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α-D-Mannosidase from *Lycopersicon esculentum* II

G. Suvarnalatha, T.N. Prabha*

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

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Abstract

Tomato (*Lycopersicon esculentum* Mill) fruit tissue was found to contain two isoforms of α-D-mannosidase (EC. 3.2.1.24), which were purified by ion-exchange chromatography on DEAE sephadex A-50 followed by gel filtration on sephadex G-100. The two isoforms were named isoform I and II (minor and major forms, respectively) which differed in their abundance, their elution profile on both ion-exchange and gel filtration column chromatography. Isoforms I and II showed 6 and 24% of the total activity, respectively. Both the isoforms had an acid pH optimum (4.5) and were thermally stable at 65° for up to 15 min. Isoform I showed a broad temp optimum between 55 and 65° whereas isoform II had an optimal activity at 65°. The K_m values of isoforms I and II for *p*-nitrophenyl-α-D-mannopyranoside were 1.11 and 1.05 mM, respectively. The activity of isoform I was inhibited by Hg^{2+} (1 mM) whereas isoform II was inhibited by Cu^{2+} (0.1 mM) and Hg^{2+} (1 mM). Both the isoforms were free of other glycosidases. Purified isoform II had a SDS M_r of ca. 38,000.

Keywords: Lycopersicon esculentum; Solanaceae; Tomato; Enzyme purification; Isoforms; α-D-Mannosidase

1. Introduction

Previously we reported on the purification profile and properties of α-D-mannosidase from *Capsicum annuum* (Priya Sethu & Prabha, 1997), the present study pertains to α-D-mannosidases of tomato, another solanaceous fruit crop. This is the first report on the enzymological characterization of this enzyme from tomato fruit.

Interestingly, α-D-mannosidase not only showed an activity peak during ripening/softening of mango, banana, papaya, bell-pepper and tomato but was also the most active enzyme amongst the glycosidases examined (unpublished data). In the literature, increased activity of α-D-mannosidase during ripening has been reported for tomato, grape, muskmelon, olive, pear and watermelon (Pharr, Sox, & Nesbitt, 1976; Ahmed & Labavitch, 1980; Nakagawa, Enomoto, Asakawa, & Uda, 1988; Watkins, Haki, & Frenkel, 1988; Fils Lyvaon & Buret, 1991; Heredia, Guillen, Jimenez, & Fernandez Bolanos, 1993; Burns & Baldwin, 1994).

This enzyme cleaves the short chain oligo-mannose residues (~8–10) present in oligosaccharides and glycoproteins. The physiological role of most of the glycosidases during ripening is not known. No correlation between α-D-mannosidase activity and physiological functions has been reported and there is no known natural substrate for this enzyme in vivo. The presence of high activity in ripening fruits prompted us to look into its properties in tomato, where two isoforms of α-D-mannosidase were identified and studied.

2. Results and discussion

The purification of α-D-mannosidase is summarized in Table 1 and Fig. 1. Fractionation of the tomato enzyme extract by chromatography on DEAE sephadex A-50 showed the presence of two peaks of α-D-mannosidase activity (Figure 1a). The gel filtration profiles of which are depicted in Fig. 1b and c. The two isoforms were named isoform I and II based on their distinct elution profiles on ion-exchange chromatography, where, isoform I and II were eluted with 0.15 and 0.2 M NaCl, respectively (Fig. 1a). Isoform I contributed a minor portion (6%) and isoform II contributed a major portion (24%) of the total activity of the enzyme present in the crude extract. α-D-Mannosidase in tomato constituted ca. 4.0% of the total protein extracted (Table 1), of which isoform I and II constituted 0.7 and 3.3%, respectively. The specific activity of isoform I was slightly higher than isoform II (Table 1). Isoforms I and II were purified 9.1 and 7.4 fold, respectively, with a recovery of 6.3 and 24.5%. Total recovery of α-D-mannosidase was 30.8%.

* Corresponding author. Present address. Biochemistry and Nutrition Department, CFTRI, Mysore-570013.

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Table 1
Summary of purification of α-D-mannosidase from tomato

Fraction	Total activity[a] (units)	Total protein (mg)	Specific activity[b] (units/mg protein)	Purification fold	Recovery (%)
Crude enzyme	153.52	158.27	0.97	1	100
Dialyzed enzyme	125.22	126.49	0.99	1.02	81.56
DEAE Column					
Minor peak I	14.24	2.04	6.98	7.2	9.27 } 38.19
Major peak II	44.39	7.89	5.62	5.8	28.91 }
Sephadex G-100 Column					
Peak I	9.74	1.10	8.82	9.1	6.35 } 30.86
Peak II	37.63	5.27	7.14	7.4	24.51 }

Values are averages of three independent experiments.
[a] One unit is equivalent to 1 μmol PNP released min^{-1}.
[b] Specific activity is expressed as 1 μmol PNP released min^{-1} mg $protein^{-1}$

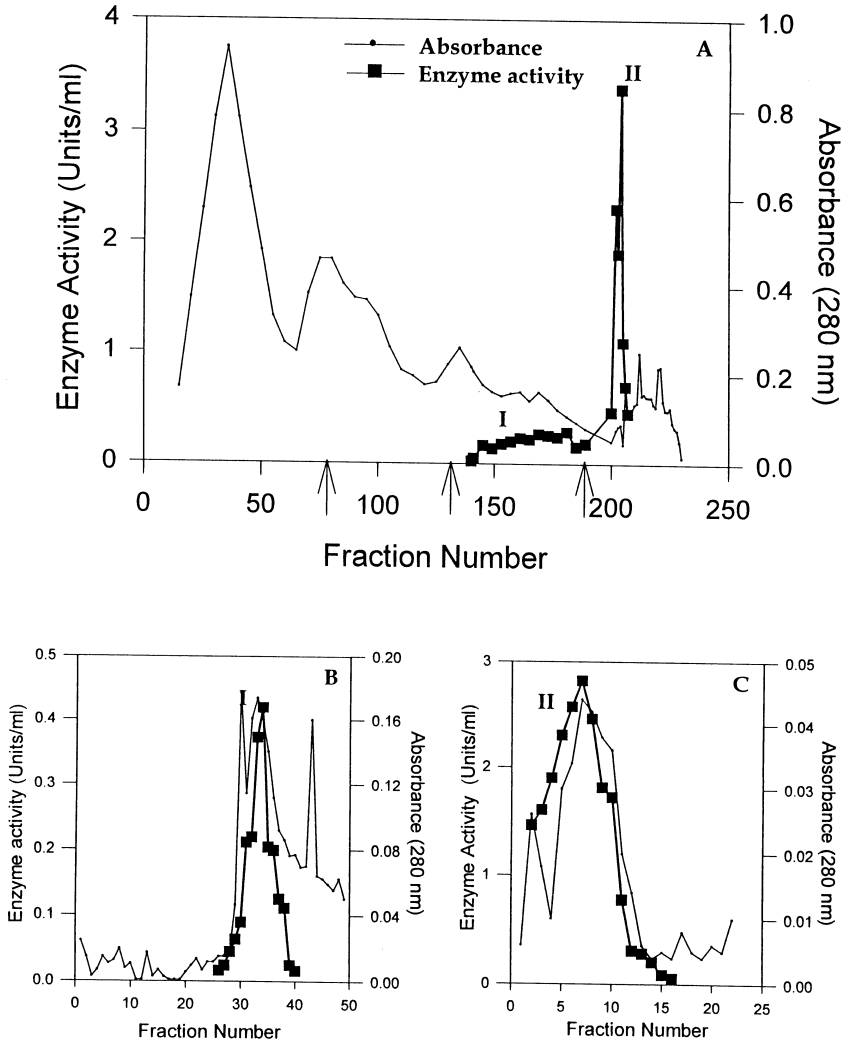

Fig. 1. Elution profile on (a) DEAE-Sephadex A-50 ion-exchange column, equilibrated and eluted with increasing concentrations of 0.05, 0.10, 0.15 and 0.2 M NaCl; pH 6.0, flow rate 0.3 ml min^{-1}. Isoform I and II eluted with 0.15 and 0.2 M NaCl, respectively. Arrows indicate the change of NaCl gradient. (b) and (c) Sephadex G-100 gel filtration, flow rate 0.3 ml min^{-1} for isoform I and II, respectively. The volume of each fraction was 3 ml (–·– is absorbance and – ■ – enzyme activity).

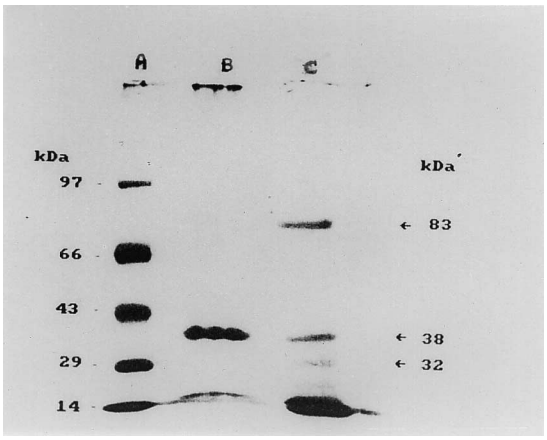

Fig. 2. SDS-PAGE of isoform II (Coomassie brilliant blue staining). (a) SDS M_r markers, (b) gel filtration column fr, (c) ion-exchange chromatography fr.

The two isoforms resolved by ion-exchange chromatography, when individually subjected to gel permeation chromatography, showed distinct separation indicating different M_r. The native M_r of isoform I and II were ca. 43,000 and 75,000, respectively as determined by gel filtration on sephadex G-100. The M_r weight of the purified isoform II on SDS-PAGE was ca. 38,000 (Fig. 2) and that of isoform I could not be determined as the amount of protein was not sufficient to locate the bands on SDS-PAGE.

The properties of both the isoforms are summarized in Table 2. Isoform I showed a narrow pH curve whereas isoform II exhibited a broad pH curve, but both had an optimal activity at pH 4.5, while the enzyme reported from *Capsicum* exhibited a pH optimum of 5.7 (Priya Sethu & Prabha, 1997). The optimal pH for both the isoforms of tomato was similar to the α-D-mannosidases from watermelon and *Phaseolus vulgaris* (Wilden & Chrispeels, 1983; Nakagawa et al., 1988). Isoform I had an optimal temp curve from 55–65° and isoform II had an optimum at 65°. Both the isoforms were stable to 65° when using a preincubation time of 15 min. The thermal optimum and stability for α-D-mannosidase from *Capsicum* were comparatively lower (Priya Sethu & Prabha, 1997).

The K_m values for *p*-nitrophenyl α-D-mannopyranoside for isoform I and II were 1.11 and 1.05, respectively, while for the enzyme from bell-pepper, the K_m was 0.7 mM indicating higher substrate affinity (Priya Sethu & Prabha, 1997). On the other hand the K_m was 2.3 mM for the enzyme from watermelon (Nakagawa et al., 1988). Enzyme activity of both the isoforms was inhibited (up to 85%) by Hg^{2+} (1 mM) and the activity of isoform II was inhibited (nearly 63%) by Cu^{2+} (0.1 mM). Isoform I was not inhibited by Cu^{2+}. Metal inhibitors of α-D-mannosidase for bell-pepper were Fe^{2+} and Cu^{2+} (Priya Sethu & Prabha, 1997); for jackbeans and papaya seeds, they were Hg^{2+} and Ag^{2+} (Ohtani & Misaki, 1983) and for watermelon, they were Cd^{2+} and Cu^{2+} (Nakagawa et al., 1988). Mannosidase from tomato was not inhibited by EDTA unlike that from watermelon (Nakagawa et al., 1988). As in bell-pepper (Priya Sethu & Prabha, 1997), glucose, galactose and mannose did not inhibit the enzyme activity in tomato. Both the isoforms were not glycosylated as shown by the absence of sugars when tested for by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) as well as by Con A binding. Both isoforms are free of other glycosidases such as β-mannosidase, α- and β-glucosidase and α- and β-xylosidases.

The activity of the α-D-mannosidase varies with the stage of development/ripening in bell-pepper, tomato, grape, muskmelon, olive, and pear (Pharr et al., 1976; Ahmed & Labavitch, 1980; Watkins et al., 1988; Fils Lyvaon & Buret, 1991; Heredia et al., 1993; Burns & Baldwin, 1994; Priya Sethu & Prabha, 1997). In tomato fruit, α-D-mannosidase was the most active glycosidase with a 11% increase in activity from the mature green stage to firm red stage (Pharr et al., 1976; Watkins et al., 1988). Watkins et al. (1988) showed quantitative differences in the activity of this enzyme during ripening but no direct association between the activity of this enzyme

Table 2
Properties of isoforms of α-D-mannosidase from tomato

Property	Isoform I	Isoform II
Elution on ion-exchange column	Eluted in 0.15 M NaCl	Eluted in 0.2 M NaCl
Abundance	Minor fr.	Major fr.
Sp activity (units)	8.82	7.14
pH optima	4.5	4.5
Temperature optima (°)	55–65	65
Thermal stability	65° for 15 min	65° for 15 min
Inhibition	inhibited by Hg^{2+}	inhibited by Hg^{2+} and Cu^{2+}
Glycosylated	no	no
Native M_r	ca. 43,000	ca. 75,000
SDS M_r	N.D.	38,000

and other glycosidases was seen. Significant changes in the activity of α-D-mannosidase associated with physiological changes were demonstrated in cotyledon tissues containing reserve proteins (Neely & Beevers, 1980; Plant & Moore, 1982). α-D-Mannosidase has been used as a vacuolar marker (Boller & Kende, 1979; Glund et al., 1984) and it has been suggested that it may be involved in the turnover of endoplasmic reticulum glycoproteins and glycolipids (Murray, 1983).

It should be noted here that we have also identified two isoforms of α-D-mannosidase from other fruits such as, banana and mango (unpublished work). This is the first report on the presence of two isoforms in ripening fruits. With the antibody of *Capsicum* mannosidase (Priya Sethu & Prabha, 1997), further studies on the immunological aspects of tomato enzyme are in progress. Three isoforms of α-D-mannosidase with different pIs have been reported in cotyledon tissues of *P. vulgaris* (Wilden & Chrispeels, 1983). The present study on α-D-mannosidase is a new addition to 'tomato literature' as well as in relation to fruit ripening. Since this is the most active enzyme in most of the fruits showing an enhanced activity during ripening and also an abundant protein in *Capsicum* (Priya Sethu & Prabha, 1997), the implication of this enzyme in fruit softening/ripening needs to be understood. Suppression of this enzyme by antisense approach may lead to a better understanding of it's role in the context of fruit ripening/softening. Very recently the importance of α-D-mannosidase in the context of anticancer therapy has been investigated (Howard, He, & Withers, 1998).

3. Experimental

3.1. Material

Matured and freshly harvested tomato fruits (*Rupali* var.) were obtained from a local market. Me_2CO dried powders prepared from the fruits served as the sources for enzyme extraction.

3.2. Purification

Me_2CO dried powder (12 g) was extracted for 10 h with 0.05 M Na–Pi buffer (pH 6.6) containing 0.25 M NaCl at 4° as described earlier (Priya Sethu & Prabha, 1997). The enzyme extract was filtered through four layers of cheese cloth and the filtrate was centrifuged at $5000 \times g$ for 30 min. The supernatant fr. was dialyzed overnight and loaded onto a column of DEAE sephadex A-50 ion-exchange column (4×40 cm). The enzyme-rich frs. were collected and pptd. with $(NH_4)_2SO_4$ (80%) individually for isoform I and II and dialyzed. The concd. enzyme frs. were loaded separately onto Sephadex G-100 (1.6×140 cm) gel filtration columns. The post gel filtration fr. was used for further studies.

3.3. Enzyme assay

This was performed at pH 5.6 in 0.1 M NaOAc buffer at 37° for 15 min in a total vol. of 3 ml as described earlier (Priya Sethu & Prabha, 1997). Enzyme activities towards 1.25 mM *p*-nitrophenyl-α-D-mannopyranoside were determined by measuring the liberated *p*-nitrophenol (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 one μmol of substrate per min. The sp. act. of the enzyme was expressed as units per mg protein. The protein was determined by the method of Sedmak and Grossberg (1977).

3.4. Optimal pH

The effect of pH on enzyme activity was determined using a range of 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–Pi buffer pH 5.7–7.8 and 0.1 M Tris–HCl buffer, pH 7.0–8.0).

3.5. Optimal temperature and thermal stability

The enzyme activity was assayed at temperatures 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%).

3.6. Inhibition study

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

3.7. Electrophoresis

SDS-PAGE was carried out as per the method of Blackshear (1964). Coomassie brilliant blue was used for staining the gels. M_r was determined by SDS-PAGE with phosphorylase b (97400), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,000) and lysozyme (14,300).

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