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AN IMMUNOLOGICALLY DISTINCT FORM OF α-D-MANNOSIDASE IN *CANAVALIA ENSIFORMIS* LEAF

KRISHNAKALI NIYOGI and MANORANJAN SINGH

Indian Institute of Chemical Biology, Jadavpur, Calcutta 700032, India

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Key Word Index—*Canavalia ensiformis*; Leguminosae; jackbean; α-mannosidase; leaf; western blot.

Abstrct—The distribution of α-mannosidase (EC 3.2.1.24) in various tissues of *Canavalia ensiformis* has been studied and a new form of the enzyme has been identified in the leaf. The new enzyme is immunologically distinct from the well-characterized seed enzyme since the polyclonal rabbit antisera against the latter, failed to cross-react with it in immuno-diffusion, immunoprecipitation and immunoblot experiments. The native enzyme has an approximate M_r of 217000 and a pH optimum near 4, and it is stimulated by Zn^{2+}. In these respects, it is similar to the seed enzyme.

INTRODUCTION

α-Mannosidase is a Zn^{2+}-dependent enzyme, widely distributed in plants [1–11], bacteria [12] and animals [13, 14]. Leguminous seeds are rich in this enzyme and much of the work on its characterization has been confined to this source. Many of the seed α-mannosidases have a native M_r of *ca* 200000; but in the cases which have been properly characterized, the subunit structures are found to be different, e.g. in *Canavalia ensiformis*, it is α_2 β_2 (M_r 66000 and 44000, respectively) [10] whereas in *Phaseolus vulgaris* it is α_2 (M_r 110000). [8]. Functionally, the enzyme is implicated, in conjunction with other glycosidases, in the degradation of polysaccharides, glycoproteins and other glyco-conjugates during seed germination to provide the nutrient and energy requirements of the growing seedling [15]. At other stages of the life cycle of the plants, it may be involved in the normal turn-over process of these macromolecules.

In *Canavalia ensiformis* (jackbean), the seed enzyme has been extensively studied [1–5, 10, 11]; but very little is known about the nature or the function of the enzyme in other tissues. In fact, even its presence in tissues other than the seed, has not been properly established. In the present communication, we report the tissue distribution and the identification, in the leaf of this plant, of another α-mannosidase which is immunologically different from the seed enzyme.

RESULTS

The distribution of α-mannosidase in C. ensiformis

The seeds contain high levels of α-mannosidase, whereas the enzyme activities are rather low in the leaves, embryonic axes and roots (Table 1).

Table 1. Distribution of α-mannosidase in various tissues of *C. ensiformis*

Tissue	α-Mannosidase (unit/g)	Sp. activity (unit/mg)
Cotyledon	36.00	0.45
Leaf	0.58	0.01
Embryonic axis	1.00	0.03
Root	0.22	0.01

Jackbean tissues, collected from the germinating seed (cotyledon and embryonic axis) and the growing seedling (leaf and root)), were homogenized in 5 volumes of 20 mM Tris–HCl, PH 7.5, and centrifuged at 16000 *g* for 30 min at 4°. The clear supernatant fluid was assayed for α-mannosidase activity and protein content were assayed as described in the text [23].

Immunological examination of α-mannosidase in various tissues of C. ensiformis

Immuno-diffusion studies with rabbit antisera against the seed α-mannosidase, and the tissue extracts of *C. ensiformis* resulted in precipitin band formation with the seed, the root and the embryonic axis (Fig. 1). However, no precipitin band could be detected either with the leaf extract or the partially purified leaf enzyme.

Further immunological characterization of the leaf α-mannosidase

The absence of a precipitin band in the case of the leaf α-mannosidase was further examined as to whether there was cross-reactivity without precipitin band formation. Incubation of the partially purified leaf enzyme, followed by further incubation with protein A (*S. aureus*) did not result in any detectable cross-reactivity, whereas there was complete precipitation of the seed enzyme in the control experiment (Table 2).

This finding is further substantiated by the results of the Western blot analysis (Fig. 2). Under the conditions of the experiment, about 70 μg of leaf enzyme could not be detected (lane 7), whereas 50 ng of the seed enzyme gave a distinct band (lane 2).

Physico-chemical properties

In order to compare the physico-chemical properties of the leaf α-mannosidase with those of the well-studied enzyme of the seed, a partially purified leaf enzyme preparation and a homogeneous preparation of the seed enzyme were subjected to PAGE, in the absence of reducing and denaturating agents. The results (Fig. 3) showed that in both cases the enzyme activity coincides with the protein band and there is no difference in the electrophoretic mobilities of the two enzyme preparations. However, the leaf enzyme preparation was not homogeneous, as revealed by SDS-PAGE analysis (data not shown).

Sucrose density gradient centrifugation revealed that both enzymes have a similar M_r of *ca* 217 000 (Fig. 4). This finding is further corroborated by gel filtration since they are eluted in the same volume from a Sephacryl S-300 column (2.2 × 115 cm) (data not shown).

Some enzymatic properties of jackbean leaf α-mannosidase

The pH-activity profile of the leaf enzyme in 0.03 M citrate–phosphate buffer (pH 3–7) showed a pH optimum of *ca* 4. In this respect, it is similar to the seed enzyme.

It is also similar to the seed enzyme as regards the effect of divalent metal ions. Thus exhaustively dialysed leaf

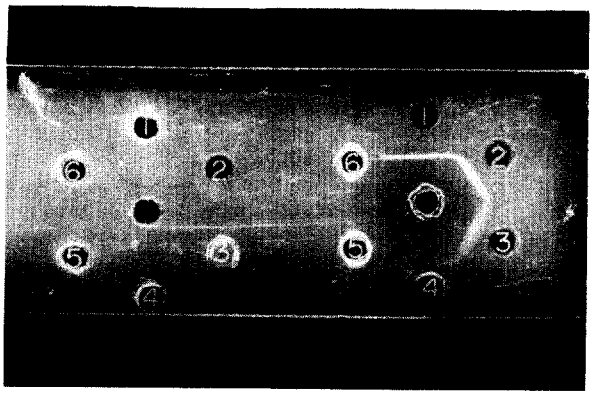

Fig. 1. Immuno-reactivity of rabbit anti-α-mannosidase (seed) with the enzyme from various tissues of *C. ensiformis*. Immunodiffusion was carried out in 1.5% agarose containing 0.2 M α-methyl D-mannoside, using rabbit anti-α-mannosidase (seed) sera and various tissue extracts of the plant. Central well: the left hand side contained non-immune sera (10 μl) and the right hand side, immune sera (10 μl). (1) Purified seed α-mannosidase (10 μg, 0.05 EU); (2) Seed extract (160 μg, 0.07 EU); (3) Embryonic axis extract (136 μg, 0.004 EU); (4) Root extract (98 μg, 0.002 EU); (5) Leaf extract (256 μg, 0.006 EU); (6) Partially purified leaf α-mannosidase (30 μg, 0.06 EU).

Table 2. Immunoprecipitation of α-mannosidase with rabbit anti-α-mannosidase (seed)

Incubation	Remaining activity EU/ml	(%)
A. Leaf α-mannosidase (2 EU/mg)		
1. 0.088 EU + buffer only	0.44	100
2. 0.088 EU + non-immune serum	0.44	100
3. 0.088 EU + immune serum	0.39	89
B. Seed α-mannosidase (3.6 EU/mg)		
4. 0.076 EU + buffer only	0.38	100
5. 0.076 EU + non-immune serum	0.38	100
6. 0.076 EU + immune serum	0	0

Leaf α-mannosidase and the seed enzyme were separately incubated overnight with 10 μl of immune sera, non-immune sera or buffer in a total volume of 100 μl at 4°, followed by 1 hr incubation at 37° after the addition of 100 μl of protein A suspension. It was then centrifuged and the remaining enzyme activity in the supernatant fluid was assayed as described in the text.

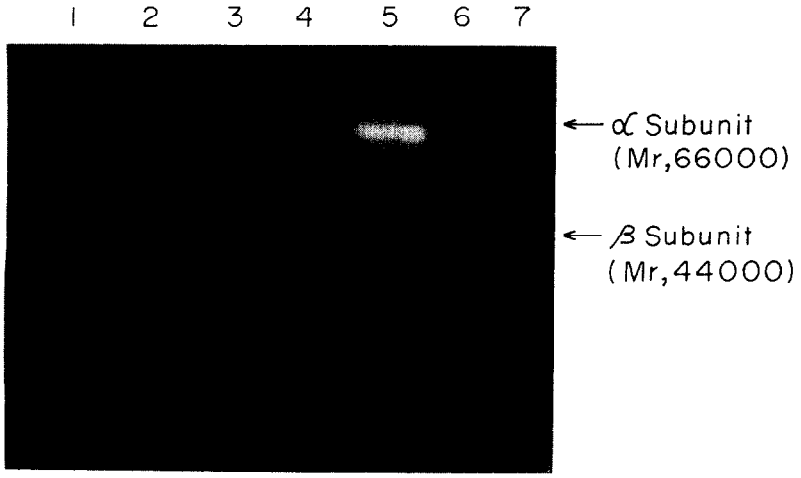

Fig. 2. Western blot analysis of α-mannosidase from the leaf of *C. ensiformis*. Partially purified leaf α-mannosidase and the seed enzyme were subjected to SDS-PAGE (12%) and Western transfer was carried out electrophoretically. It was reacted with rabbit anti-α-mannosidase (seed) sera, followed by [^{125}I]-protein A (1.5 × 10^6 cpm/ml). Purified seed enzyme: (1) 30 μg, (1 × 10^{-4} EU); (2) 54 μg, (1.8 × 10^{-4} EU); (3) 108 μg, (3.6 × 10^{-4} EU); (4) 216 μg, (7.2 × 10^{-4} EU); (5) 2.16 μg, (7.2 × 10^{-3} EU); (6) leaf extract, 0.05 EU; (7) Partially purified leaf enzyme, 0.14 EU.

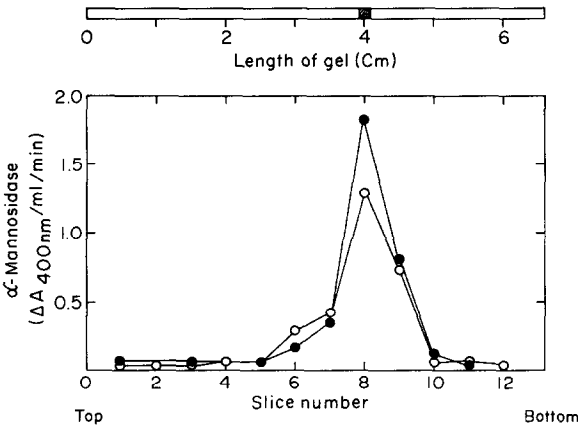

Fig. 3. Comparison of the electrophoretic mobilities of leaf and seed α-mannosidases from *C. ensiformis*. The partially purified preparation of the leaf enzyme (100 μg protein; 2 EU/mg) and a homogeneous preparation of the seed enzyme (100 μg protein) were subjected to alkaline PAGE (5%) under non-reducing and non-denaturating conditions. A set of gels was stained with Coomassie Blue and another set was sliced into 0.5 cm pieces. The gel slices were homogenised in a small volume of 20 mM Tris–HCl, pH 7.4 and kept overnight at 4°. It was centrifuged and the supernatant fluid was assayed for the enzyme activity as described in the text. The solid circle represents the seed enzyme and the open circle, the leaf enzyme. The upper panel represents the Coomassie Blue stained gels of both the preparations.

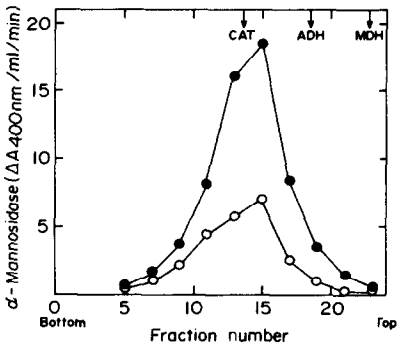

Fig. 4. Determination of M_r of the native α-mannosidase from the leaf of *C. ensiformis* by sucrose density gradient centrifugation. Partially purified leaf α-mannosidase was subjected to sucrose density gradient centrifugation at 216000 g for 8 hr at 4°, as described in the text. –●—●–, seed α-mannosidase; –○—○–, leaf α-mannosidase; CAT, catalase (M_r, 240000); ADH, alcohol dehydrogenase (M_r, 150000); and MDH, malate dehydrogenase (M_r, 62000).

enzyme preparation was stimulated by Zn^{2+} and was slightly inhibited by Mn^{2+} and Cu^{2+}, whereas Mg^{2+} and Ca^{2+} had no effect on the activity (Table 3).

Table 3. Effect of divalent metal ions on the activity of leaf α-mannosidase

Metal ion added	Enzyme activity (A_{400}/10 min)	(%)
1. None	0.66	100
2. 1.0 mM $ZnSO_4$	1.18	178.8
3. 1.0 mM $MgSO_4$	0.69	104.5
4. 1.0 mM $CaCl_2$	0.67	101.5
5. 1.0 mM $MnCl_2$	0.56	84.8
6. 0.1 mM $CuSO_4$	0.54	81.8

Partially purified leaf α-mannosidase was exhaustively dialysed against 20 mM Tris–HCl, pH 7.4, and the enzyme activity was measured as described in the text with the addition of various metal ions as indicated using 30 μg protein.

DISCUSSION

Jackbean seed is rich in α-mannosidase and the enzyme from this source has been extensively studied [1–5, 10, 11]. In the present study, we have established the occurrence of this enzyme in various other tissues of the plant even though the amounts are rather low. This is not surprising as there are previous indications that the level of the enzyme is low in the embryonic axis as compared to the cotyledon (unpublished results, cited in [16]).

On the basis of the immunological evidence presented here, the cotyledon, the embryonic axis and the root seem to have the same enzyme whereas the leaf enzyme is different since sufficiently high amounts of the latter did not cross-react with the antiserum, raised against the seed enzyme. In the immunoblot experiment, a 140-fold less amount of the seed enzyme gave rise to a distinct band, corresponding to the subunit M_r, 66000 (Fig. 2).

In this experiment, the apparent lack of proportionality in the intensities of the two bands, corresponding to the α and β subunits of the seed α-mannosidase, may be attributed to the nature of the antiserum since the β subunit is probably less antigenic than the α subunit.

In the present study, the following different criteria were chosen: (i) the immuno-diffusion experiment would reveal the reaction of the native enzyme with the antibody leading to precipitation. (ii) In the immunoprecipitation experiment, if there was any cross-reaction with the native enzyme without precipitation, the complex would be precipitated by the protein A. (iii) The immunoblot experiment would reveal any cross-reaction between the antibody and the SDS-denatured enzyme.

Since the leaf enzyme failed to cross-react with the antibody under any of these conditions, it is obvious that it is immunologically distinct from the seed enzyme. On the other hand, many properties of the leaf enzyme are similar to those of the seed enzyme. The present finding of an approximate M_r of 200000 for the native enzyme seems to be a common feature of other α-mannosidases of plant seeds [8, 10, 11]. However, in the other cases the subunit structures have been shown to be different. This has not been shown for the leaf enzyme since the preparation is only partially pure.

The physiological function of the enzyme in the plant, particularly the significance of its occurrence in distinct forms in different tissues, is not clear. While the suggested role in the degradation of glyco-conjugates in germinat-

ing seeds [15] may be important, its function at the other stages of the life cycle of the plant and in tissues other than the seed remain obscure. It is worthwhile considering the involvement of the different forms of α-mannosidase in the processing of high mannose-containing glycoprotein intermediates, generated by the dolichol phosphate pathway [17]. Recent work by Sturm *et al.* [18] has shown that the $(Man)_9$ $(GlcNAc)_2$-oligosaccharide chain attached to Asn^{341} of phasoelin is not susceptible to the well characterized jackbean seed α-mannosidase, whereas the pronase digested glycopeptide can be digested by this enzyme. Unlike in the mammalian system, the mechanism of the processing of glycoprotein intermediates in plants is yet to be worked out and none of the glycosidases involved, has been identified. From this point of view, the present identification of another form of α-mannosidase may be significant as it may be involved in other physiological role different from degradation of storage glyco-conjugates to provide nutrients to other tissues of the plant.

EXPERIMENTAL

Materials. Trizma, α-methyl-D-mannoside, *p*-nitrophenyl-α-D-mannoside, *N,N,N',N'*-tetramethylene diamine (TEMED), bis-acrylamide, 2-mercaptoethanol, Coomassie Blue R 250, Bromophenol Blue, sodium deoxycholate, Triton X-100, SDS, DTT, NAD, NADH, oxalacetate, yeast alchol dehydrogenase, catalase, malate dehydrogenase, jackbean seed α-mannosidase, Antifoam C emulsion, cross-linked polyvinylpyrrolidone (PVP), soluble protein A from *Staph. aureus*, and bovine serum albumin were purchased from Sigma Chemicals, U.S.A. Standard M_r marker proteins and Nonidet P40 (NP-40) were obtained from Bethesda Research Laboratory, U.S.A. Sephacryl S-300, Biogel P-4 and nitrocellulose paper (BA 85) were from Pharmacia, Bio-Rad and Schleicher and Schuell, respectively. Carrier-free [^{125}I], as NaI was supplied by Bhabha Atomic Research Centre, India. Staphylococcal protein A suspension from *S. aureus* (Cowan I) was prepared according to the procedure of Kessler [19]. Other chemicals were analytical grade reagents.

Plant material. Jackbean, *Canavalia ensiformis* (L) D.C., seeds and leaves were obtained from plants grown in the institute. Embryonic axes and roots were taken from seeds germinated in the laboratory under controlled conditions.

Assay of α-mannosidase. The enzyme was assayed by the procedure of ref [1], using *p*-nitrophenyl α-D-mannoside as the substrate. The enzyme was incubated in 125 mM citrate buffer, pH 4.5, with 5 mM *p*-nitrophenyl-α-D-mannoside in a total vol. of 100 μl for 10–30 min at 37°. The reaction was stopped by the addition of 900 μl 0.2 M borate buffer, pH 9.8, and the liberated *p*-nitrophenol was measured at 400 nm. An enzyme unit is defined as the amount of enzyme required to liberate 1 μmol of the product per min under the conditions of the assay.

Other enzyme assays. Other enzymes used as M_r markers were assayed by standard procedures using H_2O_2 for catalase [20], EtOH for alcohol dehydrogenase [21] and oxalacetate for malate dehydrogenase [22].

Protein assay. Protein was assayed either by the method of ref. [23] using bovine serum albumin as the standard or by the method of ref. [24].

SDS-PAGE was carried out by the procedure of ref. [25] and stained with Coomassie Blue. Alkaline PAGE was done in tubes by the same proedure but in the absence of SDS and 2-mercaptoethanol.

Sucrose density gradient centrifugation for the M_r *determination of proteins.* Sucrose density gradient was carried out according to ref. [26]. The enzymes were layered on 5 ml of a 5–20% (w/v) sucrose gradient and subjected to centrifugation at 48 000 rpm for 8 hr at 4° in a Beckmann L-8M ultracentrifuge. Tubes were punctured at the bottom and 0.2 ml fractions were collected and assayed for the enzymes.

Preparation of antiserum against seed α-mannosidase. Seed α-mannosidase was either obtained commercially or prepared by the method of ref. [1] with the minor modification of using Sephacryl S-300 for gel filtration instead of Sephadex G-200. Homogeneity of the preparation was checked by SDS-PAGE; antibody was raised in rabbits and the specificity of the antiserum was ensured by immuno-electrophoresis as described earlier [27].

Purification of leaf α-mannosidase. Young deveined jackbean leaf (fresh or frozen) was homogenized in 5 vol of 20 mM Tris–HCl, pH 8.0, containing cross-linked PVP (1 gm/160 ml) and the homogenate was filtered through several layers of cheese cloth. An Me_2CO powder was prepared by the addition of chilled (−20°) Me_2CO upto 80% saturation (v/v) in an ice-salt bath and the ppt. was recovered by centrifugation. The air-dried Me_2CO powder was extracted with 40 vol. 20 mM Tris–HCl, pH 7.5, in a blender and the extract was clarified by centrifugation at 16 000 *g* for 30min at 4°. $(NH_4)_2SO_4$ fractionation (30–70%), gel filtration on a Sephacryl S-300 and ion exhange chromatography with DE 52, yielded a preparation having a specific activity of 2 with an overall recovery of 18%.

Immuno-diffusion. In order to eliminate lectin-glycoconjugate interactions, immuno-diffusion studies were carried out in the presence of 0.2 M α-methyl-mannoside in 1.5% agarose according to the procedure of ref. [28].

Immunoprecipitation. Immunoprecipitation of α-mannosidase was carried out by overnight incubation of the enzyme with the antisera at 4° in a total vol. of 100 μl incubation mixture containing 0.1 M K-Pi buffer, pH 7.2, 0.12 M NaCl, 0.3% sodium deoxycholate and 0.6% Triton X 100, followed by 1 hr incubation at 37° after the addition of 100 μl staphylococcal protein-A suspension. It was then centrifuged in a microfuge. The supernatant soln was assayed for the residual enzyme activity.

Western Blot analysis of α-mannosidase. SDS denatured protein samples were resolved on slab SDS-PAGE (12%) in the presence of 2-mercaptoethanol and then electrophoretically transferred to nitro-cellulose paper. Immunological reactions and washings were done as described in ref. [29] with minor modifications in the washing procedure [30]. Radio iodination of protein A was carried out by the chloramine T method [31] to yield a specific activity of 1.5×10^6 cpm/μg.

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