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EVIDENCE FOR A NON-LYSOSOMAL α-MANNOSIDASE IN RAT LIVER HOMOGENATES

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SUMMARY

The α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) activities in a lysosomal fraction and a soluble cytoplasmic fraction from rat liver were compared. The lysosomal enzyme was inhibited by EDTA and this effect was reversed by Zn^{2+} but by no other metal ion. Inactivation of the cytoplasmic α-mannosidase activity on dilution and incubation in the absence of substrate was potentiated by EDTA but prevented by the addition of low concentrations of Mn^{2+}, Co^{2+} or Fe^{2+}, whereas Zn^{2+} addition had an inhibitory effect. The enzyme activities in the two fractions exhibited widely different pH optima, that of the supernatant enzyme being near neutrality. The Michaelis constant for the supernatant enzyme was considerably lower than that for the lysosomal enzyme, with the *p*-nitrophenyl glycoside as substrate. Both enzyme activities were strongly inhibited by D-mannono-(1→5)-lactone.

The results strongly suggest that the enzyme activity in the cytoplasmic fraction of rat liver homogenates is partly due to a soluble α-mannosidase distinct from the lysosomal enzyme.

INTRODUCTION

From the results of previous studies on the distribution of α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) in mammalian tissues[1,2] it has generally been assumed that this enzyme is wholly lysosomal in its intracellular location, exhibiting typically latent activity which is relieved by the surface-active agent Triton X-100. The soluble low enzyme activity in blood plasma[3] was found to have an acidic pH optimum similar to that of the particulate enzyme in tissues and in common with other lysosomal glycosidases. The physiological role of mammalian α-mannosidase is presumed to be in the digestion of glycoprotein containing mannose residues, and ARONSON AND DE DUVE[4] have shown that enriched and apparently intact lysosomal fractions from liver homogenates of rats previously treated with Triton WR1339 can release most of the mannose content of ovalbumin glycopeptide.

Nevertheless, some lysosomal glycosidases, *e.g.* β-glucuronidase (EC 3.2.1.31)[5],

α-glucosidase (EC 3.2.1.20)[6] and β-*N*-acetylglucosaminidase (EC 3.2.1.30)[7], have been shown to be present also in other subcellular locations. In this paper evidence is presented for the presence in the soluble fraction of iso-osmotic homogenates of rat liver of a 'neutral' α-mannosidase which, from its characteristics, seems most unlikely to have arisen from lysosomal particles by leakage of the enzyme.

MATERIALS AND METHODS

Preparation of lysosome-enriched and supernatant fractions from homogenates

Freshly dissected and chilled livers from adult albino Wistar rats, previously starved for 24 h, were washed with 0.25 M sucrose and treated for 15 sec in 0.25 M sucrose, containing 1 mM 2-mercaptoethanol, in a chilled Potter–Elvehjem homogenizer, with Teflon pestle, to yield a 10% (w/v) homogenate. All subsequent operations were carried out at 0–3°. The fractionation procedure (Scheme 1) which followed was an abbreviated form of the method of RAGAB *et al.*[8]; it was initially conducted on a Sorvall RC2B centrifuge with SS34 rotor. The discontinuous sucrose gradient used to obtain lysosomal Sediment II, consisted of 14 ml of 0.60 M sucrose, 12 ml of 0.45 M sucrose, and 10 ml of 0.30 sucrose in a portion of which lysosomal Sediment I (from 8–12 g liver) was resuspended. The microsomal fraction was further centrifuged on the Beckman Model L2-65B centrifuge with the 65 rotor to yield a final supernatant cytoplasmic fraction devoid of particulate matter.

All sedimented pellets were washed on the centrifuge, then resuspended in ice-cold 0.25 M sucrose, containing 1 mM 2-mercaptoethanol and 1 mM phosphate pH 6.5, with a hand-driven glass homogenizer.

Enzyme assay

Incubation mixtures (2 ml) for routine assays of centrifugal fractions were buffered with either 0.1 M acetic acid–NaOH (pH 5.0) or with 0.05 M sodium dihydrogen phosphate–NaOH (pH 6.5) and contained 1.2 mM *p*-nitrophenyl α-D-mannopyranoside (Koch-Light Laboratories), 0.25 M sucrose and 1 mM 2-mercaptoethanol, together with the suitably diluted enzyme preparation. In assays of particulate fractions, 1 mM $ZnSO_4$ was also present, and 0.1% (w/v) Triton X-100 if total enzyme activity was to be measured. Incubation at 37°, usually for 1 h, was terminated by addition of 2 ml 0.4 M glycine–NaOH buffer (pH 10.5) and the mixture centrifuged for 15 000 × $g \cdot$min. Liberated aglycon was then measured immediately at 400 nm in the Unicam SP600 spectrophotometer. Controls were included to correct for absorbance due to unhydrolyzed substrate and unsedimented protein. Preliminary experiments indicated no adsorption of *p*-nitrophenol on to sedimented protein after glycine buffer addition, and showed that 0.25 M sucrose did not appreciably affect the rate of enzymic hydrolysis but reduced the absorbance of alkaline *p*-nitrophenol by about 6% at 400 nm. In all assays the amount of substrate hydrolyzed did not exceed 10% of the total and was usually less than 3%.

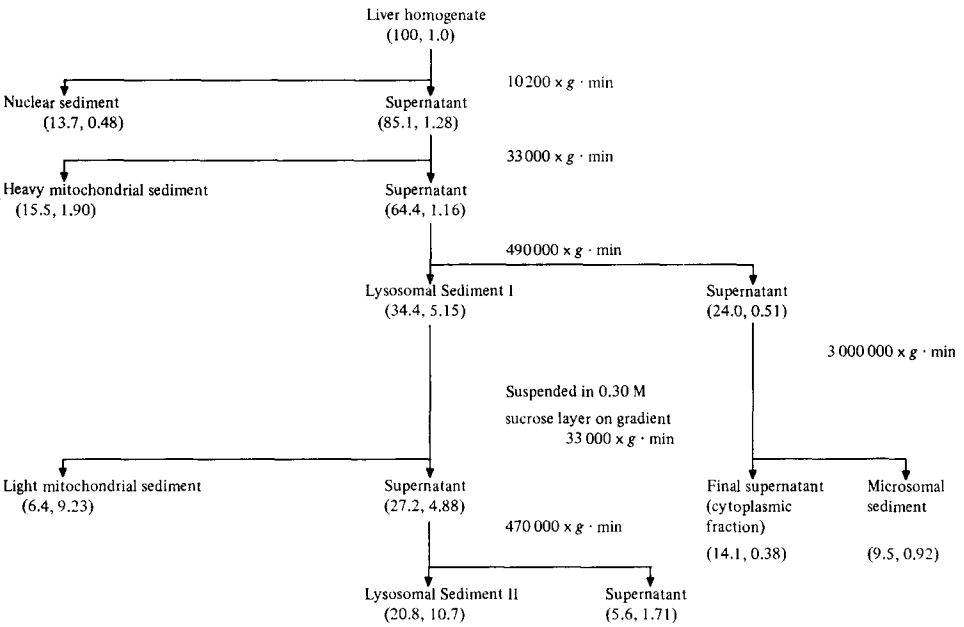
D-Mannono-(1→5)-lactone (m.p. 154–160°) was obtained by bromine oxidation of D-mannose[9], and freshly prepared aqueous solutions of this mannosidase inhibitor were used in the appropriate experiments.

Protein was estimated by the Lowry method as modified by OYAMA AND EAGLE[10], with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Homogenate fractionation

Appreciable α-mannosidase activity at pH 5.0 was found in all fractions (see Scheme 1) obtained by differential centrifugation of the crude tissue homogenate, probably indicating the heterogeneous nature of the lysosomal organelles. Recoveries of enzyme activity in the sedimented and supernatant fractions at each stage always



Scheme 1. Centrifugation procedure for liver homogenate. Values in parenthesis denote for each fraction the percentage total α-mannosidase activity at pH 5.0, then the relative specific enzyme activity, compared with the crude liver homogenate in a typical experiment. Details of sucrose gradient given in text.

exceeded 90%. Compared with similar fractionations of acid phosphatase in rat liver[8], the degree of purification of α-mannosidase in the final lysosomal sediment was appreciably lower, but the proportion of total liver enzyme activity recovered in this fraction was equivalently greater. The mean recovery of the enzyme in the lysosomal II fraction over 5 preparations was 17% of the homogenate activity. The proportion (14%) of original activity recovered in the cytoplasmic fraction was quite constant provided that the centrifugation procedure was carried out expeditiously after homogenization of fresh tissue, but if liver homogenates were allowed to stand overnight at 0° before fractionation, this value rose to 60% due to loss of lysosomal integrity and solubilization of the lysosomal enzymes.

Mean specific activity of α-mannosidase of the final lysosomal preparation was 12 nmoles *p*-nitrophenol per min per mg protein compared with 0.9 nmole *p*-nitrophenol per min per mg protein for the crude homogenate and 0.4 nmole *p*-nitrophenol per min per mg protein (at pH 5.0) for the cytoplasmic fraction.

Comparative properties of α-mannosidase in the lysosomal II and final supernatant (cytoplasmic) fractions

The apparent enzyme activity in absence of Triton X-100 of the final lysosomal fraction varied with the period of incubation during enzyme assay, being 24% of the total (Triton X-100 treated) activity for a 30-min incubation time, and 40% of total activity for 60 min incubation. Thus the latency of the lysosomal enzyme was markedly reduced after 30 min at 37° in presence of substrate. The enzyme activity of the supernatant fraction was not affected by 0.1% Triton X-100, and the rate of enzyme hydrolysis, in presence or absence of sucrose, was directly proportional to the amount of enzyme present over an 8-fold concentration range. The rate of hydrolysis of the substrate was unchanged during a 3-h incubation period; the lysosomal enzyme behaved similarly in the presence of Triton X-100. These results also indicated the absence of endogenous inhibitors of α-mannosidase, soluble or solubilised by Triton X-100, in either fraction.

The ratios of total enzyme activities at different pH values varied considerably for the different fractions obtained in the fractionation procedure. Thus the ratio of the activity at pH 5.0 to that at pH 6.5 were: for the crude homogenate, 0.57; for the lysosomal fraction, 3.3; for the supernatant fraction, 0.18 (these experiments were performed with 1 mM $ZnSO_4$ present in all assays). It can be seen that under conditions approaching physiological pH the supernatant enzyme activity contributed to most of the total α-mannosidase activity of the crude homogenate. At pH 6.5, in the absence of added zinc, the mean specific activity of the crude homogenate was 1.9 nmoles per min per mg protein, compared with 0.9 nmole per min per mg protein at pH 5.0 in the presence of 1 mM zinc. The specific activity of the enzyme in the soluble cytoplasmic fraction was 3.3 nmoles per min per mg protein at pH 6.5, and contained 75% of the total enzyme activity at this pH of the crude homogenate.

The variation of enzyme activity with pH for the lysosomal II and the supernatant (cytoplasmic) fractions is shown in Fig. 1. In common with most other lysosomal glycosidases, α-mannosidase in the lysosomal fraction had an acidic pH optimum near 5.0, whereas that of the supernatant enzyme was much closer to neutrality at pH 6.5, although a slight irregularity in the pH activity profile of the latter is noticeable to about pH 5, probably due to contamination by solubilized enzyme of lysosomal origin.

In some preliminary experiments, EDTA (0.1 mM) was added to the homo-

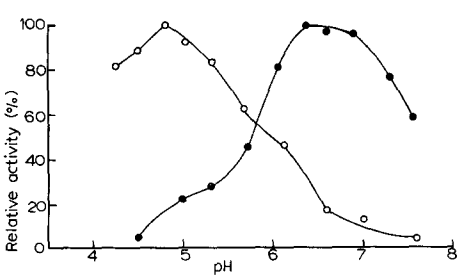


Fig. 1. Effect of pH on α-mannosidase activity of lysosomal fraction (○—○) and supernatant (cytoplasmic) fraction (●—●). Buffers were; for range pH 3.9–5.4, 0.1 M acetic acid–NaOH; for range pH 5.4–7.6, 0.05 M NaH_2PO_4–NaOH. Optimal activity of each fraction is expressed as 100.

genizing fluid, to minimise the possible inhibitory effects of heavy metal ions; it was noted, however, that the total α-mannosidase activity at pH 5.0 of the crude homogenate was then significantly reduced. The effects of EDTA and metal ions on the lysosomal and final supernatant enzyme activities were therefore studied. For these experiments, partial purification of the lysosomal enzyme was achieved by resuspending the lysosomal Sediment II in phosphate buffer (0.01 M, pH 6.5, 9 vol.), freezing the suspension at −20° overnight and removing particulate matter by centrifugation at 300 000 × g·min. Recovery of the now soluble lysosomal α-mannosidase activity was 78%. Fractionation at 0° with 20–50% (w/v) acetone (recovery of activity 57%) then gave a final product of specific activity 0.3 μmole/min per mg protein at pH 5.0. Attempts to partially purify the cytoplasmic enzyme were abandoned when its instability was noted (see below).

The very different effects of EDTA and metal ions upon the activity of the two mannosidase preparations are summarized in Table I. At the concentrations employed, the metal ions were found to change the absorption of alkaline p-nitrophenol at 400 nm by not more than 5%. The lysosomal enzyme was stable on incubation at 37° in absence of substrate but lost some activity (23%) on preincubation with 0.1 mM EDTA; this inhibition was potentiated by the presence of Fe^{3+} or Cu^{2+}

TABLE I

EFFECTS OF EDTA AND METAL IONS ON α-MANNOSIDASE ACTIVITIES OF SOLUBLE PREPARATIONS FROM LYSOSOMAL AND SUPERNATANT FRACTIONS

The enzyme preparation (1.8 ml), buffered (a) with 0.1 M acetic acid–NaOH, pH 5.0, for the lysosomal enzyme, or (b) with 0.1 M sodium maleate–NaOH, pH 6.5, for the supernatant enzyme, were incubated at 37° for 20 min before addition of substrate (0.2 ml, final concn. 1.2 mM), and incubation continued for a further 30 min. When EDTA was present, the metal ion was added 5 min prior to substrate addition.

Present in 1st incubation		*Loss of enzyme activity (%)*	
EDTA (0.1 mM)	*Metal ion (1 mM)*	*Lysosomal*	*Supernatant*
−	—	0	77
+	—	28	90
+	Li^+	29	89
+	K^+	30	87
+	Be^{2+}	34	88
+	Mg^{2+}	30	88
+	Ca^{2+}	30	91
+	Ba^{2+}	32	91
+	Al^{3+}	28	91
+	Mn^{2+}	22	−3
+	*Fe^{2+}	34	−42
+	*Fe^{3+}	75	76
+	Co^{2+}	21	−6
+	Ni^{2+}	23	93
+	Cu^{2+}	100	100
+	Zn^{2+}	−4	98
+	Ag^+	31	100
+	Cd^{2+}	32	100
+	Hg^{2+}	35	100
+	Pb^{2+}	41	93

* Concentration 0.5 mM.

but not by other heavy metal ions. Only in the presence of Zn^{2+} was the inhibition by EDTA reversed and the enzyme restored to full activity. SNAITH AND LEVVY[11,12] demonstrated that jack bean α-mannosidase and the lysosomal α-mannosidase of rat epididymis are zinc metalloenzymes, but found them to be inactivated by Cd^{2+}, Co^{2+}, Hg^{2+} and Ag^{+} as well as by Cu^{2+}.

There was a considerable loss of enzyme activity, accentuated by 0.1 mM EDTA, when the supernatant (cytosol) fraction was diluted 20-fold and incubated in the absence of substrate (Table I); inclusion of 1 mM mercaptoethanol had no effect upon this loss. Complete loss of activity was observed when the enzyme was incubated with 1 mM Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+} or Zn^{2+} in addition to EDTA. When these ions were included in the incubation of enzyme with substrate in absence of EDTA, the reduction in substrate hydrolysis during 30 min incubation at 37° was: with 1 mM Cu^{2+}, Ag^{+} or Hg^{2+}, 100%; with 1 mM Cd^{2+}, 65%; with 1 mM and 0.1 mM Zn^{2+}, 80% and 23% respectively. Enzyme activity after preincubation with EDTA was fully restored, however, by addition of 1 mM Mn^{2+} or Co^{2+} and activated (42%) by addition of 0.5 mM Fe^{2+}. In the absence of EDTA, minimum concentrations required to prevent loss of enzyme activity during dilution and incubation without substrate were: Mn^{2+}, 0.7 mM; Co^{2+}, 0.1 mM; Fe^{2+}, 0.08 mM. In contrast to the specific stabilizing effect of Zn^{2+} upon the lysosomal enzyme, addition of any of the above divalent ions thus maintained the activity of the supernatant α-mannosidase activity, for which Zn^{2+} was inhibitory.

Kinetic experiments in which the substrate concentration was varied over the range 0.24–5 mM give typical Michaelis curves of initial velocity for the lysosomal enzyme at pH 5.0 or 6.5 and for the supernatant enzyme at pH 6.5, with linear curves for double reciprocal plots. Values obtained for the Michaelis constant for α-mannosidase in the two preparations differed considerably, however; mean K_m values for four experiments were: for the lysosomal enzyme, 3.6 mM at pH 6.5 and 5.9 mM at pH 5.0; for the supernatant enzyme, 0.17 mM at pH 6.5. When examined at pH 5.0, the supernatant enzyme did not give a linear Lineweaver–Burk plot[13] but a

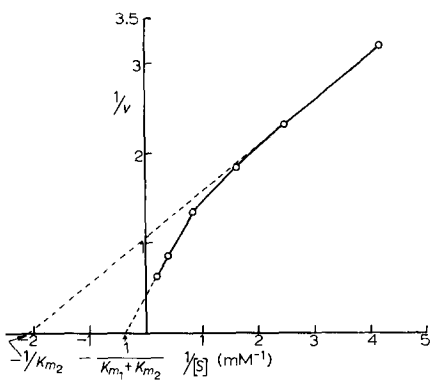


Fig. 2. Lineweaver–Burk plot[13] for variation with substrate concentration of α-mannosidase activity in supernatant (cytoplasmic) fraction at pH 5.0. Graphical derivation of K_m values are derived on assumption that two enzymes act upon the substrate and that their Michaelis constants differ considerably ($K_{m1} > K_{m2}$).

curve (Fig. 2) which could be explained by the combined hydrolytic action of two different enzymes. Based upon the assumption that these had widely differing K_m values, this curve could be divided into two linear sections from which the K_m values of the contributing enzymes could be calculated[14]; values of K_m derived thus were 0.48 mM and 2.9 mM, roughly approximating to the values found for the supernatant enzyme and the lysosomal enzyme, respectively.

Both enzyme fractions were strongly and competitively inhibited by D-mannono-(1→5)-lactone. Values of K_i obtained were with the lysosomal enzyme at pH 5.0, 39 μM (*cf.* LEVVY *et al.*[9]); with the supernatant enzyme at pH 6.5, 23 μM. The latter value is probably an overestimate, since the inhibitor undergoes spontaneous hydrolysis at pH 6.5 (ref. 9) and non-specific lactonases may also be present in the enzyme fraction[15]; for these reasons the period of incubation for assay in this experiment was reduced to 20 min.

These observations strongly suggest the presence of two distinct α-mannosidases in rat liver homogenates. The supernatant enzyme is apparently a soluble enzyme, without any latency properties and probably present in the cell cytoplasm, although it is possible that at lease some fraction of it may be attached to a subcellular particle and be solubilised during disruption of the cell. Further comparative studies, *e.g.* of differences in substrate specificity, will necessitate initial purification of the enzyme in the two centrifugal fractions; this has been achieved for the lysosomal α-mannosidase in rat epididymis[12] but difficulties have been encountered with the supernatant enzyme due to its instability; decreases of 20–50% activity on standing overnight at 0° were found with different preparations, although this was reduced to about 10% by addition of 0.1 mM Co^{2+}.

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