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# Purification and Properties of α-D-Mannosidase from Jack-Bean Meal

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1. α-Mannosidase from jack-bean meal was purified 150-fold. β-*N*-Acetylglucosaminidase and β-galactosidase were removed from the preparation by treatment with pyridine. $Zn^{2+}$ was added during the purification to stabilize the α-mannosidase. 2. At pH values below neutrality, α-mannosidase undergoes reversible spontaneous inactivation at a rate dependent on the temperature, the degree of dilution and the extent of purification. The enzyme is also subject to irreversible inactivation, which is prevented by the addition of albumin. 3. Reversible inactivation of α-mannosidase is accelerated by EDTA and reversed or prevented by $Zn^{2+}$. Other cations, such as $Co^{2+}$, $Cd^{2+}$ and $Cu^{2+}$, accelerate inactivation; an excess of $Zn^{2+}$ again exerts a protective action, and so does EDTA in suitable concentration. 4. Neither $Zn^{2+}$ nor EDTA has any marked effect in the assay of untreated enzyme. In an EDTA-treated preparation, however, $Zn^{2+}$ reactivates the enzyme during assay. 5. It is postulated that α-mannosidase is a dissociable $Zn^{2+}$–protein complex in which $Zn^{2+}$ is essential for enzyme activity.

D-Mannose is a common component of glycoproteins. The apparently universal occurrence of α-mannosidase in mammals and higher plants, as opposed to the virtual absence of β-mannosidase, suggested that, in many glycoproteins at least, the mannose residue is α-linked (Conchie, Findlay & Levvy, 1959; Levvy & McAllan, 1962). Early evidence for the α-linkage in ovalbumin glycopeptide was provided by the action of almond emulsin (Fletcher, Marks, Marshall & Neuberger, 1963; see also Clamp & Hough, 1965); emulsin appears to lack β-mannosidase activity (Conchie, Gelman & Levvy, 1968; Baumann & Pigman, 1957). Assignment of the α-configuration to mannose in glycoproteins, in particular ovalbumin, was supported by work with α-mannosidase from jack-bean meal (Li, 1967) and from the mollusc *Charonia lampas* (Muramatsu, 1967).

In early attempts to purify α-mannosidase for use as a structural reagent in the glycoprotein field, we employed rat epididymis, at that time the richest known source of this enzyme. Li (1966), however, reported that jack-bean meal was a richer and more convenient source of α-mannosidase, and examination of this type of preparation is the subject of the present paper. We developed the method described below for preparing α-mannosidase from jack-bean meal; it differs from the procedure of Li (1967) in certain important features.

As with α-mannosidase from other sources, $Zn^{2+}$ was found to be an essential component of the enzyme from jack-bean meal (Snaith & Levvy, 1968*a*,*b*). The study of the behaviour of the enzyme in partially purified and purified preparations towards $Zn^{2+}$ and other metal ions became an essential feature of the purification.

## EXPERIMENTAL

*α-Mannosidase assay.* The hydrolysis of 6mM-*p*-nitrophenyl α-mannoside in 0·125M-acetic acid–NaOH buffer, pH 5·0, at 37° was measured as described by Levvy & Conchie (1966), in the presence of 0·01% bovine albumin. The results are expressed as μg. of *p*-nitrophenol liberated/hr. $Zn^{2+}$ in the substrate, determined by atomic-absorption spectrophotometry, was equivalent to 0·375μM in the assay.

The pH for optimum activity of α-mannosidase from jack-bean meal in 0·125M-acetic acid–NaOH buffer was between 3·5 and 4·5 (cf. Li, 1967). At pH 7 there was little enzyme activity, but at pH 5 at least 90% of the maximum activity was retained. Since the enzyme was reasonably stable at all stages of purification when incubated for 1hr. with substrate at pH 5 in the absence of added $Zn^{2+}$, pH 5 was adopted for routine assay in the present work.

Muramatsu (1967) observed considerable activation of α-mannosidase from *C. lampas* with 0·3M-NaCl in the assay. We have found that this observation extends to the enzyme from the limpet *Patella vulgata* (S. M. Snaith & G. A. Levvy, unpublished work). With the enzyme from jack-bean meal (and from rat epididymis) NaCl caused no increase in activity in the assay, nor did it protect against inactivation of the enzyme beforehand. With jack-bean-meal preparations beyond stage 3 (Table 1) albumin caused up to 10% activation under routine assay conditions.

*β-N-Acetylglucosaminidase assay.* The hydrolysis of 5mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide in 0·05M-citric acid–NaOH buffer, pH 4·4, at 37° was measured in

the presence of 0·1M-NaCl and 0·01% albumin (Levvy & Conchie, 1966). Results are expressed as above. The pH was optimum for the enzyme from jack-bean meal.

*β-Galactosidase assay.* The hydrolysis of 2·5mM-*o*-nitrophenyl β-galactoside in 0·125M-acetic acid–NaOH buffer, pH 3·0, was measured as described by Conchie & Hay (1959). Results are expressed as μg. of *o*-nitrophenol liberated/hr. at 37°.

*Other glycosidase assays.* The hydrolysis of the following substrates was determined as indicated: phenyl α-galactoside (Conchie & Levvy, 1957), phenyl *N*-acetyl-α-glucosaminide (Findlay, Levvy & Marsh, 1958), 5mM-*p*-nitrophenyl α-L-fucoside (as α-mannosidase), 5mM-*p*-nitrophenyl α-glucoside (as α-mannosidase), *o*-nitrophenyl β-glucoside at pH 4·5 (Conchie, Gelman & Levvy, 1967) and phenyl β-mannoside (Levvy, Hay & Conchie, 1964).

*Protein determination.* This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin as standard. At concentrations of 2M or more in the test sample, pyridine interferes in the reaction.

At stage 2 of the purification (Table 1), the N content was 18% of the protein, estimated as albumin, indicating that the protein was slightly less chromogenic than the standard. At stage 6, the N content was 13% on the same basis, suggesting that the purified enzyme was more chromogenic than albumin.

## RESULTS

### *Purification procedure*

*General comments.* To our knowledge, in every material where α-mannosidase is present in high activity as a constitutive enzyme, β-*N*-acetylglucosaminidase and β-galactosidase are also present. Because of their potential action on glycoproteins, the removal of the latter two enzymes was given first priority in the purification procedure. β-*N*-Acetylglucosaminidase presented much greater difficulty than β-galactosidase in this respect.

Results obtained by the final procedure are illustrated in Table 1. During precipitations, water was added to correct for volume changes after mixing of the reagents. A.R. ammonium sulphate was employed. Precipitates were washed on the centrifuge with the appropriate solvent. Except at low speed (1200*g*), centrifuging was done at 0°. From stage 3 onwards, precipitates were dissolved in 0·05M-acetic acid–sodium hydroxide buffer, pH 5·0, containing 0·1M-sodium chloride and 0·1mM-zinc sulphate. This buffer solution was also employed for dialysis and for gel chromatography. There was 150-fold purification of α-mannosidase, with nearly 50% recovery.

*Stage* 1 (*acetone-dried powder*). A 20g. portion of jack-bean meal (Sigma Chemical Co., St Louis, Mo., U.S.A.) was stirred mechanically with 200ml. of ice-cold acetone for 5min. The precipitate was sedimented at 1200*g* for 10min. and dried in an air stream. It was suspended in 100ml. of ice-cold water with an Ultra-Turrax TP 18/2N disintegrator. There was no measurable loss in enzyme activity at this stage.

*Stage* 2 (*aqueous extract*). Insoluble material was sedimented as before and re-extracted twice with water. The combined extract (175ml.) was frozen and thawed, and residual debris was centrifuged off at 75000*g* for 20min.

*Stage* 3 (*ammonium sulphate fractionation*). Solid ammonium sulphate was stirred into the extract (pH 6·3) to give 45, 60 and 80% saturation. Precipitates were allowed to settle for 45min. at 0°, collected at 25000*g* for 15min., and taken up in 10ml. of buffer solution. The fraction (stage 3*b*) that separated between 45 and 60% saturation with ammonium sulphate was used for further purification.

*Stage* 4 (*dialysis*). The clear enzyme solution was dialysed against several changes of the buffer for 2–3 days at 0° in Visking dialysis tubing, and the material that sedimented in the sac was removed by centrifuging at 75000*g* for 20min. This step removed inactive material that would otherwise separate from solution on Sephadex columns.

*Stage* 5 (*treatment with pyridine*). Pyridine (0·2vol.) was added to the dialysed preparation

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

After stage 3, the enzyme was diluted for assay with 0·1mM-$ZnSO_4$. Specific activities are expressed as units/mg. of protein, with bovine albumin as protein standard.

| Stage | Description | Recovery (units/g. of meal) α-Mannosidase | β-*N*-Acetylglucosaminidase | β-Galactosidase | Specific activity of α-mannosidase |
|---|---|---|---|---|---|
| 1 | Acetone-dried powder | 750000 | 72500 | 15000 | 2900 |
| 2 | Aqueous extract | 500000 | 68000 | 15000 | 3200 |
| 3*a* | 0–45%-satd. $(NH_4)_2SO_4$ | 24000 | 1900 | 4800 | 2700 |
| 3*b* | 45–60%-satd. $(NH_4)_2SO_4$ | 455000 | 58000 | 11000 | 24000 |
| 3*c* | 60–80%-satd. $(NH_4)_2SO_4$ | 13000 | 17000 | 92 | 270 |
| 4 | Dialysis | 462000 | 60000 | 11000 | 31000 |
| 5 | Treatment with pyridine | 376000 | 0 | 0 | 250000 |
| 6 | Gel chromatography | 349000 | — | — | 441000 |

(20ml.), and the mixture was incubated at 37° for 20min. After cooling to 0°, it was centrifuged at 25000*g* for 15min. α-Mannosidase in the supernatant was precipitated at 0° with acetone at 60% (v/v) concentration. After settling for 30min. at 0°, the precipitate was collected at 25000*g* for 15min. It was taken up in the buffer solution, and insoluble material was removed at 25000*g* to give a clear colourless solution (10ml.).

*Stage* 6 (*Sephadex G-100*). The preparation was passed through a column of Sephadex G-100 at 4° and the active fractions (fractions 12–19, Fig. 1) were pooled. After precipitation with acetone as above, the enzyme was dissolved in the buffer solution (2–3ml.). There was no change in the α-mannosidase activity after storage for 3 months at 0°.

### *Removal of other glycosidases*

The results in Table 1 show that pyridine treatment freed α-mannosidase from the accompanying β-*N*-acetylglucosaminidase and β-galactosidase in jack-bean meal, and precipitated a great deal of inactive protein. Subsequent passage of the preparation through Sephadex G-100 (Fig. 1) gave a single peak of protein, corresponding to α-mannosidase. A second passage of the active material through Sephadex gave an identical chromatographic pattern for α-mannosidase, and no change in specific activity.

It was found that the effect of pyridine (2M) on β-*N*-acetylglucosaminidase in jack-bean-meal preparations (stage 4, Table 1) was to cause almost instantaneous, irreversible, inactivation, with virtually no fall in α-mannosidase activity. The experiments were so arranged that, after dilution of the preparation for assay, the pyridine concentration fell to a value that was without effect on these enzymes.

Without pyridine treatment, α-mannosidase could be partially separated from β-*N*-acetyl-

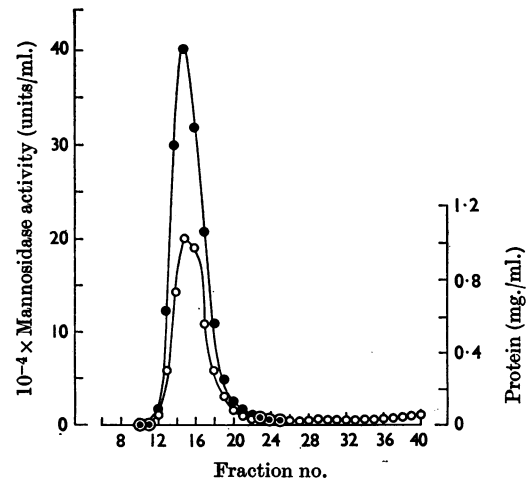


Fig. 1. Elution of α-mannosidase from a column of Sephadex G-100 (60cm. × 3cm.) with 0·05M-acetic acid–NaOH buffer, pH 5·0, containing 0·1M-NaCl and 0·1mM-$ZnSO_4$; 100ml. was run to waste and fractions (5ml.) were then collected. ●, α-Mannosidase activity; ○, protein. The column was loaded with a pyridine-treated preparation (stage 5, Table 1).

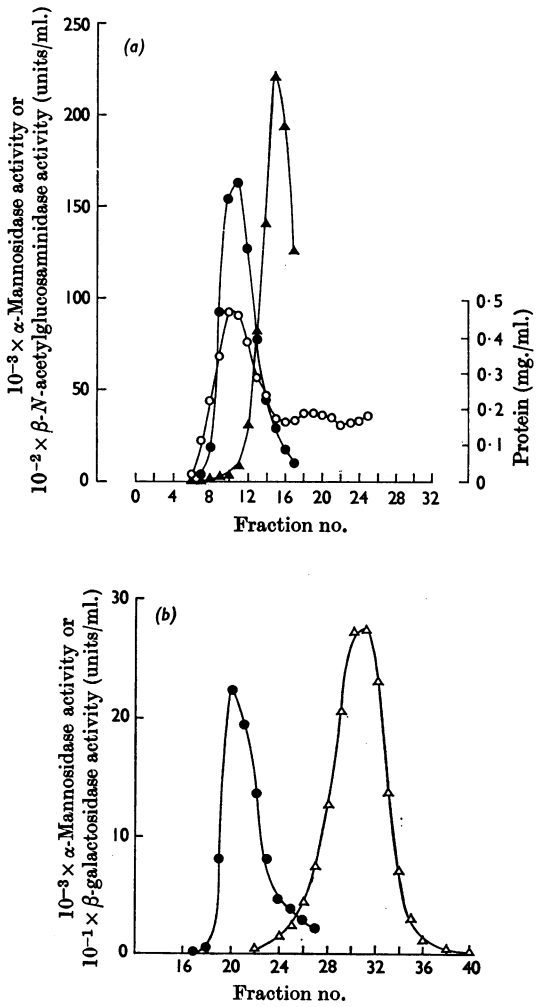


Fig. 2. Elution of jack-bean-meal enzymes from a column of Sephadex G-100 (60cm. × 3cm.) run as in Fig. 1. The preparation (stage 4, Table 1) had not been treated with pyridine. (*a*) ●, α-Mannosidase activity; ▲, β-*N*-acetylglucosaminidase activity; ○, protein. (*b*) ●, α-Mannosidase activity; △, β-galactosidase activity.

glucosaminidase on Sephadex G-100, as illustrated in Fig. 2(*a*), with a considerable increase in specific activity. This Figure closely resembles that shown by Li (1967) for the passage of a jack-bean-meal preparation through Bio-Gel P-200 at pH 6. β-*N*-Acetylglucosaminidase was almost completely removed by repeated passage of the α-mannosidase-rich fractions through Sephadex, but the overall recovery of α-mannosidase was then decreased to less than 20%. $Zn^{2+}$ did not alter the relative positions of the peaks of the two enzymes on Sephadex.

A few attempts were made to separate α-mannosidase from β-*N*-acetylglucosaminidase on DEAE-Sephadex A-50 with 0·05M-acetic acid–sodium hydroxide buffer, pH 6·0, containing 0·1mM-zinc sulphate, or 0·01M-phosphoric acid–sodium hydroxide buffer, pH 7·0. When the buffer concentration was increased tenfold or 25-fold respectively, both enzymes emerged. Use of 0·5M-pyridine–acetic acid buffer, pH 6·0, caused α-mannosidase to emerge alone (cf. Li, 1967). These experiments were discontinued when it was found that pyridine inactivated β-*N*-acetylglucosaminidase. DEAE-Sephadex appeared to offer no advantage as a means of increasing the specific activity of α-mannosidase.

Treatment with pyridine also inactivated β-galactosidase in jack-bean-meal preparations (Table 1), but removal of this enzyme from the untreated preparation on Sephadex G-100 presented no problem (Fig. 2*b*). This is in contrast with the behaviour of a similar preparation on Bio-Gel P-200 (Li, 1967).

The final product (stage 6, Table 1) was free from α-galactosidase, α-*N*-acetylglucosaminidase, α-L-fucosidase, α-glucosidase, β-glucosidase and β-mannosidase activity. None of these was present in more than trace amounts in the preparation at stage 3*b*, apart from α-galactosidase with an activity of 780 phenol units/g. of jack-bean meal.

*Inactivation of α-mannosidase and the action of* $Zn^{2+}$

*Inactivation in partially purified preparations.* At pH 5 in the absence of added $Zn^{2+}$, α-mannosidase activity fell slowly on storage at 0°, and more rapidly on incubation at 37°. This spontaneous inactivation varied in rate from preparation to preparation, but in general it increased with the degree of purification and dilution of the enzyme. It was particularly marked during and after dialysis or gel filtration in the absence of $Zn^{2+}$, often exceeding 50% overnight at 0°.

Addition of $Zn^{2+}$ (0·1–1mM) stabilized the enzyme at pH 5, and activity was slowly restored in decayed preparations at a rate dependent on the $Zn^{2+}$ concentration. The final extent of activation was variable, depending on the previous state of the preparation. $Zn^{2+}$ was the only cation found to have this action. Dialysis was possible at pH 5 without loss in activity when $Zn^{2+}$ was present.

In similar concentrations (0·1–1mM) EDTA hastened inactivation of the enzyme, and when it was in excess it reversed the action of $Zn^{2+}$. Addition of an excess of $Zn^{2+}$ to an EDTA-treated preparation, however, caused immediate reactivation. At lower concentrations (1–10μM) EDTA protected the enzyme against spontaneous inactivation.

These phenomena pointed to the presence in the enzyme preparations of deleterious cations, which could replace $Zn^{2+}$ in an active enzyme complex, but which, like $Zn^{2+}$, were sequestrated by EDTA. Screening experiments with metal ions in 10–100μM concentration showed that the following hastened inactivation: $Cu^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Ag^{+}$, $Fe^{2+}$ and $Fe^{3+}$. The effects, if any, of other metal ions were too small to have unequivocal significance. In 10μM concentration $Cu^{2+}$ and $Cd^{2+}$ gave very similar rates of decay to mM-EDTA, but reactivation by $Zn^{2+}$ was slow.

In the following paragraphs reference is made to two types of enzyme preparation, purified enzyme at stage 6 (Table 1) and partially purified enzyme at stage 3*b* (Table 1). In the latter case $Zn^{2+}$ was omitted from the buffer used to dissolve the precipitated enzyme. In the former case dilution of the concentrated, highly active, preparation decreased the $Zn^{2+}$ content to a low value (0·05–1μM) before incubation with added cations or EDTA.

Neither β-*N*-acetylglucosaminidase nor β-galactosidase was affected by $Zn^{2+}$ or EDTA.

*Reversible and irreversible inactivation.* Like many other enzymes, α-mannosidase is irreversibly inactivated at low protein concentration. This process, which is prevented by albumin, can be distinguished from the reversible inactivation that is overcome by $Zn^{2+}$. Fig. 3 shows the effects of $Zn^{2+}$ and albumin on purified enzyme that had been incubated beforehand for 17hr. at 37° and pH 5, in the presence and absence of albumin. Inactivation was greater in the albumin-free preparation. Further incubation in the presence of $Zn^{2+}$ caused a gradual restoration of activity in both types of preparation, but the difference between them did not diminish. Reversible inactivation to the extent of more than 50% could be produced in 3hr. with the purified enzyme by adding 5μM-$Cu^{2+}$, with albumin present to check irreversible inactivation.

The partially purified enzyme did not need albumin to prevent irreversible inactivation in this kind of experiment, but more than 50% of the activity was lost reversibly in 3hr. at 1:1000 dilution of the preparation at stage 3*b*. It appears that the less pure preparation contained toxic cations that were removed to a large extent during the purification procedure, probably at stage 6.

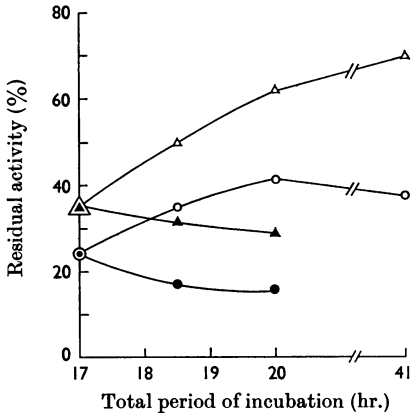


Fig. 3. Effects of albumin and $Zn^{2+}$ on the activity of α-mannosidase. α-Mannosidase (stage 6, Table 1, diluted 1:100) was incubated beforehand at 37° and pH 5 (0·05 M-acetic acid–NaOH buffer) in the presence and absence of 0·01% albumin. After 17 hr. an equal volume of 2 mM-$ZnSO_4$ in the buffer or of the buffer alone was added, along with albumin where appropriate to maintain 0·01% concentration, and incubation was continued. ●, Enzyme alone; ○, enzyme+$Zn^{2+}$; ▲, enzyme+albumin; △, enzyme+albumin+$Zn^{2+}$. The preparations were further diluted 1:80 before assay, and the results are expressed as percentages of the original activity of the unincubated enzyme preparation.

It is concluded that the effects of $Zn^{2+}$ and albumin on α-mannosidase are independent and additive.

*Effect of* pH. Table 2 shows the effect of varying the pH on the loss in activity of the purified enzyme, incubated for 17 hr. at 37° in the presence of albumin. At pH 7 the enzyme was stable in the absence of added $Zn^{2+}$, but as the pH was lowered it became progressively less stable. The action of EDTA, small at pH 7, became more pronounced as the pH fell. Addition of $Zn^{2+}$ prevented inactivation at acid pH. Almost identical results were obtained with the partially purified preparation, incubated for 3 hr. without addition of albumin.

Concentrated enzyme preparations buffered to pH 7 were relatively stable in the absence of added $Zn^{2+}$, and could be dialysed at 0° for at least 24 hr. with little inactivation. Dialysis was not possible at pH 5 unless $Zn^{2+}$ was added. At low protein concentration α-mannosidase preparations still required addition of albumin when incubated at pH 7 to prevent irreversible inactivation (cf. Fig. 3).

*Displacement curves.* $Zn^{2+}$, EDTA and a toxic cation ($Cd^{2+}$, $Cu^{2+}$ or $Co^{2+}$) were titrated against each other by incubating two-component mixtures with the partially purified enzyme for 3 hr. at pH 5 before assay. At appropriate relative concentrations, EDTA not only abolished protection by $Zn^{2+}$, but it also prevented inactivation by the other cations. In the same way, $Zn^{2+}$ and a deleterious ion were shown to counteract one another.

Table 2. *Effect on α-mannosidase activity of incubation for 17 hr. at 37° and various* pH *values*

A purified enzyme preparation (stage 6, Table 1) was incubated at 1:100 dilution in 0·125 M-acetic acid–NaOH buffers containing 0·01% albumin. Similar preparations were incubated in the presence of 1 mM-$ZnSO_4$ and 1 mM-EDTA. The solutions were further diluted 1:80 for assay. The results are expressed as percentages of the initial activity of the enzyme preparation.

| | Residual activity (%) | | |
|---|---|---|---|
| Addition ... ... | None | 1 mM-$ZnSO_4$ | 1 mM-EDTA |
| pH | | | |
| 4·0 | 50 | 104 | 0·5 |
| 4·5 | 59 | 109 | 1·0 |
| 5·0 | 73 | 102 | 1·5 |
| 6·0 | 91 | 90 | 45 |
| 7·0 | 100 | 91 | 87 |

Fig. 4 shows all the two-component mixtures that were studied; for convenience, concentrations are plotted on a logarithmic scale. Figs. 4(*d*), 4(*e*) and 4(*f*) show that the affinities of $Co^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ for the enzyme at pH 5 increase in that order; this is the order in which the residual activities in equimolar mixtures fall, relative to each other and to the controls. The protective action of 10 μM-EDTA alone against spontaneous inactivation is shown in Fig. 4(*a*), as well as its effect in potentiating activation by $Zn^{2+}$ when the latter was present in equivalent or higher concentration. When EDTA was present in excess, it inactivated the enzyme.

Figs. 4(*b*) and 4(*c*) show that increasing concentrations of $Cd^{2+}$ and $Cu^{2+}$ respectively progressively overcame the protective action of 10 μM-EDTA, until at the equivalence point inactivation became greater than in the control. Little change was seen when the concentration of EDTA was increased in the presence of 10 μM-$Cd^{2+}$ or -$Cu^{2+}$; inactivation by the cation gave way to inactivation by the chelating agent.

Although the partially purified enzyme was fairly stable at pH 7, and resistant to inactivation by EDTA, it was still susceptible to inactivation by $Cd^{2+}$ and $Cu^{2+}$, and protection of the enzyme against these cations by EDTA and by $Zn^{2+}$ could be demonstrated.

Purified enzyme gave the same results as partially purified in experiments of the type illustrated in Fig. 4. The dilution of the purified preparation was varied from 1:100 to 1:2000, and albumin was sometimes added. The incubation was usually continued for 17 hr.

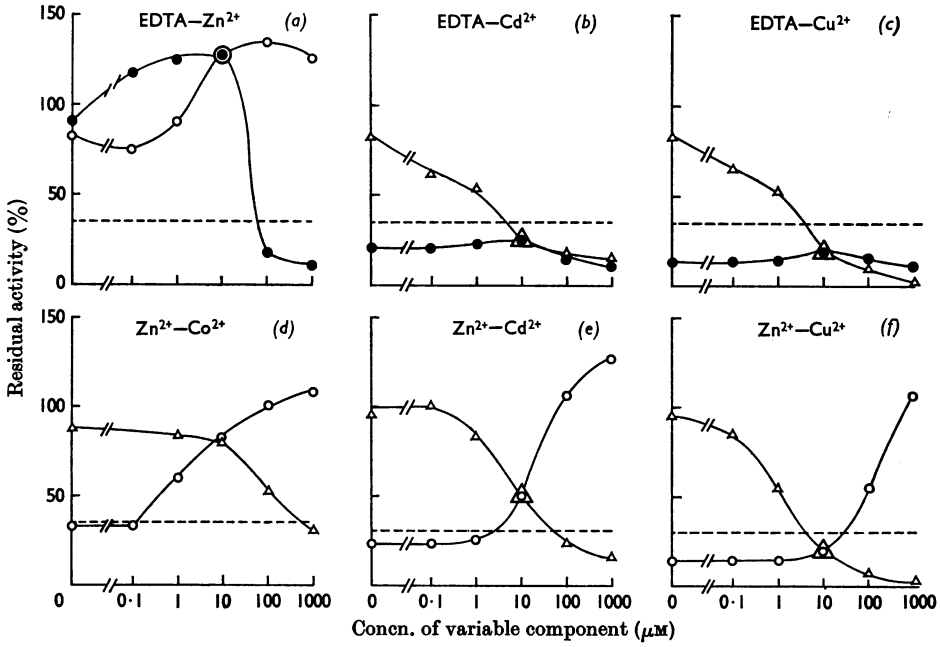


Fig. 4. Effect on α-mannosidase of incubation for 3 hr. at 37° and pH 5·0 (0·05 M-acetic acid–NaOH buffer) with different two-component mixtures. The concentration of each component was varied in the presence of a fixed concentration of the other (10 μM); residual activity with the fixed component alone is shown at the ordinate: (*a*) EDTA and $Zn^{2+}$; (*b*) EDTA and $Cd^{2+}$; (*c*) EDTA and $Cu^{2+}$; (*d*) $Zn^{2+}$ and $Co^{2+}$; (*e*) $Zn^{2+}$ and $Cd^{2+}$; (*f*) $Zn^{2+}$ and $Cu^{2+}$. ●, EDTA concentration varied; ○, $ZnSO_4$ concentration varied; △, $CdSO_4$, $CuSO_4$ or $Co(NO_3)_2$ concentration varied. Concentrations of the variable component are shown on a logarithmic scale. Results are shown as percentages of the initial activity in an untreated control; the value for this control after 3 hr. at 37° is shown by the broken line. The enzyme preparation (stage 3*b*, Table 1) had been diluted 1:1000, and after incubation it was further diluted 1:5 for assay.

*Effect of cations and of EDTA in the presence of substrate.* Neither $Zn^{2+}$ nor EDTA added to the incubation mixture caused more than small changes (10%) in α-mannosidase activity during the normal 1 hr. assay. $Zn^{2+}$ did, however, prevent the fall in the rate of hydrolysis of *p*-nitrophenyl α-mannoside that occurred when incubation was prolonged beyond 1 hr. at pH 5, whereas EDTA accelerated the fall. These effects are illustrated for the partially purified enzyme in Fig. 5. Addition of 180 μM-nitrophenol did not influence the rate of hydrolysis.

Comparison of the curves in Fig. 5 with decay curves for the same enzyme preparation incubated at pH 5 in the absence of substrate made it obvious that the substrate had a protective action on the enzyme. This is demonstrated in Table 3. Inactivation, whether spontaneous or induced by EDTA or $Cd^{2+}$, was much slower in the presence of substrate. On the other hand, the stabilizing action of $Zn^{2+}$ was enhanced by the substrate.

With purified enzyme, $Zn^{2+}$ was not required to obtain a constant rate of hydrolysis during the first 4 hr. of incubation with substrate. This agrees with the suggestion made above that toxic ions were removed during the purification.

*Action of EDTA.* The difference between age-inactivated preparations (in which trace impurities had presumably replaced $Zn^{2+}$ in the enzyme complex) and EDTA-treated preparations (in which all cations were open to sequestration) was strikingly illustrated when they were assayed in the presence of excess of $Zn^{2+}$. In the latter case there was immediate and complete recovery of activity. In the former case $Zn^{2+}$ had no effect on the activity, and when incubation was prolonged, as in Fig. 5, the rate of hydrolysis remained constant at the initial depressed value. Substrate in fact prevented the gradual restoration of activity by $Zn^{2+}$ that is shown in Fig. 3.

Reactivation by $Zn^{2+}$ in the assay of EDTA-inactivated enzyme occurred with both purified and partially purified preparations, whatever their extent of inactivation. The rate of inactivation by EDTA was greater in more dilute enzyme prepara-

tions. It was temperature-dependent: there was virtually no effect after 17hr. at 0°.

The reactivation of an EDTA-inactivated preparation during the actual enzyme assay provided a convenient and convincing test for the identity of the metal ion in the active α-mannosidase complex. Of a number of cations examined at 100 μM concentration, only $Zn^{2+}$ had this effect on the purified enzyme. The enzyme preparation was incubated with mM-EDTA and 0·01% albumin at pH 5 until it had undergone 50% inactivation, after which it was diluted so as to decrease the EDTA concentration in the assay mixture to insignificant proportions. The following ions had no effect in the assay: $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Cd^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Ba^{2+}$, $Al^{3+}$, $K^+$ and $Cr_2O_7^{2-}$. Introduction of $Ag^+$ or $Hg^{2+}$ into the assay produced further inactivation of the EDTA-treated enzyme: these ions inactivate 'normal' enzyme under assay conditions.

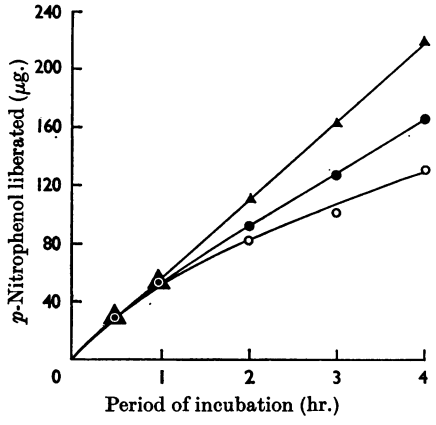


Fig. 5. Hydrolysis of 6 mM-*p*-nitrophenyl α-mannoside for various periods at 37° in 0·125 M-acetic acid–NaOH buffer, pH 5·0. ●, Enzyme alone; ▲, enzyme + 100 μM-$ZnSO_4$; ○, enzyme + 100 μM-EDTA. The enzyme preparation (stage 3*b*, Table 1) was diluted 1:10000 before addition to the incubation mixture.

## DISCUSSION

From the evidence that we have obtained it is postulated that α-mannosidase forms a dissociable enzyme–metal ion complex with $Zn^{2+}$ in which the metal ion is essential for catalytic activity. Whereas the study of the untreated preparation reveals the conflicting effects of different cations present as trace impurities, the results for the EDTA-treated enzyme (since it is not subject to this type of interference) are more readily reproduced. The most convincing evidence for the identity of the cation in the hypothetical enzyme–metal ion complex lies in the fact that it can be replaced by added $Zn^{2+}$ without any change in the activity of a fresh preparation. No other cation was found to activate the enzyme.

On the assumption that α-mannosidase forms a $Zn^{2+}$ complex, it is difficult to decide whether $Zn^{2+}$ is part of the enzyme active centre since normally it has little effect under assay conditions. Alternatively, it is possible that the cation is attached at some other site where it maintains a suitable configuration in the enzyme for the reaction with substrate. The fact that substrate can stabilize the enzyme to some extent in the absence of exogenous $Zn^{2+}$ suggests that it locks the cation within the

Table 3. *Effects of various agents on the incremental activity of α-mannosidase when incubated for various periods at* 37° *and* pH 5

The activities shown are difference measurements made at hourly intervals. In the cases where substrate is shown to be absent from the original mixture, it was added for the final hour of incubation. Results are shown as percentages of the activity in the normal 1hr. assay. The enzyme was at stage 3*b* (Table 1). In columns 1–4, it was diluted 1:10000 before addition to the assay mixture. In columns 5–8, it was diluted 1:5000 for incubation without substrate. Concentrations in the incubation mixtures were: 100 μM-EDTA, 100 μM-$Zn^{2+}$ and 10 μM-$Cd^{2+}$.

| | | Activity (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Substrate present | | | | Substrate absent | | | |
| Hourly interval | Addition ... | (1) None | (2) $Zn^{2+}$ | (3) EDTA | (4) $Cd^{2+}$ | (5) None | (6) $Zn^{2+}$ | (7) EDTA | (8) $Cd^{2+}$ |
| 0–1 | | 100 | 101 | 98 | 93 | — | — | — | — |
| 1–2 | | 73 | 110 | 62 | 58 | 43 | 92 | 22 | 19 |
| 2–3 | | 69 | 93 | 58 | 51 | 18 | 89 | 8 | 6 |
| 3–4 | | 71 | 110 | 50 | 39 | 14 | 90 | 4 | 3 |
| 4–5 | | — | — | — | — | 10 | 82 | 2 | 3 |

protein molecule. On the other hand, the action of substrate in preventing the reactivation of a decayed preparation that would otherwise occur when $Zn^{2+}$ is added suggests that it is equally effective in locking the metal ion out.

Spontaneous reversible inactivation of α-mannosidase, which is a time- and temperature-dependent process, could arise in two ways: dissociation of such a protein–$Zn^{2+}$ complex, or displacement of $Zn^{2+}$ by other cations present as trace impurities. Though both ways are possible, the behaviour of inactivated enzyme in the presence of substrate and $Zn^{2+}$ is more readily explicable in terms of the latter. It is only necessary to postulate that substrate can combine with inactive enzyme molecules containing a foreign cation. Inactivation by EDTA too is in contrast with the spontaneous inactivation. When all the bivalent cations in the preparation have been sequestrated by EDTA, addition of $Zn^{2+}$ results in immediate reactivation, even in the presence of substrate. Presumably the substrate cannot combine with the enzyme in the absence of bivalent cation.

Treatment with pyridine affords a convenient method of obtaining α-mannosidase in jack-bean-meal preparations free from the other glycosidases that could hinder its use as a structural reagent for glycoproteins. The success that Li (1967) had in freeing α-mannosidase from β-*N*-acetylglucosaminidase on a column of DEAE-Sephadex could have arisen from inactivation of the latter enzyme during the elution of the former with pyridine–acetic acid buffer. In our own purification procedure β-galactosidase activity also is destroyed by pyridine. Unlike β-*N*-acetylglucosaminidase, however, β-galactosidase can be readily separated from α-mannosidase on Sephadex G-100. From the results of Li (1967) it appears that the peaks for all three enzymes overlap when they are eluted from Bio-Gel P-200. Li (1967) commented on the instability of α-mannosidase at pH values below 6, even in a concentrated state. This difficulty we have to some extent overcome by adding $Zn^{2+}$.

Direct comparison of the efficiency of our purification procedure with that of Li (1967) is difficult, particularly since he assayed his enzyme at 25°. However, our purification factor is only 150, compared with his value of 525. We did not succeed in getting his final fourfold increase in specific activity on DEAE-Sephadex. Nevertheless, we consider our product to be pure enough for structural studies on glycoproteins, and the yield is good.

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