 15 min. The K_m for *p*-nitrophenyl α-D-mannopyranoside was 0.7 mM. The enzyme activity was inhibited nearly 95% by Fe^{2+} and Cu^{2+} at 0.1 mM. The preparation was free of other glycosidases. Antibodies were raised against the purified enzyme for further studies on the physiological function of α-D-mannosidase in fruit systems. This is the first report on purification to homogeneity and characterization of α-D-mannosidase from fruit systems. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

α-D-Mannosidase and β-D-galactosidase have been used as probes in the structural elucidation and functional studies of biologically important glycoproteins and glycolipids containing α-linked D-mannose and/or β-D-galactose residues as their major carbohydrate components [1, 2]. These glycosidases have been reported from different sources, e.g. plants [3, 4], fungi [5, 6], yeast [7] and animal tissues [8, 9].

In our previous study on the behaviour of carbohydrate hydrolases in *C. annuum* during textural softening, α-D-mannosidase was found to show consistently increased activity with softening/ripening [10]. When the activity was expressed on a protein basis, again only α-mannosidase activity increased during ripening and was also predominant among the carbohydrate hydrolases screened.

The activity of α-D-mannosidase was reported to increase with ripeness in tomato [11, 12], grape [13], muskmelon [14], olive [15] and pear fruit [16]. However, this enzyme has not been purified and characterized from ripening fruits. Mannans from plant sources contain linear chains of (1 − 4)-linked β-D-mannose residues as their main structural features [17] but the relationship between α-D-mannosidase activity and physiological function in fruits is not known. This enzyme was purified to electrophoretic homogeneity from bell pepper, and characterized in order to elucidate its properties.

RESULTS AND DISCUSSION

α-D-Mannosidase constituted *ca* 11% of the total protein extracted (Table 1). Its abundance in the crude protein extract is shown by the PAGE results (Fig. 1(A)). This was further confirmed by extracting proteins with buffers of varying pH and ionic strength and subjecting them to PAGE (data now shown).

The enzyme was purified nearly nine-fold with a recovery of 27%. The overall purification process of α-D-mannosidase is summarized in Table 1. The elution profiles of the enzyme from gel filtration, ion-exchange and HPLC columns are represented in Figs 2(A)–(C). The fractions collected after ion exchange chromatography and HPLC were subjected to PAGE and the protein bands detected by silver staining (Fig. 1(B) and (C)). The post ion-exchange fraction was found to have two minor contaminants which were removed when the enzyme was finally purified on HPLC. The homogeneity of the HPLC fraction was demonstrated electrophoretically on PAGE (Fig. 1(C)) and SDS–PAGE (Fig. 1(D)).

The M_r of the purified α-D-mannosidase as ascertained by HPLC-gel filtration was *ca* 43 000 (Fig. 2(D)). The M_r on SDS–PAGE was *ca* 23 000 (Fig. 1(D)). The enzyme had an optimal activity at 50° and was stable to 60° in a preincubation time of 15 min. It showed a broad pH curve exhibiting maximum activity

*Author to whom correspondence should be addressed.

Table 1. Purification of α-D-mannosidase from *Capsicum annuum*

Fraction	Total activity* (Unit)	Total protein (mg)	Specific activity† (Unit/mg)	Fold purification	Recovery (%±0.5)
Extract from Me_2CO dry powder	99.2	167.9	0.59	1	100
$(NH_4)_2SO_4$ (30–80%) saturation	45.6	27.4	1.66	2.8	46
Gel filtration on Sephadex G-100	42.4	15.1	2.80	4.7	43
Ion-exchange on DEAE Sephadex A-50	28.7	6.4	4.45	7.5	29
HPLC-gel filtration	26.9	5.1	5.24	8.9	27

The values are averages of three independent experiments.
*1 Unit is equivalent to 1 μmol PNP released min^{-1}.
†Specific activity is expressed as 1 μmol PNP released min^{-1} mg $protein^{-1}$.

at pH 5.7. The K_m value for *p*-nitrophenyl α-D-mannopyranoside was 0.7 mM. Enzyme activity was inhibited (nearly 95%) by Fe^{2+} and Cu^{2+} at 0.1 mM, whereas the metal inhibitors for the same enzyme in the case of jack beans and papaya seeds were Hg^{2+} and Ag^{+} [18]. There was no end product inhibition since the enzyme activity was not inhibited by mannose or its analogues (glucose and galactose). The enzyme, when checked for sugars by the phenol sulphuric acid method [19], showed no response implying that it is not glycosylated. The purified α-D-mannosidase was found to be free of other glycosidase activities such as β-mannosidase, α- and β-glucosidase, α- and β-galactosidase and α- and β-xylosidase. It could be stored at 0° for several months without appreciable loss in activity, but was inactivated by lyophilization.

Ouchterlony double diffusion showed a clear single line of precipitate at the junction. The antibody was also cross-checked by performing the enzyme assay after immunoprecipitation.

The presence of α-D-mannosidase has been frequently used as a vacuolar marker [20, 21], and significant alterations in the activity of this enzyme associated with metabolic changes have been demonstrated in tissues containing reserve proteins [22, 23]. A lysosomal location for α-D-mannosidase in *Pisum sativum* seeds

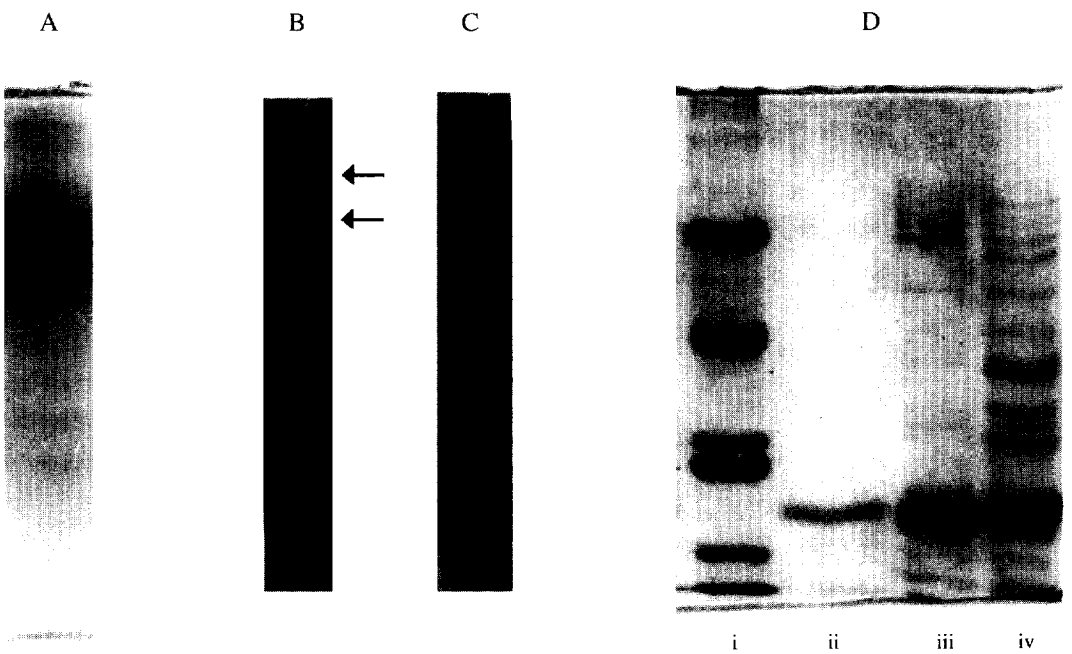

Fig. 1. PAGE (silver staining) of: (A) crude enzyme extract; (B) ion-exchange chromatography fraction (minor contaminants are indicated by arrows); (C) final HPLC fraction; (D) SDS–PAGE of (i) SDS M_r markers, (ii) HPLC fraction, (iii) ion-exchange chromatography fraction, (iv) crude enzyme extract.

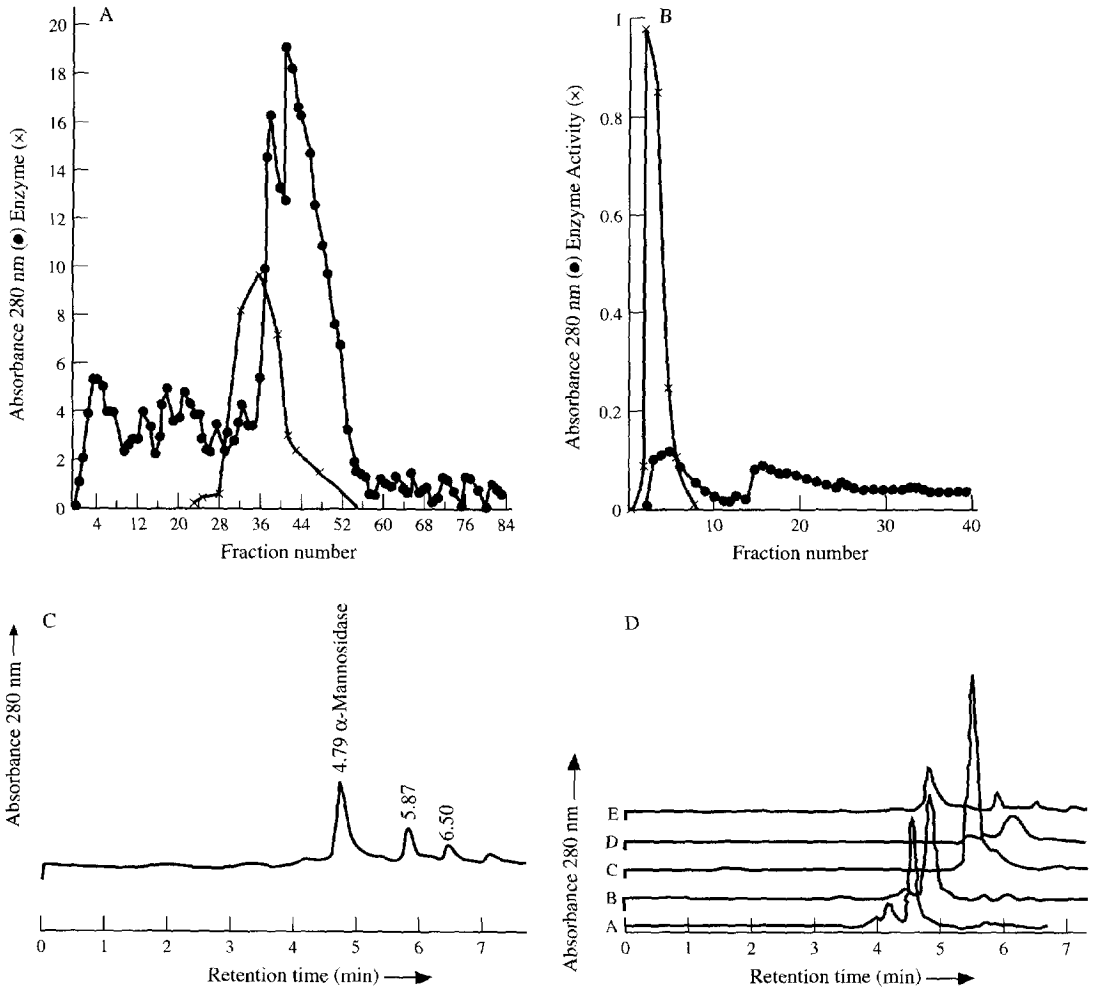

Fig. 2. Elution profile on: (A) Sephadex G-100 gel filtration column, flow rate 0.32 ml min^{-1}; (B) DEAE–Sephadex A-50 ion-exchange column, equilibrated and eluted with increasing concentrations of (0.05, 0.10 and 0.15 M) NaCl, pH 6 — the enzyme eluted with 0.15 M NaCl. (C) HPLC-gel filtration column, mobile phase was 0.2 M Na_2HPO_4, pH 7 and detected by UV at 280 nm, flow rate 2 ml per min. (D) M_r determination of HPLC-gel filtration column with standards (RT, retention time in minutes; 1 min = 100 sec). (a) Bovine serum albumin, RT 4.51; (b) ovalbumin, RT 4.79; (c) chymotrypsin, RT 5.47; (d) ribonuclease, RT 6.09; (e) α-D-mannosidase, RT 4.79.

was proposed by Murray [24], where it may be involved in the turnover of endoplasmic reticulum glycoproteins and glycolipids.

It was reported that α-D-mannosidase was the most active glycosidase in tomato fruit, with an 11% increase in activity between the mature green stage and the firm ripe stage [11, 12]. In grape fruit, the enzyme activity was initially low but steadily increased with advanced maturity [13], whereas in olive fruit, the activity was absent during the first phase of ripening but appeared in the completely ripe stage [15]. In pear fruit, there was a five-fold increase in mannosidase activity in the cell wall during ripening [16]. In our previous study, isoenzymes of α-D-mannosidase were detected and characterized in mango, banana and papaya (unpublished observation). It is conceivable that α-D-mannosidase isoenzymes are involved in the processing of glycoproteins in ripening fruits by way of deglycosylation.

EXPERIMENTAL

Material. Matured capsicum was freshly harvested from a local farm. Me_2CO dried powder prepared from the fruits served as the starting material for enzyme purification.

Purification. Me_2CO dried powder (10 g) was extracted for 12 hr with 0.05 M Na-P_i buffer (pH 6.6) containing 0.25 M NaCl at 4°. The resulting suspension was passed through 4 layers of cheese cloth and the filtrate was centrifuged at 5000 *g* for 30 min. The clarified filtrate was fractionated with $(NH_4)_2SO_4$ (30–80%). The protein fr. so precipitated, containing α-D-mannosidase was dialysed and then loaded onto a column of Sephadex G-100 (1.6 × 140 cm). The α-D-mannosidase-rich fr. was concd and applied on DEAE–Sephadex A-50 ion-exchange column (3.6 × 40 cm). The post-ion-exchange fr. was subjected to further purification using HPLC-gel filtration column (Zorbax

Bio series GF-250, 9.4 mm i.d. × 25 cm, packed with zirconia-stabilized silica).

Enzyme assay. All enzyme reactions were performed at pH 5.6 in 0.1 M NaOAc buffer at 37° for 15 min in a total vol. of 3 ml. Enzyme activities towards 1.25 mM *p*-nitrophenyl α-D-mannopyranoside were determined by measuring the liberated *p*-nitro-phenol (PNP) at 420 nm after the addition of 0.5 M Na_2CO_3 to the reaction mixt. One unit of enzyme activity was defined as the amount of enzyme which hydrolysed 1 μmol of substrate per min. The sp. act. of the enzyme was expressed as units per mg protein, which was determined by the method of ref. [25].

Electrophoresis. Native as well as SDS–PAGE were carried out according to the method of ref. [26]. Both Coomassie brilliant blue and silver [27] staining were used for the location of the protein bands.

M_r *determination.* M_r of the enzyme was determined by HPLC-gel filtration (GF-250 as given above) with bovine serum albumin (M_r 67 000), ovalbumin (43 000), chymotrypsin (25 000) and ribonuclease (13 700) as standards. M_r was also determined by SDS–PAGE with α-lactalbumin (14 200), trypsin (20 100), carbonic anhydrase (29 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), egg albumin (45 000) and bovine serum albumin (66 000) as standards.

Optimal pH. The effect of pH on enzyme activity was found using buffers (0.1 M Na-citrate buffer, pH 3.0–4.0; 0.1 M NaOAc buffer, pH 4.0–5.6; 0.1 M Na-P_i buffer, pH 5.7–7.2 and 0.1 M Tris-Cl buffer, pH 7.0–8.0).

Optimal temp. and temp. stability. The enzyme activity was assayed at temps ranging from 20 to 80° and compared with the activity at 37°. The stability of the enzyme was measured by preincubating the enzyme at different temps for 15 min and assaying the remaining activity at the optimal temp. The activity of the untreated enzyme was used as the control (100%).

Inhibition study. The effect of EDTA (10 mM) and metal ions (Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2-}, Ca^{2+}, Mn^{2+} and Zn^{2+}) on activity was studied by incubating the enzyme in buffer in the presence of 0.1–1.0 mM of each test metal ion for 30 min prior to the addition of the substrate.

Immunization of rabbits. The purified enzyme in Na-P_i buffered saline (PBS-0.1 M Na-P_i buffer containing 0.85% NaCl, pH 7.2) was used as the antigen. Rabbits were immunized with a primary dose and three booster doses of *ca* 300 μg each. Intramuscular injections (with equal vol. of Freund's complete adjuvant) were administered at 15-day intervals. The antiserum was sepd from the blood 2 days after the fourth injection and subjected to $(NH_4)_2SO_4$ fractionation (60%) for immunoglobulin preparation. The precipitated immunoglobulin fr. was dissolved in PBS and dialysed against PBS before use.

Ouchterlony double diffusion. Agarose gels (2 mm thick) were made with 1% agarose in 0.05 M PBS containing 0.1% NaN_3 as a preservative. Wells were punched using a template and a cork borer. Double diffusion was carried out at 25° for 12 hr. Purified enzyme prepn in 0.05 M PBS adjusted to identical protein concn was used as the antigen.

Cross-checking of the antibody. To a known vol. of the antibody, antigens were added in excess to absorb the antibodies. The supernatant fluid obtained after centrifugation at 3600 *g* for 20 min at 4° was used for the enzyme assay. For the control, globulins of preimmunized serum were treated with antigen. The ppt. obtained above was washed with 0.05 M PBS, dissolved in Tris-Cl buffer, pH 10.2 and the assay repeated.

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