Purification and Properties of α -D-Mannosidase from the Germinated Seeds of *Medicago sativa* (Alfalfa)

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(Received 11 June 1979)

α -Mannosidase of *Medicago sativa* (alfalfa) was purified 1340-fold. The purification method included dialysis of the crude extract against a citrate/phosphate buffer, pH 3.9, $(\text{NH}_4)_2\text{SO}_4$ precipitation, hydroxyapatite chromatography, chromatography on Sephadex G-200 and finally a preparatory electrophoresis on polyacrylamide-gel gradient by Doly & Petek's [(1977) *J. Chromatogr.* 137, 69–81] method. Each step of purification was checked by polyacrylamide-gel disc electrophoresis. The purified enzyme showed a single band, corresponding to α -mannosidase activity. α -Mannosidase has a mol.wt. of 230 000 as estimated by Hedrick & Smith's [(1968) *Arch. Biochem. Biophys.* 126, 155–164] method and also by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn [(1969) *J. Biol. Chem.* 244, 4406–4412]. The enzyme comprises four subunits of different molecular weight. Optimum pH and K_m values were determined with *p*-nitrophenyl α -D-mannoside as substrate. When incubated at a temperature between 20 and 62°C before assay, α -mannosidase initially shows an increase in activity. α -Mannosidase is stable when the pH is about neutrality. It can be inactivated by several metal ions, including Zn^{2+} . At a pH below 5 the enzyme undergoes irreversible inactivation. The presence of EDTA at acid pH considerably enhances the inactivation of the enzyme. This inactivation due to EDTA can be specifically reversed by incubation with Zn^{2+} .

α -Mannosidases are widely distributed in Nature. Purified from various vegetable and animal species, the enzymes can serve as a structural reagent for those glycoproteins having D-mannose as a common constituent. Interest in these enzymes was increased by the discovery that α -mannosidase deficiency in mammals leads to a hereditary disease that can be related to other lysosomal storage diseases such as Hurler's, Tay–Sachs and Sandhoff's diseases. The mannosidosis described in man by Öckerman (1967, 1969a) and also observed in Angus cattle (Öckerman, 1969b; Hocking *et al.*, 1972; Jolly, 1971) is characterized by a visceral accumulation of mannose-rich oligosaccharides and a decreased α -mannosidase activity that has a more neutral pH optimum than normal (Öckerman, 1968; Kjellman *et al.*, 1969; Hultberg, 1970). Marsh & Gourlay (1971) extracted an acid lysosomal activity and a neutral cytoplasmic activity from rat liver. Carroll *et al.* (1972) resolved the α -mannosidase activity of the human liver into three components: two activities, A and B, absent in cases of mannosidosis,

and a neutral activity, C, which remained at a normal value. Dewald & Touster (1973) discovered an intermediate activity in the Golgi apparatus of rat liver. Various lysosomal acid activities, neutral cytoplasmic and intermediate activities were later revealed in the tissues and fluids of mammals. Many of them were isolated and some used as a tool for analytical purposes. The purified enzyme obtained from human liver removed the α -linked non-reducing terminal mannose residue from a trisaccharide isolated from the urine of a patient affected with mannosidosis (Phillips *et al.*, 1976). Hydrolysis by *Canavalia ensiformis* (jack bean) α -mannosidase of eight oligosaccharides isolated in the urine of patients affected with mannosidosis liberated mannose and the disaccharide $\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}$ (Strecker *et al.*, 1976). These oligosaccharides are derived from oligomannosidic-type glycoproteins that are not catabolized in cases of deficiency of the lysosomal acid enzyme (Montreuil, 1975). α -Mannosidases from seeds resemble the acid α -mannosidases of mammals in having their maximum activity at

approx. pH 4–4.5 and in often being Zn^{2+} -dependent. Indeed it seems that the acid α -mannosidases of mammals are frequently activated by Zn^{2+} , whereas the neutral enzymes are more likely to respond to activation by Co^{2+} , Fe^{2+} and Mn^{2+} (Marsh & Gourlay, 1971; Snaith, 1977; Shoup & Touster, 1976). The relation between Zn^{2+} and enzymic activity has been studied in detail for several acid α -mannosidases of mammals (Snaith, 1977; Snaith & Levvy, 1969; Opheim & Touster, 1978), as well as for the enzymes of *Phaseolus vulgaris* (kidney bean) (Paus & Christensen, 1972; Paus, 1977) and *C. ensiformis* (Snaith, 1975).

The present paper describes the purification and the determination of some physical properties of the α -mannosidase of *Medicago sativa* (alfalfa). The results obtained indicate that *M. sativa* contains an α -mannosidase that is active at acid pH. The effect of metal ions, in particular Zn^{2+} , on the activity of the purified enzyme was studied. It seems that the enzymic protein contains a specific Zn^{2+} ion that is necessary for its activity.

Materials and Methods

Materials

Dry seeds of *Medicago sativa* (alfalfa, variety Europe) were supplied by Vilmorin, Paris, France. Hydroxyapatite was prepared by the method of Tiselius *et al.* (1956), as modified by Levin (1962). Sephadex G-200 was obtained from Pharmacia, *p*-nitrophenyl α -D-mannoside from Baker, Deventer, Holland, and Coomassie Brilliant Blue from Sigma, St. Louis, MO, U.S.A. Bovine serum albumin was fraction V from Koch-Light, Colnbrook, Bucks., U.K. α -Phosphorylase *a* and pepsin were obtained from Worthington Biochemical Corp., Freehold 2, NJ, U.S.A., and trypsin from Armour Laboratories, London E.C.1, U.K. The Diaflo PM10 ultra-filtration membranes and ultrafiltration apparatus were produced by Amicon Corporation, Oosterhout, Holland. The salts used to study the action of cations on α -mannosidase came from Prolabo, Paris, France, Merck, Darmstadt, Germany, and Johnson and Matthey, London E.C.1, U.K. EDTA (disodium salt) was from Prolabo.

Assay of enzyme activity

For standard α -mannosidase activity determinations, the incubation mixture contained, in 125 μ l final volume, 8 mM-*p*-nitrophenyl α -D-mannoside, 25 μ l of McIlvaine (1921) buffer and 50 μ l of suitably diluted enzyme. Unless otherwise mentioned, the incubation was performed at 37°C for several minutes and was stopped by addition of 3 ml of 0.2 M- Na_2CO_3 . Liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm by using a Beckman DBG T instrument with a digital dis-

play. Hydrolysis was linear with time during assay. One unit of the enzyme was defined as the amount of enzyme that liberated 1 μ mol of mannose/min at 37°C. The specific activity is expressed as units per mg of protein. The concentrations of enzyme used in presence of cations and EDTA are indicated in the legends to the Tables and Figures.

Protein determinations

Proteins were determined by the Zamenhof (1957) micromethod, with crystalline bovine serum albumin as standard.

Gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed as described by Maizel (1964) on 7% (w/v) polyacrylamide disc gels in 0.025 M-Tris/0.2 M-glycine buffer, pH 8.6. The gels were stained with Coomassie Brilliant Blue (0.25%, w/v) in methanol/acetic acid/water (9:2:9, by vol.) for 2 h, then destained in a solution containing 25% (v/v) methanol and 7% (v/v) acetic acid in water at 40°C for 24 h.

Molecular-weight determinations

The molecular weight of α -mannosidase was determined by the electrophoretic method of Hedrick & Smith (1968) with Tris/glycine buffer, pH 8.6. Acrylamide concentrations used for gels were 5.5, 6.5, 7.5 and 8.5% (w/v). Bovine serum albumin was used as the molecular-weight standard. Gels were stained and destained as described above. The molecular weights of subunits were established by the method of Weber & Osborn (1969) with the following reference proteins: phosphorylase *a* (mol.wt. 94 000), serum albumin monomer (68 000), pepsin (35 000), trypsin (23 000). Electrophoresis was performed on 5.5% (w/v) acrylamide gels for 18 h at 20°C. A 3 mA current was applied on each gel (5 mm diam., 110 mm long). Gels were stained for 5 h in 0.25% Coomassie Brilliant Blue before being destained as described above.

Polyacrylamide gradient electrophoresis

Preparative polyacrylamide gradient electrophoresis was performed as described by Doly & Petek (1977).

Purification of α -mannosidase

Extraction. Dry seeds of *M. sativa* (200 g) were soaked in water for 16 h and germinated on damp filter paper in the dark for 48 h. The crude extract was prepared by grinding in a Waring blender in a solution of 0.9% (w/v) NaCl. The extract was centrifuged for 30 min at 13 000 g and the pellet discarded. The supernatant (fraction I) was dialysed for 24 h against a 0.2 M-sodium citrate/sodium phosphate buffer, pH 3.9. After centrifugation, the proteins

of the supernatant (fraction II) were precipitated by 80%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained was dissolved in 80ml of water, dialysed for 48h against water and centrifuged. The clear supernatant (about 120ml; fraction III) was chromatographed directly on hydroxyapatite.

Column chromatography. The enzyme solution (fraction III) was chromatographed on a column (200mm $\times$ 20mm) of hydroxyapatite previously equilibrated with 5mM-potassium phosphate buffer, pH 6.0. After washing with buffers of increasing molarities (50mM, 100mM, 150mM), the whole of the α -mannosidase activity was eluted with 300mM-buffer. The collected fraction was concentrated on a Diaflo membrane to a volume of 4ml and dialysed for 24h against water (fraction IV). It was then chromatographed on a column of Sephadex G-200 and eluted with 10mM-potassium phosphate buffer, pH 6.0. The active fraction (fraction V) was concentrated as described above.

Preparative electrophoresis. The enzyme preparation was further purified by preparative electrophoresis on acrylamide gradient gels (Doly & Petek, 1977). The acrylamide gradient used was prepared by successive polymerizations of 5ml of 6.5%, 5ml of 7.5% and 22ml of 8.5% acrylamide solution. Each gel (15cm $\times$ 1.6cm) was loaded with 0.15ml of fraction V (400 μ g of protein). The electrophoresis was carried out at 4°C with a constant voltage of 100V for 25h in Tris/glycine buffer, pH 8.6. One of the preparative gels was sliced into discs 1mm thick, on which the α -mannosidase activity was revealed by incubation with *p*-nitrophenyl α -D-mannoside. The portion containing the α -mannosidase activity was then cut out of each of the other gels, eluted as described by Doly & Petek (1977) and dialysed against water (fraction VI).

The successive steps of this purification are summarized in Table 1.

Homogeneity of the purified preparations. The

protein composition of the various fractions was determined during purification by polyacrylamide-gel electrophoresis. Fraction VI always contained a single protein band, showing that at this stage of purification the α -mannosidase preparation is perfectly homogeneous. This homogeneity was also confirmed when the molecular weight was evaluated by Hedrick & Smith's (1968) technique; whatever concentration of acrylamide was used in the gels, only one band was detected.

Results

Stability

The purified enzyme preparations are stable in a neutral or slightly alkaline medium. In phosphate/citrate or acetate buffers at pH values below 5.0, the purified α -mannosidase undergoes gradual and irreversible inactivation. When stored at pH 5.0 or slightly above and at 4°C, the enzyme shows no appreciable variations in activity for several weeks. At the same pH, the enzyme is equally stable in an acetate medium as in a phosphate/citrate medium. Stabilization increases with the dilution of the buffer. Purified solutions obtained from fraction VI have been kept frozen at -20°C after dialysis, then thawed and stored at 4°C.

Molecular weight and subunits

The disc-electrophoretic method of Hedrick & Smith (1968) for estimation of molecular weight gave a value of 220 000. When examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by Weber & Osborn's (1969) method, the enzyme showed four protein bands corresponding to four distinct subunits of mol.wts. 75 000, 60 000, 50 000 and 45 000 (Fig. 1). The total (230 000) agrees with the total obtained by Hedrick & Smith's (1968) method. Figs. 2(a) and 2(b) show photographs of the gels.

Table 1. *Purification of α -mannosidase*

The substrate was *p*-nitrophenyl α -D-mannoside. The incubation mixture contained 50 μ l of diluted enzyme, 50 μ l of substrate and 25 μ l of McIlvaine buffer. The pH of the assay was 4.1. After 3 min the reaction was stopped by adding 3 ml of 0.2 M-sodium carbonate. The liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm.

Fraction	Volume (ml)	Total activity (μ mol of mannose/min)	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
I. Crude extract (200g of seeds)	770	1740	11 000	0.158		
II. After acid dialysis, pH 3.9	750	1292	5450	0.237	75	1.5
III. After 80%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis	66	635	521	1.22	37	7.7
IV. Column 1 (hydroxyapatite)	4	550	20.4	17.25	20	109
V. Column 2 (Sephadex G-200)	1.2	184	3.3	55.5	10	350
VI. Preparative electrophoresis	5.5	51.7	0.240	215	3	1350

Fig. 1. Estimation of the molecular weights of the subunits of α -mannosidase of *Medicago sativa*

α -Mannosidase was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described in the Materials and Methods section. Reference proteins were: phosphorylase *a* (mol.wt. 94 000), serum albumin monomer (68 000), pepsin (35 000) and trypsin (23 000). The arrows correspond to the positions of the subunits.

Kinetic data

Influence of pH. The effect of pH on the activity of α -mannosidase was studied by using 10 mM-*p*-nitrophenyl α -D-mannoside in a 0.1 M-sodium citrate/phosphate buffer ranging from pH 3.0 to 8.0. The pH-activity profile showed a maximum around pH 4.1. With *p*-nitrophenyl α -D-mannoside as a substrate, the enzyme follows Michaelis-Menten kinetics, and the Lineweaver-Burk plot gives a K_m of 2.3 mM.

Thermal activation. The effect of preincubation of the enzyme at several temperatures is demonstrated in Fig. 3. After various preincubation times, portions of the enzyme preparation were added to substrate solutions at 37°C. Examination of the curves showing the relation between residual activity and preincubation time indicates that the activity is at first enhanced if preincubation occurs below 60°C. The thermal activation of the enzyme may be significant; when left at room temperature for 3 days, samples of the enzyme increase their activity by about 40% before undergoing gradual inactivation.

Action of cations. In a neutral medium, α -mannosidase is inactivated by a number of metal ions (e.g. Fe^{2+} , Fe^{3+} , Hg^{2+} , Zn^{2+} , Cu^{2+} , Cd^{2+} , Ag^+ , in increasing order of effectiveness). Fig. 4 shows the gradual inactivation of an enzyme solution (2 $\mu\text{g}/\text{ml}$) at pH 5.0 and 4°C by these various cations (1 mM). Fig. 5 shows the inactivation at 4°C and pH 5.0 of a solution containing 3 μg of α -mannosidase/ml by Zn^{2+} used at various concentrations. After 5 h of incubation at 4°C with 500 μM - Zn^{2+} , the enzyme did

Fig. 2. Polyacrylamide-gel electrophoresis of purified α -mannosidase

(a) On 7.5% (w/v) polyacrylamide gel. (b) Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969). For procedural details, see the Materials and Methods section.

not lose any noticeable activity, whereas, when incubated for same length of time with 2.5 mM- Zn^{2+} , its activity decreased by about 60%. Inactivation by Zn^{2+} and other cations is irreversible and leads to a 100% loss of activity.

Action of EDTA. It was pointed out above that α -mannosidase of *M. sativa* is unstable in acid media, as are other α -mannosidases (Paus & Christensen, 1972; Saita *et al.*, 1971; Snaith & Levvy, 1968, 1969). The presence of EDTA considerably increases the inactivation of the enzyme in acid media. Incubation of purified α -mannosidase with EDTA leads to gradual inactivation, which can be total. This inactivation is temperature-dependent, and is very slow at 4°C; it is a function of the acidity of the medium. When maintained at pH 7, the enzyme is not inactivated. When the enzyme is partially inactivated, neither its K_m nor its

Fig. 3. Effect on activity of preincubation of α -mannosidase at various temperatures

Solutions of purified α -mannosidase were incubated respectively at 40°C (O), 52°C (Δ), 56°C ($\square$), 59°C ($\bullet$), 62°C ($\blacktriangle$), 65°C ($\blacksquare$) in McIlvaine (1921) buffer at pH 5.5. At the time indicated, samples were taken and the activity measured at 37°C and pH 4.1, as indicated in the Materials and Methods section. The activity is expressed as a percentage of the original activity of the enzyme stored at 4°C.

Fig. 4. Gradual inactivation of α -mannosidase by metal ions

Solutions containing 2 μ g of purified enzyme/ml were incubated at pH 5.0 and 4°C with 1 mM-metal ion solutions. The various salts used are specified in the Materials and Methods section. After various incubation times, samples were taken and the activity was measured at 37°C at pH 4.1 with *p*-nitrophenyl α -D-mannoside under the standard conditions previously described (see the Materials and Methods section). Key to symbols: O, Fe^{2+} ; Δ , Fe^{3+} ; $\square$, Hg^{2+} ; $\bullet$, Zn^{2+} ; $\circ$, Cu^{2+} ; $\blacktriangle$, Cd^{2+} ; $\blacksquare$, Ag^{+} . Results for Ca^{2+} , Ba^{2+} , Mn^{2+} , Ni^{2+} and Co^{2+} at 22 h are shown as horizontal bars.

electrophoretic behaviour is modified. It therefore seems improbable that we are dealing with some new form of partially inactivated enzyme. More likely, incomplete attack by EDTA leaves some residual native enzyme with its own characteristic properties. Fig. 6 shows inactivation in phosphate/citrate medium at 20°C [(a) at pH 4.0, (b) at pH 5.0] of α -mannosidase incubated with and without EDTA (disodium salt; 400 μ M) as compared with the control activity of an aqueous solution of the enzyme. One can distinguish, on the one hand, inactivation due to the instability of the enzyme, and, on the other, inactivation due to the chelating properties of EDTA in this medium. In a phosphate/citrate medium at pH 4.0, the enzyme incubated under these conditions totally loses its activity over a period of about 20 h. In a phosphate/citrate medium at pH 5.0, incubation with 400 μ M-EDTA leads only to a loss of 37% of the activity in 22 h, and the control enzyme without EDTA retains 88% of its activity.

Re-activation by Zn^{2+} . By incubation with various cations, we attempted to restore the activity of a solution of α -mannosidase that had been almost totally inactivated by EDTA in an acid medium. Among the cations used (Zn^{2+} , Ca^{2+} , Ba^{2+} , Mn^{2+} ,

Ni^{2+} , Fe^{2+} , Fe^{3+} , Cd^{2+} , Cu^{2+} , Co^{2+} , Ag^{+} , Al^{3+} , Hg^{2+} , Be^{2+}), only Zn^{2+} had a re-activating effect. After incubation with 500 μ M- Zn^{2+} for 20 min at 20°C, α -mannosidase that had previously lost 83% of activity through the action of 400 μ M-EDTA for 72 h at pH 5.0 and 20°C, regained 95% of the activity of the corresponding control enzyme (Fig. 6). This re-activation is Zn^{2+} -specific.

The experiment described in Fig. 7 shows the specific re-activation of α -mannosidase by Zn^{2+} used at various concentrations. An enzyme solution was inactivated by 400 μ M-EDTA for 48 h in a phosphate/citrate medium at pH 4.6 and 20°C, then dialysed against water for 48 h at 4°C, with the result that the residual activity was 7.7%. A control solution was treated the same way, but without EDTA. The residual activity was 55%. Zn^{2+} was added at various concentrations, for 1 h at 20°C, to portions of the EDTA-inactivated enzyme. Under the conditions of the experiment, the addition of Zn^{2+} led to the restoration of 94% of the corresponding activity of the control enzyme. On the other hand, the activity of the control enzyme was not affected by incubation with 40 μ M- Zn^{2+} under the same conditions. Higher concentrations of Zn^{2+} led to some inactivation of the control enzyme as

Fig. 5. Inactivation of α -mannosidase by Zn^{2+} as a function of time and concentration

Solutions containing $3 \mu\text{g}$ of enzyme/ml were incubated at 4°C with ZnSO_4 at concentrations of 0.5 mM (O), 1 mM (Δ) or 2.5 mM ($\square$), for various lengths of time. Samples were taken and activity was measured as described in the text.

Fig. 6. Inactivation of α -mannosidase by preincubation with EDTA

Solutions containing $2 \mu\text{g}$ of purified enzyme/ml were incubated at 20°C with (Δ) and without (O) $400 \mu\text{M}$ -EDTA (a) in McIlvaine (1921) buffer, pH 4.0, (b) in McIlvaine buffer, pH 5.0, for various lengths of time. Samples were taken and activity was measured at 37°C in McIlvaine buffer, pH 4.1, with *p*-nitrophenyl α -D-mannoside as substrate, as indicated in the Materials and Methods section. The activities were compared with those corresponding to an aqueous solution of the enzyme in the same dilution kept at the same temperature for equal lengths of time. Re-activation ($\blacktriangle$) by adding 1 mM - Zn^{2+} for 20 min to a sample inactivated 83% by EDTA is indicated (in b) by the broken arrow.

well as the EDTA-treated enzyme. Both the above types of experiments show that inactivation by EDTA is due to the splitting off of a specific metal

Fig. 7. Re-activation by Zn^{2+} of EDTA-inactivated enzyme

A solution ($5 \mu\text{g}/\text{ml}$) of purified enzyme was pre-incubated with 0.4 mM -EDTA at 20°C in McIlvaine buffer, pH 4.6, until the residual activity was 7.7%, then dialysed against water during 48 h at 4°C . A reference enzyme solution was similarly treated but without EDTA, and retained 55% of the activity of the original untreated enzyme. Portions of EDTA-inactivated enzyme were incubated for 1 h at 20°C with various concentrations of ZnSO_4 . Dilution during this incubation was 1:4. Samples were then withdrawn and the activities measured at pH 4.1 with *p*-nitrophenyl α -D-mannoside as substrate and under standard conditions (see the Materials and Methods section). Restored activities are expressed in relation to the activity of the reference enzyme.

ion (Zn^{2+}). These results suggest that α -mannosidase of *M. sativa* is a metalloenzyme containing zinc.

Discussion

The molecular weight of α -mannosidase of *M. sativa*, estimated at 220 000 by the electrophoretic method of Hedrick & Smith (1968), is within the range of variation of the molecular weights of many α -mannosidases of seeds. This value is in reasonable agreement with that calculated from the sum of the molecular weights of the subunits (230 000) as demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The subunit composition has been frequently described for α -mannosidases of various origins. In seeds, the *C. ensiformis* enzyme is a tetramer made up of two 66 000-mol.wt. subunits and two 44 000-mol.wt. subunits (Shepherd & Montgomery, 1976). *P. vulgaris* enzymes are dimers composed of two 110 000-mol.wt. subunits (Paus, 1977). In mammals, the existence of subunits has also often been observed. The acid enzyme of pig kidney is a dimer made up of two 42 000-mol.wt. subunits (Okumura & Yamashina, 1973), the A

acid form from the human kidney is a tetramer containing two 58000-mol.wt. subunits and two others with a mol.wt. of 30000 (Marinković & Marinković, 1976); the neutral enzyme of rat liver is a tetramer each of whose subunits has a mol.wt. of 110000 (Shoup & Touster, 1976). The α -mannosidase of *M. sativa* is unusual in comprising four subunits of different molecular weights. A similar example was noted by Opheim & Touster (1978). They pointed out that electrophoresis of the acid enzyme of rat liver in the presence of sodium dodecyl sulphate gives three bands corresponding to mol.wts. of 80000, 55000 and 40000.

The K_m value 2.3 mM measured from the Lineweaver-Burk plot with *p*-nitrophenyl α -D-mannoside at optimum pH is of same order of magnitude as for other α -mannosidases of seeds with this substrate, and the optimum pH agrees well with those of other seed enzymes.

The effect of temperature, i.e. the temporary enhancement of activity by preincubation at high temperatures, has not been reported for other glycosidases.

The study of inactivation of α -mannosidase and of its reversion leads to the conclusion that α -mannosidase is a zinc-containing protein; although the α -mannosidase is not stable at acid pH, as observed for other α -mannosidases of various origins (Snaith & Levvy, 1968, 1969; Saita *et al.*, 1971; Paus & Christensen, 1972) the presence of EDTA (at acid pH) considerably enhances the inactivation of the enzyme, and the only cation able to restore α -mannosidase activity after inactivation by EDTA is Zn^{2+} . The fact that α -mannosidase of *M. sativa* cannot be restored to the same activity as the original enzyme after treatment with EDTA in an acid medium is due to the instability of the enzyme in such a medium. This effect becomes more apparent as the EDTA incubation time increases. The effect of the acidity of the medium is the same in the phosphate/citrate medium as in the acetate medium and is greater in both cases with concentrated buffer. It is, however, significant that when the EDTA-inactivated enzyme is exposed to the action of Zn^{2+} , it recovers about 95% of the activity of the control enzyme. Although it was thus established that α -mannosidase requires Zn^{2+} for activity, the enzyme is susceptible to gradual inactivation at pH 5 by several metal ions, including Zn^{2+} when used at high concentrations. For the Zn^{2+} -re-activation experiment illustrated in Fig. 6, which includes a rather long dialysis, consideration should be given to the probable effect, during the dialysis, of residual toxic cations on the EDTA-inactivated enzyme as well as the control enzyme. This would explain the relatively low activity in relation to that of the original enzyme (55%). The attacks by EDTA and by the toxic

ions probably take place at different sites on the molecule.

The role played by Zn^{2+} in the enzymic reaction has not yet been elucidated. The zinc atoms may be involved in subunit interactions, but the rapid re-activation and the similarity of the K_m and the electrophoretic behaviour of both native and EDTA-inactivated enzymes suggests that the removal of Zn^{2+} does not bring about important changes in the conformation of the molecule. Zn^{2+} could act as a ligand for the substrate, although the enzyme can be inactivated by EDTA even in the presence of *p*-nitrophenyl α -D-mannoside. It must be noted that, in contrast with what has been observed with several α -mannosidases (Saita *et al.*, 1971; Snaith & Levvy, 1968, 1969), Zn^{2+} does not prevent the loss of enzymic activity due to maintenance of the enzyme at acid pH. Zn^{2+} does not activate the native enzyme, as observed for the α -mannosidase of the vetch *Vicia sativa* (Petek & Villarroja, 1968). Nor does Zn^{2+} reverse inactivation by Cu^{2+} , as it does in the case of the *C. ensiformis* enzyme (Snaith, 1975) and of the rat epididymis enzyme (Snaith & Levvy, 1969).

That Zn^{2+} is necessary for the activity of acidic α -mannosidase has frequently been reported, whereas the neutral activity is often related to the presence of other ions such as Co^{2+} , Mn^{2+} or Fe^{2+} (Marsh & Gourlay, 1971; Shoup & Touster, 1976; Snaith, 1977). In mammals the restoration of activity of EDTA-treated enzymes by Zn^{2+} has been observed for the lysosomal α -mannosidases of rat liver (Opheim & Touster, 1978) and of rat epididymis (Snaith, 1975). A similar method has demonstrated the presence of zinc in several α -mannosidases of seeds. Detailed studies concern some purified enzymes such as the two isoenzymes of *P. vulgaris* (Paus & Christensen, 1972; Paus, 1977) and the *C. ensiformis* enzyme (Snaith, 1975). The zinc content of these molecules has been measured and a value of two atoms per molecule has been determined for each of the isoenzymes just mentioned. For the α -mannosidase of *M. sativa*, further experiments are necessary to increase our knowledge on the structure of the enzyme, its zinc content, and the role of this metal in the enzymic reaction.

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