Purification and Properties of α -D-Mannosidase from the Limpet, *Patella vulgata*

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1. α -Mannosidase from the limpet, *Patella vulgata*, was purified nearly 150-fold, with 40% recovery. β -N-Acetylglucosaminidase was removed from the preparation by treatment with ethanol. The final product was virtually free from β -galactosidase. 2. Limpet α -mannosidase was assayed at pH 3.5 and at this pH it was necessary to add Zn^{2+} for full activity. At pH 5, the enzyme had the same activity in the presence or absence of added Zn^{2+} . 3. On incubation at acid pH, the enzyme underwent reversible inactivation, which was prevented by adding Zn^{2+} . 4. EDTA accelerated inactivation and the addition of Zn^{2+} at once restored activity. No other cation was found to reactivate the enzyme. 5. Cl^- had an unspecific effect on hydrolysis by limpet α -mannosidase. It increased the rate of reaction with substrate. The anion did not prevent or reverse inactivation by EDTA. 6. It is concluded that α -mannosidase is a metalloenzyme or enzyme-metal ion complex, dissociable at the pH of activity, and that it requires Zn^{2+} specifically.

The purification and properties of α -D-mannosidase (EC 3.2.1.24) from jack-bean meal (Snaith & Levvy, 1968) and from rat epididymis (Snaith & Levvy, 1969) have been described. In both cases, the enzyme behaved as a dissociable metalloenzyme or enzyme-metal ion complex, in which Zn^{2+} was essential for catalytic activity.

Conchie & Levvy (1957) found that the limpet, *Patella vulgata*, was a rich source of α -mannosidase, and is readily available, in the British Isles at least. Muramatsu (1967) and Muramatsu & Egami (1967) have studied α -mannosidase from *Charonia lampas* and from *Turbo cortunus* respectively. It was found that sodium chloride accelerated hydrolysis by the enzyme from both these molluscs. This did not happen with α -mannosidase from jack-bean meal and from rat epididymis (Snaith & Levvy, 1968).

The enzyme from *P. vulgata* has now been studied and purified. It is completely specific for Zn^{2+} in the catalytically active form, and it portrays in an exaggerated fashion the effects of competing toxic cations, compared with the enzyme from jack-bean meal or from rat epididymis. Limpet α -mannosidase is relatively stable, and the rate of reaction is increased in the presence of Cl^- .

EXPERIMENTAL

Substrates

Melting points are corrected.

p-Nitrophenyl N-acetyl- β -D-glucosaminide. This compound was conveniently prepared by condensing aceto-

chloroglucosamine (1-chloro-1-deoxy-2,3,4,6-tetra-acetyl- α -D-glucosamine) (Horton & Wolfson, 1962) with *p*-nitrophenol in aqueous alkali and acetone (Leaback & Weissman, 1963). A better yield was claimed by Begbie (1969) who did the condensation in methyl sulphoxide containing NaH.

N-Acetylglucosamine (33g) was suspended in acetyl chloride (66ml) in a 500ml flask fitted with reflux condenser and drying tube. Reaction, initiated by adding three drops of water, became vigorous after 1h, but no cooling was needed and it abated after 3h. After 2 days at room temperature, dichloromethane (330ml) was added to the flask, and the solution was slowly poured with stirring into ice-water (800ml). The organic layer was washed with saturated aq. NaHCO_3 (total 300ml), and dried over $\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$ and charcoal. It was concentrated to 130ml and diluted with ether (400ml). The crystals of acetochloroglucosamine were filtered off after 7h at 0°C and dried (33g, m.p. 116–117°C).

A solution of acetochloroglucosamine (105g) and *p*-nitrophenol (80g) in acetone (1litre) was mixed with *m*-NaOH (580ml) and left at 0°C for 3 days. The crystalline glycoside tri-*O*-acetate was washed with water (45g, m.p. 233–234°C). It was de-*O*-acetylated in boiling methanol-chloroform (1:1, v/v) (20ml/g of acetate) with the addition of 0.1M-sodium methoxide in methanol (0.5ml/g of acetate). The liquid was at once allowed to cool and left overnight at 0°C. The glycoside, which separated in almost quantitative yield, was filtered off, dried and triturated on the filter with acetone. It was used without recrystallization [m.p. 206–207°C; $[\alpha]_D^{20}$ –16°, *c* 0.5 in acetone-water (1:1, v/v)].

p-Nitrophenyl α -D-mannoside. Commercial D-mannose employed previously was predominantly the β -isomer and yielded a crystalline penta-acetate (Levy & Conchie, 1966). The α -D-mannose now available yields only a syrup, and this was employed exactly as described by

Levy & Conchie (1966) for the preparation of the mannoside from the crystalline penta-acetate. To obtain the syrup, mannose (24g) was added in small portions over 30 min to a mixture of acetic anhydride (200 ml) and pyridine (260 ml) cooled in ice-salt. The mixture was stirred until all the mannose was in solution and kept at 0°C for 2 days. It was poured slowly with stirring into ice-water (1 litre) and stirring was continued for 45 min. The penta-acetate was extracted with chloroform (total 450 ml) and the solution was washed thoroughly with water (total 3.5 litres). After drying over CaCl_2 , the chloroform was removed and the residue was thoroughly dried (52 g).

o-Nitrophenyl β -D-galactoside. This was made as described by Conchie & Hay (1963).

Methods

α -D-Mannosidase assay. The hydrolysis of 6 mM *p*-nitrophenyl α -D-mannoside in 0.125 M acetic acid-NaOH buffer, pH 3.5, at 37°C was measured as described by Levy & Conchie (1966), in the presence of 0.1 mM ZnSO_4 and 0.1 M NaCl. One unit is the amount of enzyme liberating 1 μ g of *p*-nitrophenol/h.

Other glycosidase assays. Preparations were examined for the presence of other glycosidases as described by Snaith & Levy (1968, 1969). One unit is the amount of enzyme liberating 1 μ g of aglycone/h at 37°C.

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. The final product of the purification procedure (Table 1) had the same chromogenic value as albumin on an N basis. The crude supernatant (stage 2, Table 1) had an N content of 27% of the protein, measured as albumin, pointing to the presence of less chromogenic nitrogenous material that was removed during purification.

RESULTS

Purification procedure

General comments. Considerable difficulty was encountered in early attempts to purify limpet

α -mannosidase. With assays at pH 5, there were wide variations in the activity (units/g of tissue) of the starting material. This variability extended to the specific activity (units/mg of protein) and it could not be eliminated by several stages of purification. Later it was found that the product could be activated at a low pH, and it was realized that the differences in specific activity arose largely from firmly bound interfering material, probably toxic cations. Assay at pH 3.5 avoided this difficulty to a large extent.

As with α -mannosidase from other sources, the removal of β -N-acetyl-D-glucosaminidase (EC 3.2.1.30) and β -D-galactosidase (EC 3.2.1.23) was made a primary objective: these two enzymes were not subject to the same interference as α -mannosidase. Results obtained by the final procedure are illustrated in Table 1.

During precipitations, water was added to correct for volume changes after the reagents had been mixed. Precipitates were left to settle at 0°C for 30 min before centrifugation at 35 000g for 15 min at low temperature (below 10°C). The enzyme precipitated by acetone at stage 3 was taken up in 0.05 M acetic acid-sodium hydroxide buffer, pH 5.0, containing 0.1 mM zinc sulphate and 0.1 M sodium chloride, and this Zn^{2+} -containing buffer was added after treatment with ethanol at stage 4. After fractionation with ethanol at stage 5, the enzyme was dissolved in 0.1 M acetic acid-sodium hydroxide buffer, pH 6.0, containing 0.1 M sodium chloride, and it was passed through Sephadex G-100 (stage 6) in this buffer. At pH 6, the limpet enzyme was stable on the column without addition of Zn^{2+} . There was nearly 150-fold purification of α -mannosidase with 40% recovery. The final specific activity compared satisfactorily with the values obtained on purification of the enzyme from jack-bean meal (Snaith & Levy, 1968) and from rat epididymis (Snaith & Levy, 1969).

Table 1. Purification of α -mannosidase from the limpet, *Patella vulgata*

Assays were done at pH 3.5, in the presence of Zn^{2+} and Cl^- . Specific activities are expressed as units/mg of protein, with bovine serum albumin as protein standard.

Stage	Description	Recovery (units/g of tissue)			Specific activity of α -mannosidase
		α -Mannosidase	β -N-Acetylglucosaminidase	β -Galactosidase	
1	Homogenate	330 000	150 000	68 000	3800
2	Supernatant	310 000	150 000	66 000	5600
3(a)	0-25% Acetone	23 000	5500	2400	890
3(b)	25-40% Acetone	270 000	16 000	11 000	32 000
3(c)	40-60% Acetone	8300	95 000	44 000	620
4	Treatment with 60% ethanol	210 000	1800	7100	31 000
5(a)	0-25% Ethanol	23 000	340	850	4100
5(b)	25-35% Ethanol	4500	18	0	53 000
5(c)	35-45% Ethanol	180 000	93	460	360 000
5(d)	45-60% Ethanol	37 000	330	4800	43 000
6	Gel chromatography	130 000	10	92	550 000

Fig. 1. Elution of α -mannosidase from a column (60 cm $\times$ 3 cm) of Sephadex G-100 with 0.1M-acetic acid-NaOH buffer, pH 6.0, containing 0.1M-NaCl; 100ml was run to waste and fractions (5ml) were then collected. ●, α -Mannosidase activity; ○, protein; ▲, β -N-acetylglucosaminidase activity; △, β -galactosidase activity. The enzyme was a preparation different from that shown in Table 1. It was at stage 5(c) (Table 1) and fractions 26–30 were retained.

Stage 1 (homogenate). Freshly dissected limpet visceral humps were suspended in cold water (5ml/g of moist tissue) with an Ultra-Turrax TP18/2N disintegrator and the suspension was stored at -20°C .

Stage 2 (supernatant). Insoluble material was removed from the thawed homogenate (100ml) by centrifuging in the cold at 25000g for 15 min. The sediment was washed by centrifugation with an equal volume of cold water that was added to the supernatant. The pH (about 6.3) was adjusted to 5 with two or three drops of 3M-hydrochloric acid, and 10mM-zinc sulphate was added to give a final concentration of 0.1mM.

Stage 3 (acetone fractionation). The supernatant was cooled and ice-cold acetone was added to give in turn 25, 40 and 60% (v/v) concentration, and each precipitate was suspended in the Zn^{2+} -containing buffer solution, to give a final volume of 20ml. The fraction (stage 3b) that separated between 25 and 40% acetone concentration was used for further purification.

Stage 4 (treatment with 60% ethanol). Ethanol was added to give 60% (v/v) concentration. All the α -mannosidase activity was thereby thrown out of

solution and the suspension was kept at 27°C for 2h. The ethanol concentration was then lowered to 25% by the addition of ice-cold Zn^{2+} -containing buffer.

Stage 5 (ethanol fractionation). Insoluble material (stage 5a) was removed from the suspension by centrifugation, and ice-cold ethanol was added to the cooled supernatant to give 35, 45 and 60% concentration. In each case, the sediment was taken up in the buffer at pH 6 (10ml).

Stage 6 (Sephadex G-100 chromatography). The preparation at stage 5(c) (35–45% ethanol) was passed through a column of Sephadex G-100 at room temperature (Fig. 1) and the enzymically active fractions were pooled. The enzyme was precipitated at 0°C with 60% (v/v) acetone and dissolved in 1–2ml of the Zn^{2+} -containing buffer, pH 5.0. The preparation was stable at 0°C for several weeks at least.

Removal of other enzymes

α -Mannosidase from jack-bean meal (Snaith & Levvy, 1968) and from rat epididymis (Snaith & Levvy, 1969) was completely freed from the accompanying β -N-acetylglucosaminidase by incubating the preparation with pyridine. In the case of the limpet preparation, destruction of β -N-acetylglucosaminidase by pyridine was always accompanied by a similar drop in α -mannosidase activity. Advantage was therefore taken of an observation that the addition of ethanol to a limpet preparation led under suitable conditions to selective inactivation of β -N-acetylglucosaminidase. There was wider separation of the peaks for α -mannosidase and residual β -N-acetylglucosaminidase on Sephadex G-100 at pH 6 than at pH 5, and at room temperature than at 0°C .

Treatment of the jack-bean-meal and rat epididymal preparations with pyridine led also to incidental inactivation of β -galactosidase. This did not occur when limpet preparations were treated with ethanol, and in the main removal of β -galactosidase had to be accomplished during the fractionation of α -mannosidase. The double peak for β -galactosidase in Fig. 1 is particularly noteworthy, since passage of the preparation through Sephadex G-100 led to an increase of almost threefold in its total β -galactosidase activity. It is fortunate that the major peak ran well behind that of α -mannosidase. Possibly some change in the ionic environment activated one or both of the β -galactosidase components towards the nitrophenol substrate.

Apart from α -mannosidase, β -N-acetylglucosaminidase and β -galactosidase, limpet visceral hump possesses the following glycosidase activities in significant amount: α -glucosidase, β -glucosidase, α -galactosidase, β -mannosidase, α -N-acetylglucosaminidase and β -glucuronidase (Conchie & Levvy,

1957); α -L-fucosidase (Levy & McAllan, 1961); β -D-fucosidase (Levy & McAllan, 1963). It is also rich in 1-aspartamido- β -N-acetylglucosamine amidohydrolase (Conchie & Strachan, 1969). None of these enzymes was present in our final product, except β -glucuronidase, with an activity of about 1300 units/g, compared with an initial value of about 100 000. Limpet β -glucuronidase had the same elution profile as α -mannosidase from Sephadex G-100. It was an advantage of our final procedure that dialyses were eliminated, since crude limpet preparations rapidly rupture dialysis tubing: this does not happen with the purified α -mannosidase preparation, but the enzyme itself is then unstable during dialysis.

Properties of the enzyme

Study of the usual variables that influence the velocity of hydrolysis was complicated by the

Fig. 2. Hydrolysis at different pH values of 6mM-*p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer. At pH 2.8, 0.125M-acetic acid solution was employed, and at pH 2.3, 0.125M-acetic acid adjusted with H₂SO₄. The crude enzyme (stage 2, Table 1) was diluted 1:250 and incubated with substrate for 1h alone (Δ) and in the presence of 0.1mM-ZnSO₄ ($\blacktriangle$), 0.1M-NaCl ($\circ$) and 0.1mM-ZnSO₄+0.1M-NaCl ($\bullet$). The α -mannosidase activity is expressed as a percentage of the maximum value obtained in the presence of Zn²⁺+Cl⁻.

effects of three other factors: (1) the action of Cl⁻ on the rate of reaction, (2) the need for excess of Zn²⁺ in the assay to maintain the enzyme in its catalytically active form, and (3) the effect of very firmly bound toxic cations that may prevent the enzyme from exhibiting its full activity.

Like α -mannosidase from jack-bean meal and from rat epididymis, the limpet enzyme showed greater stability at pH 5 than at lower pH values. Unlike the enzyme from the other two sources, however, it only exhibited a small proportion of its optimum activity at pH 5, and its kinetics of action were anomalous at this pH value.

Effect of pH on activity. Fig. 2 shows pH optima for the hydrolysis of *p*-nitrophenyl α -mannoside by the crude limpet enzyme (stage 2, Table 1) and illustrates the effects of different factors that are enlarged upon below. Both Zn²⁺ and Cl⁻ had to be added for the enzyme to display its potential activity to the full, and Cl⁻ caused a rise in the pH for optimum activity to pH 3.5. At the optimum, the addition of Zn²⁺ had a considerable effect upon the activity, whereas at pH 5 it had none. Experiments with the purified enzyme (stage 6, Table 1) gave curves that were similar in every respect to those shown in Fig. 2.

Dissociation and activation. α -Mannosidase from jack-bean meal and from rat epididymis, incubated in the absence of substrate, became increasingly unstable as the pH was lowered below 6. Inactivation was accelerated by EDTA and prevented by Zn²⁺. Addition of excess of Zn²⁺ to the EDTA-treated preparations at once restored their original activity. Irreversible inactivation at low protein concentration was minimized by adding albumin. Pure rat epididymal α -mannosidase displayed irreversible inactivation above pH 6 under all conditions. Neither type of preparation showed more than 10% activation by Zn²⁺ in the normal assay at pH 5.

Similar experiments were done with limpet α -mannosidase. Table 2 shows the results when the preparation was assayed in the usual way at pH 3.5. Reversible inactivation became evident as the pH was lowered, and Zn²⁺ and EDTA had their expected effects. In the absence of Zn²⁺, maximum stability was exhibited at pH 6, whereas Zn²⁺ was required at lower pH values, and irreversible inactivation was seen at pH 7.

There was an important difference when the assays were done at pH 5 (Table 3). There appeared to be marked activation when the preparation was incubated with Zn²⁺ at an acid pH value. Preparations treated with EDTA and then assayed in the presence of excess of Zn²⁺ showed this to an even greater extent. Activation by Zn²⁺ varied directly with the degree of inactivation in EDTA.

From these observations, which applied equally to the purified enzyme or to crude tissue extracts, it

Table 2. *Effect on α -mannosidase activity of incubation for 3 h at 37°C and various pH values: assay at pH 3.5*

A purified enzyme preparation (stage 6, Table 1) was incubated at 1:500 dilution in 0.1M-acetic acid-NaOH buffer containing 0.01% albumin. Similar preparations were incubated in the presence of 1mM-ZnSO₄ and 1mM-EDTA. The solutions were then further diluted 1:10 for the assay, which was done under the conditions described in the Experimental section except for assay of the EDTA-treated enzyme in the absence of Zn²⁺ (column 3). The results are expressed as percentages of the initial activity of the enzyme preparation, assayed with (columns 1, 2 and 4) or without (column 3) added Zn²⁺.

Addition ...	Residual activity (%)			
	None	1mM-ZnSO ₄	1mM-EDTA	
			Assay without Zn ²⁺	Assay with Zn ²⁺
pH	(1)	(2)	(3)	(4)
3.0	67	31	3	99
3.5	51	107	5	110
4.0	62	100	34	109
4.5	69	101	41	96
5.0	81	97	67	99
6.0	93	94	75	94
7.0	30	28	51	61

Table 3. *Effect on α -mannosidase activity of incubation for 3 h at 37°C and various pH values: assay at pH 5*

The same diluted enzyme preparation was incubated as described in Table 2. The solutions were further diluted 1:2 for assay, which was done in 0.125M-acetic acid-NaOH buffer, pH 5.0, in the presence of 0.1M-NaCl; ZnSO₄ was included in the assay mixture only in column 4. The results are expressed as percentages of the initial activity of the enzyme preparation assayed at pH 5 (the value at this pH is normally unaffected by Zn²⁺). This control value was only 13.5% of the value obtained when the enzyme was assayed at pH 3.5 in the presence of Zn²⁺, in absolute terms.

Addition ...	Residual activity (%)			
	None	1mM-ZnSO ₄	1mM-EDTA	
			Assay without Zn ²⁺	Assay with Zn ²⁺
pH	(1)	(2)	(3)	(4)
3.0	129	96	6	315
3.5	84	251	13	350
4.0	90	236	52	282
4.5	91	190	60	206
5.0	116	155	90	160
6.0	101	104	96	103
7.0	22	21	40	44

was concluded that much of the enzyme protein is firmly bound to toxic cations. Only at a pH value where there is considerable dissociation can they be displaced at all quickly by excess of Zn²⁺. This happens during assay at the pH optimum (pH 3.5; Fig. 2). Assay at pH 5 slows equilibration between the different cations in the mixture.

Table 3 shows that maximum activation was attained when excess of Zn²⁺ was added after incubation of the enzyme at 37°C in 1mM-EDTA and 0.01% albumin at pH 3.5. The rate of activation was governed by the degree of dilution, and the enzyme

at stage 6 (Table 1) had to be diluted 100- to 500-fold before incubation with EDTA became fully effective in 3 h. Such EDTA-treated preparations gave pH-activity curves in the presence of excess of Zn²⁺ similar to those shown in Fig. 2, except in that the slope was more gradual on the alkaline side of the peak. The preparations could be concentrated after the EDTA treatment by addition of acetone to give 60% (v/v) concentration, and extraction of the enzyme from the precipitate, along with variable quantities of EDTA and albumin, in the Zn²⁺-containing buffer, pH 5.0.

Fig. 3. Hydrolysis of 6 mM-*p*-nitrophenyl α -mannoside for various periods at 37°C in 0.125 M-acetic acid-NaOH buffer containing 0.1 M-NaCl and 0.1 mM-ZnSO₄. A purified enzyme preparation that had been activated with EDTA (see the text) was incubated at pH 3.5 (●) and at pH 5.0 (○), in 1:25 000 and 1:10 000 dilution respectively. The enzyme preparation was also incubated at pH 5.0 (▲), without prior activation, in 1:2500 dilution.

Effect of duration of hydrolysis. After activation with EDTA as described above, the enzyme gave a velocity curve in the presence of Zn²⁺ and Cl⁻ that was linear for 3 h at pH 5 (Fig. 3). At pH 3.5, a fall in the rate of hydrolysis was already apparent after 2 h. This may have been due to irreversible inactivation of the enzyme protein, but could equally well have arisen from displacement of toxic cations from their EDTA complexes by the relatively high concentration of Zn²⁺. Fig. 3 also illustrates the abnormal type of velocity curve that was obtained at pH 5 with non-activated enzyme preparations, whether or not Zn²⁺ or Cl⁻ was added. Incubation of the diluted enzyme at pH 5 evidently led to slow activation (cf. Table 3), even though substrate was present.

Effect of Cl⁻. Fig. 4 shows the effect of adding sodium chloride at various concentrations during the assay of limpet α -mannosidase. At pH 3.5 there was optimum activity in the presence of 0.1 M-sodium chloride. A similar curve was obtained with a preparation activated beforehand with EDTA as described above, but when this preparation was assayed at pH 5 its activity curve showed a broad plateau, followed by a gradual decline, as the sodium chloride concentration was increased. Assay of a non-activated preparation at pH 5 showed a continuous increase in activity with the sodium chloride concentration up to 0.5 M. This last is the type of curve that Muramatsu (1967) and Muramatsu & Egami (1967) obtained with their molluscan preparations.

Fig. 4. Effect of various concentrations of NaCl on the activity of α -mannosidase during a 1 h assay in 0.125 M-acetic acid-NaOH buffer. A purified enzyme preparation was assayed at pH 3.5 in the presence of 0.1 mM-ZnSO₄ (●) and at pH 5.0 without Zn²⁺ (○). An enzyme preparation, previously activated by incubation with EDTA (see the text), was assayed at pH 5.0 in the presence of 0.1 mM-ZnSO₄ (▲). Results are expressed as percentages of the activity of each preparation assayed in the absence of NaCl.

Table 4. *Effects of various anions on the activity of α -mannosidase during a 1 h assay at 37°C*

α -Mannosidase activity was assayed in 0.125 M-acetic acid-NaOH buffer, pH 5.0, without Zn²⁺. Salt concentrations in the incubation mixture were 100 mM except for 50 mM-Na₂SO₄. Results are shown as percentages of the enzyme activity assayed without added salt. A partially purified enzyme preparation was used, which was made by an earlier variant of the final procedure (Table 1); the enzyme was precipitated at 40–50% (v/v) ethanol concentration after a preliminary ammonium sulphate fractionation.

Addition	Relative activity (%)
None	100
NaCl	220*
KCl	220*
KBr	156
KI	156
KF	101
NaNO ₃	163
Na ₂ SO ₄	100

* Identical effects were produced when the assays were done in acetic acid-KOH buffer.

Table 4 shows that the increase in the rate of reaction caused by sodium chloride was due to Cl⁻ and not to Na⁺. Certain other univalent anions behaved like Cl⁻. These assays were done at pH 5 to avoid the need to add a zinc salt.

The effect of Cl⁻ on the rate of reaction could be clearly distinguished from the absolute dependence

Fig. 5. Effect of EDTA, ZnSO_4 and NaCl on the activity of α -mannosidase. A partially purified preparation, as described in Table 4, was diluted 1:100 and incubated for various periods at 37°C in 0.125M-acetic acid-NaOH buffer, pH 5.0, containing 1mM-EDTA. ●, Enzyme alone (1); ▲, enzyme+1.1mM- ZnSO_4 (2); ■, enzyme+100mM-NaCl (3). The solutions were further diluted 1:4 before assay, which was done at pH 5.0 in the presence of the same concentrations of EDTA, ZnSO_4 or NaCl as the pre-incubation. Solutions (1) and (2) were also assayed in the presence of 0.1mM-NaCl (○ and △ respectively) and solution (3) in the presence of 1.1mM- ZnSO_4 (□). The results are expressed as percentages of the original activity in the unincubated enzyme preparation assayed without NaCl.

of the enzyme on Zn^{2+} for catalytic activity (cf. Tables 2 and 3). The preincubations (Fig. 5) and the subsequent assays were done at pH 5 to retard cation exchange. The unincubated controls showed about 100% increase in the rate of reaction when Cl^- was added to the assay medium. This difference was maintained in parallel assays with and without Cl^- throughout the course of inactivation by EDTA and activation by Zn^{2+} , and Cl^- did not check the EDTA inactivation. Fig. 5 illustrates an additional point, already demonstrated in Table 3, that as inactivation in EDTA increased, so did the ultimate activity in the presence of excess of Zn^{2+} .

Experiments of this type were also done in which inactivation was produced by addition of a toxic cation, such as Cd^{2+} (1mM). Unlike Zn^{2+} , Cl^- afforded no protection during the preincubation, but in its presence all assay results were elevated to the same degree.

Effect of Zn^{2+} and other cations. With α -man-

nosidase from jack-bean meal and from rat epididymis, the specificity for Zn^{2+} could be demonstrated by adding various cations to preparations that had been inactivated by pretreatment with EDTA. Only Zn^{2+} restored the activity of the enzyme in the assay. This was also the case in experiments with purified limpet enzyme that had been incubated at 37°C with 1mM-EDTA for 3 h at pH 5, the cations being added in about 0.1mM excess. Enzyme activity was assayed at pH 5 to minimize cation exchange during the incubation with substrate.

The specificity of the limpet enzyme for Zn^{2+} could also be demonstrated by replacing the zinc sulphate in the assay medium at pH 3.5 with other metal salts (cf. Fig. 2 and Table 2). No other cation was found to increase α -mannosidase activity.

Activation by excess of Zn^{2+} was immediate in an EDTA-treated limpet preparation, assayed at pH 5 (cf. Table 3). If the preparation was incubated for several hours at pH 5 after the addition of Zn^{2+} , but before addition of substrate, a gradual fall in the α -mannosidase activity was observed, pointing to the release of toxic cations that had been bound to EDTA.

Limpet α -mannosidase again resembled the enzyme from the other two sources in that Cu^{2+} , Cd^{2+} and Co^{2+} (10–1000 μM) caused inactivation at pH 5 in the absence of substrate except when Zn^{2+} was present in excess.

The additional observation was made that dilute limpet α -mannosidase preparations (stage 6, Table 1, at 1:100 dilution) were stable for several months at 0°C , after they had been incubated in 1mM-EDTA and 0.01% albumin at pH 3.5 (excess of Zn^{2+} being added during assay). This bears out the belief that spontaneous reversible inactivation of α -mannosidase is largely due to toxic cations in the preparations. Jack-bean-meal α -mannosidase was nearly as stable as the limpet enzyme under similar circumstances, but the enzyme from rat epididymis underwent rapid irreversible inactivation.

Effect of varying the concentration of enzyme and of substrate. The crude enzyme (stage 2, Table 1) was employed in the following experiments. Up to about 10% hydrolysis of the substrate, *p*-nitrophenyl α -mannoside, the reaction rate was directly proportional to the amount of enzyme used, under the conditions of assay described in the Experimental section.

Fig. 6 shows the effect of varying the substrate concentration in the presence of Zn^{2+} and Cl^- under the usual conditions of assay (given in the Experimental section), and in the presence of Zn^{2+} alone and Cl^- alone. Inspection of the curves reveals that Zn^{2+} increased the affinity of the enzyme for the substrate, whereas the main effect of Cl^- was to increase the rate of hydrolysis. Derivation of K_m ,

Fig. 6. Effect of substrate concentration on rate of hydrolysis of *p*-nitrophenyl α -mannoside in 0.125 M-acetic acid-NaOH buffer, pH 3.5, at 37°C by a crude limpet preparation (stage 2, Table 1): ●, in the presence of 0.1mM-ZnSO₄ and 0.1mM-NaCl; ▲, in the presence of 0.1mM-NaCl only; ○, in the presence of 0.1mM-ZnSO₄ only.

by the method of Lineweaver & Burk (1934), gave a value of 4.5mM in the presence of Zn²⁺ and Cl⁻, and 12mM in the presence of Cl⁻ alone: there was no difference in the maximum velocity. In the presence of Zn²⁺ alone, K_m was 1.6mM and the maximum velocity was only one-quarter of that with Zn²⁺ and Cl⁻.

In the equation

where E is the enzyme, S the substrate and $K_m = (k_2 + k_3)/k_1$, it is evident that addition of Zn²⁺ increased k_1 , whereas addition of Cl⁻ increased k_3 , and that k_3 was significantly large compared with k_2 . The results are in accord with the conclusions already arrived at on other grounds for the respective roles of the two ions in the catalytic action of α -mannosidase.

DISCUSSION

Except for the effect of Cl⁻ on the rate of reaction, α -mannosidase from the limpet is like the enzyme from jack-bean meal and from rat epididymis in that Zn²⁺ is required to maintain it in the catalytically active form. It differs from the enzyme from the latter two sources in having a lower pH optimum, at which the protein-Zn²⁺ complex is more freely dissociable, and in binding toxic cations very firmly at pH 5–6. In most respects, the limpet compares satisfactorily with jack-bean meal as a

source of α -mannosidase for use as a structural reagent (cf. Conchie, Hay, Strachan & Levvy, 1969).

With regard to the status of α -mannosidase as a metal-dependent enzyme, the preparations from all three sources can be considered together. According to Vallee's classification (Vallee, 1955; Vallee & Coleman, 1964), α -mannosidase might be regarded as a dissociable enzyme-metal ion complex, since it requires the addition of the functional cation during or after purification in order to maintain its activity. Metalloenzymes, on the other hand, reach a limiting value for their functional metal content during purification, without any loss in activity.

α -Mannosidase has been studied at a more acid pH than has been customary for the recognized metalloenzymes. By working at a more alkaline pH, if that were feasible, it might be found to behave more like a metalloenzyme. Whether α -mannosidase should be regarded as an enzyme-metal ion complex, or as a metalloenzyme that is dissociable at the pH of activity, it is clear that Zn²⁺ is essential for the integrity of the catalytically active molecule.

The specific action of Zn²⁺ is different from the unspecific effect of Cl⁻ on the activity of limpet α -mannosidase. Cl⁻ acts solely upon the rate of reaction with substrate. The distinction between the effects of Zn²⁺ and Cl⁻ in increasing the activity of the limpet enzyme is clearly shown by the inactivation experiments in Fig. 5 and by the kinetic experiments in Fig. 6.

Many enzymes display a form of 'metal activation' that is analogous to the action of Cl⁻ on limpet α -mannosidase. The difference between the two ways in which a dissociable cation can increase the activity of an enzyme has been somewhat obscured by the arbitrary division that has been made between metalloenzymes and enzyme-metal ion complexes. A more fundamental distinction would be one based on the mode of action of the metal ion, rather than on the dissociability of the active enzyme complex.

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