Enzymic characteristics of the isoenzymes of rat epididymal neutral α -mannosidases and their changes during development *in vivo*

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Three isoenzymic species of neutral α -mannosidase (I, II and III) were partially purified from rat epididymis and characterized. Calcium phosphate gel preferentially adsorbed acidic α -mannosidase activity, thereby removing the acidic enzyme from the neutral mannosidases. The neutral enzymes, which are of cytosolic origin, require Co^{2+} for activity, and Mn^{2+} can substitute partially for Co^{2+} . Mg^{2+} or Ca^{2+} had little effect on the activity of the isoenzymes, whereas Zn^{2+} ($100\text{ }\mu\text{M}$) was a potent inhibitor of the mannosidases. The K_m values of mannosidases I, II and III for Co^{2+} were 10, 10 and 2.7 mM respectively. There was marked alteration of the specific activity of the neutral α -mannosidases during epididymal development *in vivo*. The specific activities of mannosidases I and II were relatively high in the young (24 days) rats, and during the subsequent development the specific activities decreased markedly (approx. 2–3-fold). On the contrary, mannosidase III, which showed relatively low specific activity during 24–45 days of age, increased markedly (approx. 2-fold) as the animals reached adulthood.

An acidic α -mannosidase, derived from lysosomes, activated by Zn^{2+} and having maximal activity at pH around 4.5, has been demonstrated in several tissues, including epididymis (Snaith & Levvy, 1969, 1973; Conchie & Findlay, 1959). A non-lysosomal neutral α -mannosidase, which is activated by Co^{2+} and is maximally active at around neutral pH, has been demonstrated in the cytosol of several tissues (Shoup & Touster, 1976; Marsh & Gourlay, 1971). Unlike ram epididymis (Bullock & Winchester, 1973), it was not possible to demonstrate the occurrence of neutral α -mannosidase activity in rat epididymis when the enzymic activity was measured in the crude tissue extract at various pH values (Snaith, 1973, 1977). More recently, however, it has been possible to demonstrate neutral α -mannosidase activity in rat epididymis by isolating an $(\text{NH}_4)_2\text{SO}_4$ fraction of the enzyme that is largely devoid of the acidic enzyme (Snaith, 1977). Evidence has been provided for the presence of three α -mannosidase isoenzymes (pH optima 5.2, 6.5 and 7.3) in rat epididymis by measuring enzymic activity of the $(\text{NH}_4)_2\text{SO}_4$ fraction of the enzyme at various pH values. However, Snaith (1977) failed to separate physically the isoenzymic species by using several chromatographic techniques. By using DEAE-cellulose chromatography, Bullock & Winchester

(1973) were able to resolve ram epididymal neutral α -mannosidase activity into two enzymic species. However, little is known about the characteristics of these isoenzymes.

The present study resolved for the first time three isoenzymic species of the epididymal neutral α -mannosidase, and their enzymic characteristics were elucidated. The profile of the isoenzymes altered markedly during epididymal development *in vivo*. A preliminary account of this study has been reported (Dutta & Majumder, 1980).

Materials and methods

Chemicals

p-Nitrophenyl α -D-mannopyranoside, DEAE-cellulose, Triton X-100 and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calcium phosphate gel was prepared as described by Colowick (1955); a gel preparation that was aged for at least 1 year was used in the present study. All other reagents were of analytical grade.

Animals

Male albino rats obtained from the Institute's Animal House were used. Unless otherwise specified, epididymides were obtained from adult rats (90–110 days of age).

Isolation of epididymal neutral α -mannosidases

Epididymides were minced thoroughly and dispersed in 0.25M-sucrose/0.1% Triton X-100/10% (v/v) glycerol/5mM-2-mercaptoethanol (10ml/g of tissue) with gentle stirring for 5min to extract epididymal fluid containing spermatozoa present in the epididymal lumen. The supernatant fluid, containing spermatozoa and epididymal plasma, was removed by decantation, and the step was repeated twice. By this washing procedure approx. 98% of epididymal fluid was removed from the epididymides (Biswas & Majumder, 1982). The washed tissue was then homogenized in the same buffer (1g/10ml) and the homogenate was filtered through three layers of cheese-cloth.

The homogenate was centrifuged at 30000g for 30min, and the sediment was discarded. The resulting supernatant (Step 1) was dialysed against 0.25M-sucrose/20mM-sodium phosphate (pH 8.0)/0.1% Triton X-100/5mM-2-mercaptoethanol/10% glycerol/1mM- CoCl_2 (buffer A). The dialysed enzyme preparation was treated with calcium phosphate gel (approx. 8mg/mg of protein). The suspension was stirred for 30min and then centrifuged at 30000g for 10min. The resulting supernatant was dialysed extensively against buffer B, which is the same as buffer A except that phosphate buffer is replaced with 20mM-Tris/HCl, pH 7.5 (Step 2). The enzyme preparation was then applied to a column (1.5cm $\times$ 2.0cm) of DEAE-cellulose previously equilibrated with buffer B. The column was washed with 40ml of the equilibrating buffer before further elution with a linear gradient of Tris/HCl, pH 7.5 (20–800mM) in a total volume of 150ml. The flow rate was 30ml/h, and the volume in each fraction was 2.0ml (Step 3).

All the steps of the enzyme purification were carried out in the cold (0–6°C). Protein contents of the samples were measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay of neutral α -mannosidase

The standard assay mixture contained 50 μmol of Tris/HCl, pH 7.5, 4 μmol of CoCl_2 , 1 μmol of *p*-nitrophenyl α -D-mannopyranoside, 2.5mg of Triton X-100 and enzyme in a total volume of 0.43ml. The incubation was performed at 37°C for 20min, and the reaction was stopped with the addition of 1.0ml of 50mM-NaOH, when the final pH of the mixture increased to approx. 11.2. The liberated *p*-nitrophenol in the mixture was measured at 410nm in a Gilford spectrophotometer. The absorbance of *p*-nitrophenol was maximal at pH > 10.5, and its ϵ_{410} was $1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ under the standard assay conditions.

Systems lacking enzyme preparation served as blanks. One unit of the enzymic activity was

defined as the amount of the enzyme that catalysed the liberation of 1nmol of *p*-nitrophenol from *p*-nitrophenyl α -D-mannopyranoside under the standard assay conditions. The data shown are means of duplicate determinations differing by less than 10%. Unless otherwise specified, the data shown are representative of three or four experiments.

Results

Detection of neutral α -mannosidase activity in crude tissue extract

As shown in Fig. 1, as in previous studies (Snaith, 1973, 1977) it was not possible to demonstrate the presence of the neutral enzyme in the crude rat epididymal extract when the activity was measured with or without Zn^{2+} . However, when the assays were performed in the presence of Co^{2+} as the bivalent metal ion, a distinct peak of neutral mannosidase activity was detected in the epididymal extract, the activity being maximal at pH 7.5.

Fig. 1. Assay of neutral α -mannosidase activity in the crude extract of rat epididymis

Standard assay conditions were used for the assay of α -mannosidase activity in the 30000g supernatant (Step 1 of the purification) of the rat epididymis, except for the variation in the pH and omission (○) or specified additions of bivalent metal ions: CoCl_2 (5mM, Δ) or ZnCl_2 (1mM, $\bullet$). For pH ranges 4.0–7.0 and 7.5–9.0 sodium glycerophosphate/HCl (50mM) and Tris/HCl (50mM) buffers were used respectively.

Fig. 2. DEAE-cellulose chromatography pattern of epididymal neutral α -mannosidases

A sample (100 μ l) of each fraction (2.0 ml) was assayed for mannosidase activity (O) under the standard assay conditions. For details of chromatography, see the text. —, [Tris/HCl]; arrow indicates start of gradient.

Table 1. Summary of purification of neutral α -mannosidases from rat epididymis

Neutral α -mannosidases were isolated from 7 g of rat epididymis, and the enzymic activities of the various fractions were measured under the standard assay conditions.

Fractions	$10^{-3} \times$ Activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Homogenate	50.6	1400	36.1	100	1
Step 1: 30000g supernatant	34.4	930	36.9	68	1
Step 2: Calcium phosphate gel adsorption	29.5	126.4	233.7	58.4	6.4
Step 3: DEAE-cellulose chromatography					
Mannosidase I	5.8	25.2	229.4	11.4	6.3
Mannosidase II	5.4	26.4	206	10.7	5.7
Mannosidase III	14.5	6.4	2268	29	62.8

The data show that the presence of exogenous Co^{2+} is essential for the assay of the neutral enzyme in the crude tissue extract.

Enzyme purification

Calcium phosphate gel selectively adsorbed the acidic α -mannosidase (assayed at pH 4.1 with acetate buffer) and thus removed the acidic enzyme from the epididymal extract. This preparation of the neutral enzyme was resolved by DEAE-cellulose chromatography into three distinct peaks of activity (Fig. 2). Mannosidase I was not retained by the column. Fractions containing mannosidase I (fractions 3–9), II (fractions 23–30) and III (fraction 34–41) were pooled together and dialysed against buffer B containing 30% glycerol. These enzyme preparations were stored at -10°C and used for the present study unless otherwise specified. Under the above conditions of storage the enzymic activities were stable at least for 2 months.

Results of a typical enzyme purification procedure are shown in Table 1. Mannosidases I, II and III were purified approx. 6-, 6- and 62-fold respec-

tively. The isoenzyme preparations showed their characteristic elution profiles on rechromatography on DEAE-cellulose. The presence of mercaptoethanol, sucrose, CoCl_2 , Triton X-100 and glycerol in the enzyme purification and preservation media markedly stabilized the activity of all the isoenzymic species with special reference to enzyme II.

Properties of neutral α -mannosidases

The amount of *p*-nitrophenol liberated from the synthetic substrate *p*-nitrophenyl- α -D-mannopyranoside by the isoenzymes of epididymal neutral α -mannosidase increased linearly with time for at least 20 min under the standard assay conditions. A proportional increase in the activity of the neutral enzymes was observed with as much as 55 units of the enzymes. The activities of all the isoenzymes were lost nearly completely when heated at 80°C for 10 min. The K_m values of the isoenzymes for the synthetic substrate were nearly the same (approx. 2.0 mM). Mannosidase II was maximally active at pH 7.0, whereas both the enzymes I

Fig. 3. Effect of Co^{2+} concentration on the activity of epididymal neutral α -mannosidases

Standard assay conditions were used, except for the variation in the amount of the bivalent metal ion. Best-fit curves as judged by eye were drawn.

and III showed a pH optimum of 7.5 under the standard assay conditions when the concentration of the synthetic substrate was 2.3 mM.

Metal ions

Co^{2+} or Mn^{2+} (5 mM) markedly activated all the isoenzymes of neutral α -mannosidase. Mg^{2+} or Ca^{2+} (1–5 mM) had little effect on the activity of the mannosidases. The thiol reagents (1 mM) *p*-chloromercuribenzoate, HgCl_2 or PbCl_2 strongly inhibit (100%) the activity of all the isoenzymes, indicating that the thiol groups of the enzymes are essential for their activity. Zn^{2+} (100 μM) inhibited nearly completely the activity of all the isoenzymic species. However, mannosidases I and II showed higher sensitivity to Zn^{2+} than did mannosidase II.

The effect of different concentrations of Co^{2+} on the mannosidase activities is shown in Fig. 3. The K_m values of mannosidases I, II and III were approx. 10, 10 and 2.7 mM respectively.

Subcellular distribution

As shown in Table 2, most (approx. 85%) of the total enzymic activity of the tissue was recovered in the soluble fraction of the cells. Treatment of epididymal homogenate with 0.1% Triton X-100 had no appreciable effect on the neutral α -mannosidase activity, indicating that there may not be any latent mannosidase activity in the organelles. The data strongly suggest that the neutral α -mannosidase isoenzymes that were characterized in the present study are of cytosolic origin.

Epididymal-fluid contamination

Assay of mannosidase activity in epididymal fluid, which was present as a minor contaminant

Table 2. Subcellular distribution of neutral α -mannosidase activity in rat epididymis

Epididymides were homogenized by the procedure described in the Materials and methods section, except that Triton was omitted from the medium. The subcellular organelles were then isolated by differential ultracentrifugation (Majumder & Turkington, 1971). The particulate fractions were washed twice with the homogenization buffer and finally dispersed in the same medium. Neutral α -mannosidase activity of each fraction was then assayed under the standard assay conditions. Approx. 90% of the activity present in the homogenate was recovered in the subcellular fractions.

Fractions	Neutral α -mannosidase activity		
	(Units)	(Units/mg of protein)	Distribution (%)
Nuclei	820	8.1	4.8
Mitochondria	470	11.8	2.8
Microsomal	1480	61.6	8.4
Cytosol	14920	86.8	84

(2%) in the washed epididymides, revealed that the fluid contributed little (approx. 0.2%) towards the observed total cytosolic neutral α -mannosidase activity of the epididymis.

Activity of neutral α -mannosidases during epididymal development

As shown in Fig. 4, the specific activity of the enzyme was relatively high in young rats (24 days). Subsequently, with the onset of puberty there was a marked decrease in the specific activity of the enzyme until approx. 45 days of age ($P < 0.001$). The specific activity of the mannosidase, however, increased further as the animals attained adulthood ($P < 0.01$).

Fig. 5 shows that in the immature epididymis (24 days of age) mannosidase I was the dominant form of the isoenzyme, whereas during adulthood mannosidase III was the major isoenzyme. The specific activities of both the isoenzymes I and II were relatively high in the young rat (24 days), but during the subsequent development of the tissue the specific activities of the isoenzymes decreased progressively until approx. 45 days of age ($P < 0.01$). Minimal activities of these isoenzymes were maintained as the animals attained adulthood. The developmental pattern of the isoenzyme III, however, was markedly different from those of the other isoenzymes. There was little change in the specific activity of mannosidase III during 24–45 days of age. However, as the animals attained adulthood (90 days) the specific activity of mannosidase III increased markedly (approx. 2-fold; $P < 0.001$). Most of this increase occurred

Fig. 4. Specific activity of neutral α -mannosidase during development of rat epididymis

The enzyme activity in the epididymal extract (Step 1 of enzyme purification) was measured by the standard assay method. The data shown are means $\pm$ S.D. for five experiments (except for 55-day-old rats, where there were two experiments). Numbers of animals used for each experiment were 16–20 for 24–36 days of age and 6–9 for 45–95 days of age.

between 45 and 55 days of age, i.e. before sexual maturity.

Discussion

Earlier study failed to resolve multiple forms of neutral α -mannosidase in the rat liver, testis and epididymis, presumably owing to instability of the isoenzymes during the fractionation procedure (Snaith, 1977). The present study demonstrates for the first time resolution of the three isoenzymic species of neutral α -mannosidase in rat epididymal cytosol. Altered conditions of the purification procedure and stabilization of the enzymic activity in complex media containing sucrose, Triton X-100 etc. have perhaps made possible the separation of the isoenzymes. The characteristics of the cytosolic isoenzymes are markedly different from those of the lysosomal acidic enzyme (Snaith & Levvy, 1969; Opheim & Touster, 1978). It seems unlikely that the observed epididymal neutral α -mannosidase isoenzymes are derived from Golgi membrane or microsomal fractions, because the enzymic characteristics of the epididymal neutral enzymes are clearly different from those of the α -mannosidases derived from rat liver Golgi mem-

Fig. 5. Neutral α -mannosidase isoenzyme profiles during rat epididymal development

Enzymic activities were isolated from rat epididymides of various stages of development (10–20 rats at 24–45 days; 3–9 rats at 55–95 days) and then fractionated by DEAE-cellulose chromatography by the procedure described in the Materials and methods section. α -Mannosidase activities were measured under the standard assay conditions. Specific activities of the isoenzymes were calculated on the basis of the protein contents in the epididymal extracts (Step 1 of enzyme purification). Values were corrected for the loss of enzyme activity (5–20%) during the isolation procedure. The data shown are means $\pm$ S.D. for five experiments (except for 55-days-old rats, where there were two experiments).

branes (Tulsiani *et al.*, 1977) and rabbit liver microsomal fractions (Forsee & Schulzbach, 1981).

The present study reports for the first time the developmental pattern of neutral α -mannosidase in epididymis. The observation that the specific activity of mannosidase III increases specifically after 45 days of age, as the rats attained adulthood (Fig. 5), suggests that the isoenzyme may have a role in the attainment of sexual maturity of the organ. It is noteworthy that, unlike the neutral α -mannosidases, the specific activity of the acidic α -mannosidase increases from a very low value in infancy to a very high value in adulthood (approx. 9-fold) in rats and mice (Conchie & Hay, 1959; Conchie & Findlay, 1959). At present little is known about the physiological significance of the

isoenzymic forms of the neutral enzyme. It is possible that these enzymic species may differ in their specificity towards various naturally occurring glycoproteins and thus may serve specific functions. Specific alterations in the activities of the various isoenzymes of mannosidase during epididymal development suggest that these enzymic species may have a role in cell proliferation and differentiation in epididymis and in spermatozoal maturation.

It seems possible that epididymis, a well-known secretory organ, may release cytosolic neutral α -mannosidases in the lumen. Since the neutral glycosidases are highly active at the physiological pH, it is likely that neutral α -mannosidase and perhaps other neutral glycosidases of the reproductive fluids (rather than the acidic glycosidases) may cause alteration of glycosyl side chains of the glycoproteins derived from the spermatozoal surface (Olson & Hamilton, 1978) and the surrounding reproductive fluids, and thus may modulate spermatozoal physiology. Male reproductive organs and their secretions are rich sources of Zn^{2+} (Byar, 1974). Differential sensitivity of the isoenzymes to Zn^{2+} suggests that Zn^{2+} may have a regulatory role on the neutral mannosidase.

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