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Characterization of Jack-Bean α-D-Mannosidase as a Zinc Metalloenzyme

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1. Two methods were used to obtain α-mannosidase free from unbound Zn^{2+}, (*a*) by removal of excess of metal ion from preparations purified in the presence of Zn^{2+} and (*b*) by purification under conditions that eliminate the need to add Zn^{2+}. 2. The purified enzyme is homogeneous on ultracentrifugation, polyacrylamide-gel electrophoresis and gel chromatography. 3. The molecular weight is estimated to be 230000. 4. The enzyme contains between 470 and 565 μg of zinc/g of protein, corresponding to between 1.7 and 2 atoms of zinc/enzyme molecule. The contents of other metals are much lower. 5. The enzyme is inactivated by chelating agents and activity is restored by Zn^{2+}. 6. No other metal ion was found to replace Zn^{2+} with retention of activity. Some bivalent metal ions, e.g. Cu^{2+}, rapidly inactivate the enzyme. 7. The results indicate that jack-bean α-mannosidase exists naturally as a zinc–protein complex and may be considered as a metalloenzyme.

Zn^{2+} ion has been found to maintain the activity of preparations of α-mannosidase (EC 3.2.1.24) from many plant and animal tissues (see, e.g. Snaith & Levvy, 1968*a*; Petek & Villarroya, 1968; Okumura & Yamashina, 1970; Saita *et al.*, 1971; Paus & Christensen, 1972; Sukeno *et al.*, 1972; Suzuki & Kushida, 1973; Avila & Convit, 1973). In many cases the action of Zn^{2+} has not been studied in great detail, but it was always the only cation that prevented loss of enzymic activity, and, where investigated, the only cation to restore activity to enzyme preparations inactivated by chelating agents.

The tissues in which α-mannosidase is found in highest activity are all tissues rich in zinc, and the relationship between Zn^{2+} and enzymic activity has been studied for the enzyme from three different sources (Snaith & Levvy, 1968*b*, 1969; Snaith *et al.*, 1970). In each instance the enzyme was active at a slightly acid pH value, but in this pH region preparations gradually lost activity unless a Zn^{2+} salt was added to maintain stability. Instability was particularly marked during attempts to purify the enzyme but was overcome by the addition of Zn^{2+}. For this reason it was not certain that α-mannosidase occurred naturally as a zinc–protein.

Metal-dependent enzymes have been broadly defined as either metalloenzymes or metal–enzyme complexes on the basis of their apparent stability constants (Vallee, 1955; Malmström & Rosenberg, 1959; Vallee & Coleman, 1964; Vallee & Wacker, 1970). In metalloenzymes the metal was said to be so firmly bound that it was not removed from the protein during the isolation procedure and the purified protein contained a stoicheiometric quantity of metal. On the other hand, in metal–enzyme complexes the metal was more loosely bound and metal ion had to be added to the protein after isolation to restore enzymic activity. It was suggested that the enzymes might not exhibit specific affinity for a particular metal ion, thus making it difficult to identify the biologically significant metal. Also the degree of metal binding might be a function of metal concentration, leading to less-rigid chemical stoicheiometry. In practice there is no clear distinction between the two groups and the second group has become to some extent confused with metal-ion activation, where enzyme activity is a function of the concentration of free metal ions in solution (Malmström & Rosenberg, 1959) and there is a residual, low activity even in the absence of the activator.

From these definitions it was not possible to classify α-mannosidase as either a metalloenzyme or a metal–enzyme complex. The need to add Zn^{2+} to stabilize the enzyme during purification suggested that it might perhaps be a metal–enzyme complex, but in all other respects α-mannosidase resembled a metalloenzyme. The purpose of the work described in this paper was to clarify the position and to establish whether α-mannosidase exists in nature as a metalloprotein.

Although it is inactive at pH values above neutrality (Snaith & Levvy, 1968*b*) jack-bean α-mannosidase is remarkably stable between pH 7 and 10. By maintaining the preparation almost continuously at pH 8, a method of purification was developed from the original procedure (Snaith & Levvy, 1968*b*) which enables the enzyme to be isolated without the need to add Zn^{2+} to prevent loss of activity at any stage. It

was also possible to remove all free Zn^{2+} from enzyme preparations made by the original procedure. The metal-ion requirements of α-mannosidase, purified by both methods, were studied and its metal–protein composition was determined.

Materials and Methods

α-Mannosidase assay

The hydrolysis of 6mM-*p*-nitrophenyl α-D-mannoside in 0.125M-acetic acid–NaOH buffer, pH5.0, at 37°C was measured as described by Levvy & Conchie (1966); 0.1mM-$ZnSO_4$ and 0.01% bovine serum albumin were included in the assay mixture, except where otherwise stated. One unit of α-mannosidase activity liberates 1 μmol of nitrophenol in 1min under these conditions.

Protein determination

The method of Lowry *et al.* (1951) was used with bovine serum albumin as standard.

Metal analysis

A Techtron atomic absorption spectrophotometer model AA 1000 (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.) was used. Samples for analysis were prepared by either (*a*) dry ashing or (*b*) wet ashing. Some purified enzyme preparations were also analysed without ashing (*c*). (*a*) Samples in silica crucibles were dried under i.r. lamps, heated gently over electric burners until charred and ashed overnight in a muffle furnace at 450°C. If a white ash was not obtained, a few drops of conc. HNO_3 (BDH Chemicals Ltd., Poole, Dorset, U.K.; Aristar grade) were added, evaporated under the i.r. lamps and the crucibles were returned to the muffle furnace for a further 2h. If necessary this treatment was repeated. The ash was dissolved in 2.5% HCl (BDH Chemicals Ltd.; Aristar grade). Standard metal solutions were also prepared in 2.5% HCl. (*b*) Samples were digested with HNO_3 (4ml), $HClO_4$ (BDH Chemicals Ltd.; 'low lead'; 1ml) and H_2SO_4 (BDH Chemicals Ltd., 'low lead'; 0.25ml). Digests were boiled to remove all remaining $HClO_4$ and HNO_3 and diluted to 10ml with water. Standard metal solutions were prepared in 2.5% H_2SO_4. (*c*) HCl was added to the samples to give a concentration of 2.5%. The protein concentration of the unashed enzyme solution results in an enhanced reading for metal concentration, but correction was made for this effect by the addition of a corresponding amount of albumin to the metal standards. Analyses were done in duplicate or triplicate and appropriate controls were done for the metal content of all reagents used. Throughout this work stringent precautions were taken to avoid contamination. To avoid loss of zinc, care was needed to prevent overheating during charring (*a*) and when $HClO_4$ and HNO_3 were boiled off (*b*). For the purified enzyme more reproducible results were obtained when samples were analysed without ashing, but this method was not suitable at earlier stages of purification.

Ultracentrifugation

Sedimentation-velocity studies were performed at 20°C in a Spinco model E analytical ultracentrifuge, with a schlieren optical system. An An-D rotor was used and a 12mm cell with double-sector epoxy centrepiece and quartz windows. Rotor speeds were 59780 and 52640 rev./min, and the protein concentration of samples was 1g/100ml. Photographs were taken at 4- and 8-min intervals.

High-speed sedimentation-equilibrium experiments were performed at 20°C by the method of Yphantis (1964), with a Rayleigh interference optical system. An An-J rotor was used and a 12mm, six-channel epoxy-centrepiece cell with sapphire windows. Rotor speeds were 12590 and 13410 rev./min and protein concentrations were between 0.01 and 0.05g/100ml.

Before centrifugation purified α-mannosidase preparations were dialysed against 0.15M-NaCl, adjusted to pH8 with NaOH at 4°C for 24h.

Amino acid analysis

A Technicon amino acid autoanalyser was used. Hydrolysis was for 18h at 137°C in 6M-HCl.

Electrophoresis

The homogeneity of the enzyme protein was examined by disc electrophoresis on polyacrylamide gel, with gel concentrations from 3 to 10% (w/v) acrylamide and acrylamide/methylenebisacrylamide ratios of 20:1 and 30:1 (w/w). Buffers at pH7 (0.01M-sodium phosphate) and pH9 (0.05mM-Tris–HCl) were used. The molecular weight of α-mannosidase was estimated by the method of Hedrick & Smith (1968) by using gel concentrations of 4–8% and the Tris–HCl, Tris–glycine buffer system at pH9 described by Smith (1968). Mobilities were expressed relative to Bromophenol Blue.

Protein bands were stained with either Naphthalene Black (0.1% in 7%, v/v acetic acid) or Coomassie Brilliant Blue R (0.25% in methanol–acetic acid–water; 5:1:5, by vol.). α-Mannosidase activity was detected by soaking gels in 6mM-*p*-nitrophenol α-mannoside in 0.1M-acetic acid–NaOH buffer, pH5.0, for 30min, then in 0.4M-glycine–NaOH buffer, pH10.5, until the yellow colour developed.

Gel chromatography

The molecular weight of α-mannosidase was estimated by gel chromatography on Sephadex G-200 by the method described by Andrews (1965). The column (26mm × 600mm) of gel was equilibrated

with 0.05M-acetic acid–NaOH buffer, pH5.0, containing 0.1M-NaCl and 0.1mM-$ZnSO_4$. Blue Dextran [Pharmacia (G.B.) Ltd., London W.5, U.K.] was used to determine the void volume of the column and was run alternately with the protein standards so that correction for slight changes in the column volume could be made. The sample volume was 1ml (about 5mg of protein) and 2ml fractions were collected. Protein peaks were located by extinction at 280nm and by protein assay.

Protein standards

The following standards were used for molecular-weight estimations: bovine serum albumin (68000), ovalbumin (45000), γ-globulin (160000), cytochrome *c* (12400), aldolase (160000), catalase (230000) and xanthine oxidase (290000).

Results

Enzyme purification

Table 1 gives typical results for the purification of α-mannosidase from jack-bean meal [supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] by two different methods.

Method 1. The enzyme was purified through stages 1–6 of the procedure described by Snaith & Levvy (1968*b*). The product (2–3ml; 15–20mg of protein) was dialysed against 0.01M-glycine–NaOH buffer, pH8.0 (5×1 litre) over 3 days at 4°C. Any insoluble material was removed by centrifuging at 75000*g* for 15min, and the enzyme solution was passed through a column (26mm×600mm) of Sephadex G-100 equilibrated with the pH8 buffer solution. The active fractions were pooled.

This material could be used without concentration for metal analysis and to study the catalytic properties of the enzyme. The solution could be concentrated for ultracentrifugation studies or for storage by precipitation with acetone at 60% (v/v). α-Mannosidase is not precipitated with acetone at pH8 and it was necessary to lower the pH to 5 by the addition of 1M-acetic acid–NaOH buffer, pH5.0, to give 0.1M-acetate before addition of acetone. After settling for 30min at 4°C the precipitate was collected by centrifuging at 25000*g* for 15min. It was taken up in the minimum volume (2–3ml) of 0.1M-glycine–NaOH buffer, pH8. The recovery of enzyme activity on precipitation with acetone in the absence of excess of Zn^{2+} was rather variable, but was usually between 85 and 95%.

Method 2. This method is a modification of Method 1. The enzyme preparation was maintained at pH8 throughout the purification procedure, except for stage 5 and the acetone precipitation at the end of stage 6. Comparison of the various stages indicated that losses in activity were not appreciably greater under these conditions. Dialysis and gel chromatography could be successfully carried out without the addition of Zn^{2+} to the solutions. Details of the purification are as follows and typical results are given in Table 1.

α-Mannosidase was extracted from jack-bean meal with water and fractionated with $(NH_4)_2SO_4$ as in Method 1. The fraction separating between 45 and 60% (w/v) saturation was dissolved in 0.01M-glycine–NaOH buffer, pH8.0, and dialysed against this buffer solution (4×1 litre) over 2 days at 4°C. The buffer concentration was increased to 0.1M-glycine to ensure that all the enzyme remained in solution and dialysis was continued for 17h. Inactive material that sedimented in the sac was removed by centrifugation at 75000*g* for 20min.

In Method 1 other glycosidases present in α-mannosidase preparations were conveniently completely inactivated and precipitated by incubation in 2M-pyridine solution for 20min at 37°C (stage 5). At pH8 this procedure led to severe losses of α-mannosidase activity. However, if the pH of the dialysed solution was lowered rapidly to 5 by the addition of 1M-acetic acid–NaOH buffer, pH5.0, to give 0.1M-acetate before addition of the pyridine (0.2vol.), the treatment could be done with only a slightly greatly (10%) loss in α-mannosidase activity than occurred in Method 1. (No detectable fall in α-mannosidase activity would be expected on incubation at pH5 for only 20min at 37°C at the high protein concentration of the dialysed preparation whether Zn^{2+} was added or not.) The pyridine-treated

Table 1. *Purification of α-mannosidase from jack-bean meal*

For details see the text.

Purification stage	Recovery of α-mannosidase (units/g of meal)	Specific activity of α-mannosidase (units/mg of protein)
1. Meal	89.9	0.36
Method 1		
2. Aq. extract	71.9	0.54
3. 45–60%-satd. $(NH_4)_2SO_4$	63.1	2.88
4. Dialysis, pH5+Zn^{2+}	62.4	4.28
5. Pyridine treatment	54.8	24.5
6. Gel chromatography, pH5+Zn^{2+}	42.6	49.8
7. Dialysis, pH8	38.0	41.5
8. Gel chromatography, pH8	34.1	45.0
Method 2		
2. Aq. extract	77.3	0.54
3. 45–60%-satd. $(NH_4)_2SO_4$	71.6	3.00
4. Dialysis, pH8	66.2	3.41
5. Pyridine treatment	48.0	25.7
6. Gel chromatography, pH8	44.1	41.5

preparation was cooled in ice and insoluble material removed by centrifugation at 25000*g* for 15min. α-Mannosidase was precipitated from the supernatant with acetone at 60% (v/v). The precipitate was left to settle for 30min at 4°C and collected by centrifuging at 25000*g* for 15min. The enzyme was extracted from other insoluble material with 0.01M-glycine–NaOH buffer, pH8.0, and dialysed against several changes of the same buffer at 4°C.

The dialysed enzyme solution (15–20mg of protein) was passed through a column (26mm×600mm) of Sephadex G-100 equilibrated with the same buffer solution at 4°C. The active fractions were pooled, and if concentration of the protein was required this was done by precipitation with acetone at pH5 as described above for Method 1, stage 8.

The final specific activity of α-mannosidase was similar to that obtained by Method 1, and the products from the two purification procedures were indistinguishable. α-Mannosidase prepared by both of these methods has been used to study the properties of the enzyme.

In general the specific activities of preparations made by Methods 1 and 2 were only about 75% of the maximum previously achieved at stage 6 of Method 1 (Snaith & Levvy, 1968*b*). The value for the final specific activity in that paper is 53 when expressed in the units used in the present paper, although higher values were occasionally obtained. There was usually a small fall in activity when excess of Zn^{2+} was removed from pH5 preparations. It would appear that in a medium containing no free Zn^{2+}, it was possible for some of the active zinc to be displaced from the enzyme protein by toxic cations present in the preparations and for some dissociation of the complex to occur.

The effect of including $ZnSO_4$ in the assay medium for the purified α-mannosidase was to increase the activity by about 10%. This was probably due to some dissociation of the enzyme complex occurring under the conditions of assay. In preparations containing excess of zinc, either endogenous or exogenous, the activation effect obtained was considerably smaller, usually only 2 or 3% (Snaith & Levvy, 1968*b*).

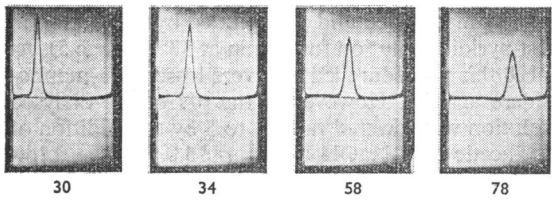

Fig. 1. *Sedimentation-velocity patterns for α-mannosidase at different times after reaching speed*

The photographs were taken at a phase-plate angle of 60°. The protein concentration was 1g/100ml in 0.15M-NaCl, pH8.0. The direction of sedimentation is from left to right.

Properties of purified α-mannosidase

Homogeneity. A single protein peak corresponding to enzymic activity was obtained when preparations of the purified enzyme were passed a second time through Sephadex G-100 and also through G-200. Two tests were used to check the homogeneity of the preparations. (1) Only one protein band could be detected when preparations were subjected to electrophoresis on polyacrylamide gel under different conditions of pH, gel concentration and with different ratios of acrylamide/methylene bisacrylamide. Gels could be stained to show a band of α-mannosidase activity. If the position of the band was marked with a piece of fine wire and the gels were subsequently stained for protein, the protein band coincided with that for α-mannosidase. (2) Sedimentation-velocity studies on the ultracentrifuge showed that 93% or

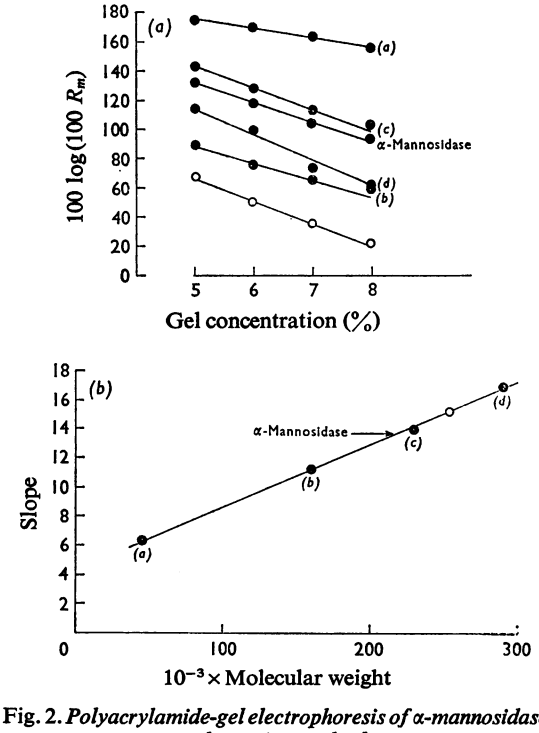

Fig. 2. *Polyacrylamide-gel electrophoresis of α-mannosidase and protein standards*

(*a*) Plots of relative mobility (R_m) against gel concentration. (*b*) Plot of negative slope against molecular weight. The gels were prepared with an acrylamide/methylene bisacrylamide ratio of 30:1 (w/w). For further details see the text. (*a*) ovalbumin, (*b*) aldolase, (*c*) catalase, (*d*) xanthine oxidase; ○, results for rat preputial-gland β-glucuronidase.

more of the purified α-mannosidase preparations sedimented as one peak with traces of both lower- and higher-molecular-weight material. The sedimentation coefficient ($s_{20,w}$) was 9.3S. Fig. 1 shows a series of photographs obtained during a sedimentation-velocity experiment. The very small peaks caused by the contaminants can just be discerned in the first frame. In this experiment the main component was 95% of the total protein.

Molecular-weight determination. This has been done by three distinct methods: by ultracentrifugation, by electrophoresis and by gel chromatography.

Two samples of purified α-mannosidase were subjected to high-speed sedimentation-equilibrium studies. Molecular weights were determined by using a value for the partial specific volume of the protein ($\bar{v}$) of 0.726. This value was calculated from the amino acid composition by the method of Cohn & Edsall (1943). Molecular weights were obtained as follows:

Sample 1 (Method 1). $\bar{M}_w$ 230000, $\bar{M}_z$ 233000 (main component 95%)
Sample 2 (Method 2). $\bar{M}_w$ 233000, $\bar{M}_z$ 242000 (main component 93%)

For each sample three different protein concentrations were used and plots of logarithm (fringe displacement) against the distance from the axis of rotation were linear. This is another indication of the homogeneity of the samples.

Fig. 2 shows the results obtained for the measurement of the molecular weight of α-mannosidase by polyacrylamide-gel electrophoresis. Similar results were obtained when the ratio of acrylamide/methylene bisacrylamide was 20:1 (w/w). The molecular weight was found to be between 220000 and 245000.

The third method used to estimate the molecular weight of α-mannosidase was gel chromatography on Sephadex G-200. The column was calibrated with cytochrome *c*, bovine serum albumin, γ-globulin, aldolase, catalase, xanthine oxidase and urease. A plot of elution volume against log(mol.wt.) was made, and the molecular weight of α-mannosidase estimated from the graph was between 240000 and 260000. Similar results were obtained when the column was equilibrated with 0.01M-glycine–NaOH buffer, pH 8.0. This value is rather higher than those obtained by ultracentrifugation and electrophoresis. From the results of the three experiments, the molecular weight of α-mannosidase was considered to be about 230000.

Effects of Zn^{2+}, chelating agents and toxic metal ions. Table 2 shows the effect of incubating the purified enzyme at 37°C, at pH 8 and at pH 5. The enzyme is completely stable at pH 8 and neither Zn^{2+} nor EDTA have any effect. When incubation was at pH 5 the enzyme preparation lost activity, but this could be prevented by the addition of Zn^{2+}. EDTA increased the rate of inactivation at this pH and the only cation found to restore α-mannosidase activity after EDTA inactivation was Zn^{2+}. Re-activation was instantaneous and was not prevented by the presence of the substrate (see Snaith & Levvy, 1968*b*). α-Mannosidase could also be reversibly inactivated by other chelating agents such as nitrilotriacetic acid and diethylene triaminepenta-acetic acid at pH 5. In each case the effects were overcome by the addition of an excess of Zn^{2+}.

Table 2. *Effects of Zn^{2+}, EDTA and Cu^{2+} on the stability of α-mannosidase*

A purified enzyme preparation (Method 1) was incubated for 3h at 37°C in 0.01M-sodium phosphate buffer, pH 8, or in 0.1M-acetic acid–NaOH buffer, pH 5, containing 0.01% albumin (α-mannosidase activity about 0.12 unit/ml). Similar solutions were incubated in the presence of 1mM-$ZnSO_4$, 1mM-EDTA, 5μM-$CuSO_4$, or 5μM-$CuSO_4$+1mM-$ZnSO_4$. After incubation the solutions were diluted 1:5 for assay and 0.5ml was included in an assay volume of 4ml. The results are expressed as percentages of the initial activity of the enzyme preparation.

	Residual activity (%)	
	Assay with Zn^{2+}	Assay without Zn^{2+}
Initial activity	100	96
Incubation at pH 8		
Addition:		
None	129	—
1mM-$ZnSO_4$	129	—
1mM-EDTA	125	120
5μM-$CuSO_4$	5	—
5μM-$CuSO_4$+1mM-$ZnSO_4$	91	—
Incubation at pH 5		
Addition:		
None	66	63
1mM-$ZnSO_4$	102	—
1mM-EDTA	100	37

Although stable to incubation at pH 8 and resistant to the effect of EDTA, α-mannosidase was still very susceptible to inactivation by certain bivalent metal ions at this pH, for example Cu^{2+} and Cd^{2+}. Table 2 shows that as little as 5μM-$CuSO_4$ could almost completely inactivate the enzyme. Inactivation was largely prevented by a considerable excess of $ZnSO_4$. Zn^{2+} could also reverse Cu^{2+} inactivation of α-mannosidase, as shown in Fig. 3. The rate of inactivation of α-mannosidase by toxic cations was considerably faster at pH 8 than it was at pH 5, 10μM-$CuSO_4$ causing about 80% inactivation in 2h at 37°C and pH 5 compared with over 95% at pH 8 (Fig. 3). This was also true of re-activation by Zn^{2+} (Fig. 3). The increase in activity which occurred on

incubation of the control enzyme solution at pH8 (Table 2 and Fig. 3) was an effect seen with most preparations and seemed to be due to reversal of inactivation which took place during purification.

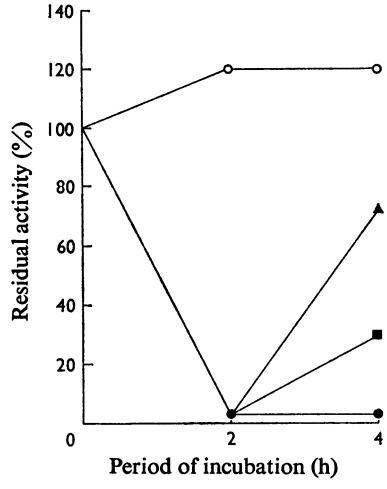

Fig. 3. *Effect of Cu^{2+} and Zn^{2+} on the activity of α-mannosidase*

α-Mannosidase was incubated at 37°C and pH8 (0.01M-sodium phosphate buffer containing 0.01% albumin) in the presence (●) and absence (○) of 10μM-$CuSO_4$. After 2h, an equal volume of 2mM-$ZnSO_4$ in buffer was added to samples of the Cu-inactivated enzyme, the pH of one sample was kept at 8 (▲) and the other was lowered to 5 (■). A third sample was diluted with buffer alone (●). Incubation was continued for 2h. The preparations were diluted a further 1:5 before assay at pH5 and 0.5ml was included in an assay volume of 4ml. The results are expressed as percentages of the original activity of the unincubated enzyme preparation.

Where very large rises in activity were obtained these coincided with unexpected losses in activity during the purification procedure and corresponding unusually low values of specific activity.

Many bivalent cations have little or no effect on the activity or stability of purified α-mannosidase. Zn^{2+} was the only one found to stabilize the enzyme and to re-activate it after inactivation either by EDTA or by a toxic cation. The results obtained with other metal ions were similar to those described previously (Snaith & Levvy, 1968*b*). It is noteworthy that Co^{2+} caused slight inactivation of jack-bean α-mannosidase. The enzyme from human blood is activated by this ion (Courtois & Mangeot, 1970), and it has recently been found that there is an α-mannosidase in the liver and testis of the rat which is also strongly activated by Co^{2+} (Snaith, 1973).

Metal content. Table 3 presents metal analysis of ten different preparations of jack-bean α-mannosidase. Analyses were done on material prepared by both Methods 1 and 2. The zinc values were rather variable but the ratio zinc/protein lay in the range 1.66 to 2.00 atoms of zinc/enzyme molecule, based on a molecular weight of 230000 for α-mannosidase. Other metals measured were at a relatively low concentration, corresponding to considerably less than 0.5 atom/enzyme molecule. There seemed to be no consistent difference in the zinc content of preparations made by the two methods and the amounts of contaminating metals were also similar.

Typical results for the metal content and α-mannosidase activity of the various fractions obtained at each stage of purification are given in Table 4. As the specific activity of the preparation increased, so did the zinc content of the protein. There were no obvious trends in the results for other metals expressed per g of protein, but if the metal content was calculated

Table 3. *Activity and metal content of purified α-mannosidase preparations*

For details see the text. N.D., Not detected.

Preparation		Specific activity of α-mannosidase (units/mg of protein)	Metal content (μg/g of protein)							Zinc content [g-atoms/mol (mol.wt. 230000)]
			Zn	Cu	Fe	Mn	Ni	Cd	Ca	
Method 1										
Dry ashed	1.	39.6	540	60	80	10	30	4	—	1.91
	2.	43.2	520	30	40	10	30	<2	—	1.84
	3.	45.6	500	50	30	13	37	9	—	1.77
Not ashed	1.	39.6	550	40	80	—	—	—	—	1.95
	4.	48.0	530	87	<50	—	—	—	—	1.88
	5.	45.0	485	60	<50	—	—	—	—	1.72
	6.	46.8	500	90	<50	—	—	—	—	1.77
Method 2										
Dry ashed	7.	42.0	500	65	90	15	60	—	—	1.77
	8.	45.6	480	90	75	23	<30	5	—	1.70
	9.	55.2	470	20	<50	<10	<10	—	N.D.	1.66
	10.	42.0	565	80	<50	—	—	—	—	2.00

Table 4. *Metal analysis of fractions during purification of jack-bean α-mannosidase*

For details see the text.

Purification stage	Recovery of α-mannosidase (units/g of meal)	Recovery of Zn (μg/g of meal)	Specific activity of α-mannosidase (units/mg of protein)	Metal content (μg/g of protein)						
				Zn	Cu	Fe	Mn	Ni	Cd	Co
1. Meal	89.9	19	0.36	76	32	100	30	20	1	2
2. Aqueous extract	79.1	10	0.55	71	34	83	36	26	1	3
Method 2										
3. 45–60%-satd. $(NH_4)_2SO_4$	77.9	2.6	3.24	106	110	37	36	45	2	1
5. Pyridine treatment	51.6	1.1	16.8	360	90	27	55	60	4	0
6. Gel chromatography	52.8	0.63	42.0	500	65	90	15	60	—	—
Method 1										
8. Gel chromatography, pH8	42.0	0.57	39.6	540	60	80	10	30	4	—

per g of original material at each stage, there was a steady decrease during purification.

Except for some of the purified preparations shown in Table 3, the results in both Tables 3 and 4 were obtained by the dry-ashing procedure. Samples at each stage of purification were also analysed by acid digestion, and the results covered the same range.

Discussion

The difficulties experienced in early attempts to purify α-mannosidase were the result of working at a slightly acid pH, the pH value at which the enzyme is active. These difficulties could be overcome by the addition of Zn^{2+}. Snaith & Levvy (1969) suggested that the use of a higher pH might enable purification to be carried out without the need to add Zn^{2+}. In the present work, by maintaining a pH value above neutrality it has been possible to isolate the enzyme as a zinc–protein complex of defined composition. The activity of the enzyme entirely depends on the presence of zinc, as shown by the effects of chelating agents and toxic metal ions.

The molecular weight of jack-bean α-mannosidase was estimated to be about 230000. This is rather higher than the values reported for α-mannosidases from other sources. The enzyme from *Phaseolus vulgaris* was reported to have a molecular weight of 200000 by polyacrylamide-gel electrophoresis (Paus & Christensen, 1972) and that from soya bean 180000 by gel chromatography on Sephadex G-200 (Saita *et al.*, 1971).

In the course of the experiments with polyacrylamide-gel electrophoresis the molecular weight of β-glucuronidase (EC 3.2.1.31) from rat preputial gland (Snaith & Levvy, 1967) was found to lie between 240000 and 260000. Two protein bands were obtained when the purified preparations were subjected to electrophoresis, but only one, the slower-moving component, showed β-glucuronidase activity with phenolphthalein glucuronide.

All the α-mannosidase preparations analysed had zinc contents lying between 1.7 and 2 atoms/enzyme molecule (mol.wt. 230000) and low contents of other metals examined. An ultimate value of 2 atoms of zinc is postulated, the lower values being due possibly to the introduction of small amounts of various inactive metal ions during the purification procedure. This suggestion is also reflected by the rather low specific activities usually obtained when the enzyme is purified in the absence of excess of Zn^{2+} (Method 2) or freed from excess of Zn^{2+} in the final stages of purification (Method 1). The results obtained for the metal content of the enzyme, prepared by both methods of purification, were in the same range, indicating that there can be no firm binding of additional Zn^{2+} ions during purification of the enzyme in the presence of excess of Zn^{2+}.

The instability of α-mannosidase below neutral pH must be to a large extent due to dissociation of the protein–zinc complex, as displacement of zinc by endogenous toxic metal ions would occur both at pH8 and 5. However, if inactivation was only due to a simple dissociation effect, it would be expected that the addition of Zn^{2+} to the assay medium would cause immediate reactivation in the same way as is seen after inactivation with EDTA (see Table 2). This point remains to be clarified. The stabilizing effect of the substrate under assay conditions has already been discussed (Snaith & Levvy, 1968*b*).

At one time it was considered that the metal could not be removed from a metalloenzyme without irreversible damage to the protein molecule. It is now known that the active cation can be reversibly detached from many metalloenzymes by working at a suitably acid pH or by the use of a chelating agent. Metal chelates are less-readily dissociated at alkaline pH than at acid pH values and most well-characterized metalloenzymes are stable in neutral or slightly alkaline solution. These enzymes are also catalytically active in this pH region. In the case of α-mannosidase, activity is shown at a slightly acid pH and here the

active protein–metal complex readily dissociates. If a distinction must be made between metalloenzymes and metal–enzyme complexes the results obtained indicate that α-mannosidase may best be considered as a zinc metalloenzyme, but it is suggested (Snaith & Levvy, 1973) that it may be more useful to distinguish between metal-containing enzymes and metal-activated enzymes. There can be little doubt that α-mannosidase in jack bean is a metal-containing enzyme and occurs naturally as a zinc–protein. It is noteworthy that no other metal ion has been found to activate the enzyme protein. α-Mannosidase is unusual among metalloenzymes in this respect. Most metalloenzymes can be activated to some extent by several metal ions.

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