Isolation of β -*N*-acetylhexosaminidase from rabbit semen and its role in fertilization

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β -*N*-Acetylhexosaminidase was purified from the rabbit seminal plasma by a three-step procedure involving hydroxyapatite, Sephadex G-200 and concanavalin A–Sephadex chromatography. The specific activity of the purified preparation was 56 μ mol/min per mg of protein, which represented a 226-fold purification and a 54% yield of the enzyme activity. The purified enzyme was electrophoretically homogeneous. The homogeneous enzyme showed optimal activity at pH 4.0. The apparent K_m value and V_{max} were 1.4 mM and 56 μ mol/min per mg of protein respectively. Metal ions such as Ag^+ and Hg^{2+} and *p*-chloromercuribenzoate strongly inhibited the enzyme activity. The treatment of rabbit ova with a mixture of β -*N*-acetylhexosaminidase and arylsulphatase A results in the swelling of the zona pellucida.

β -*N*-Acetylhexosaminidase (EC 3.2.1.30) catalyses the hydrolysis of *N*-acetylgalactosamine or *N*-acetylglucosamine from β -glycosidic linkages of glycoproteins, glycosaminoglycans and glycolipids (Walker, 1966; Davidson, 1970; Hayashi, 1977, 1978; Kobata, 1979). In mammalian tissues, this enzyme exists in two major molecular forms, A and B, which can be separated by electrophoretic and ion-exchange chromatographic methods (Robinson & Stirling, 1968; Price & Dance, 1972; Swallow *et al.*, 1976). The enzyme has been extensively purified from various sources by using both the conventional and affinity chromatographic procedures (Srivastava *et al.*, 1974; Verpoorte, 1974; Geiger & Arnon, 1976; Marinkovic & Marinkovic, 1977; Wiktorowicz *et al.*, 1977). β -*N*-Acetylhexosaminidases A and B have very similar kinetic properties when assayed with synthetic substrates, but can be clearly distinguished from each other by their activities towards certain oligosaccharides (Robinson & Stirling, 1968; Bearpark & Stirling, 1978). β -*N*-Acetylhexosaminidase A appears to be a tetramer composed of two α - and two β -polypeptide chains and the hexosaminidase B is composed of four presumably identical β -chains (Geiger & Arnon, 1976).

The occurrence of β -*N*-acetylhexosaminidases in mammalian testes, semen and epididymis is well known (Woollen *et al.*, 1961; Leaback & Walker, 1967; Allison & Hartree, 1970; Majumder & Turkington, 1974). The enzyme has been purified

from the bull epididymis and semen (Pokorny & Glaudemans, 1975; Khar & Anand, 1977) and the rabbit sperm cytoplasmic droplets (Farooqui & Srivastava, 1979a). The present paper describes (1) a rapid method for the isolation of homogeneous β -*N*-acetylhexosaminidase from rabbit seminal plasma of much higher specific activity and greater yield than reported so far, (2) the kinetic properties of the enzyme and (3) the biological action of purified β -*N*-acetylhexosaminidase on the zona pellucida of the rabbit ova.

Materials and methods

The following materials were obtained from indicated sources: *p*-nitrophenyl β -*N*-acetyl-D-glucosaminide and -galactosaminide, Coomassie Brilliant Blue G-250, Fast Violet B and hyaluronidase from Sigma Chemical Co., St. Louis, MO, U.S.A.; Sephadex G-200 and concanavalin A–Sephadex from Pharmacia, Uppsala, Sweden; acrylamide, *NN*-methylenebisacrylamide from Eastman Chemical Co., Rochester, NY, U.S.A.; hydroxyapatite, Bio-Gel HTP from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Collection of semen

Two successive ejaculates from 50 rabbits were collected in an artificial vagina, twice weekly. Each time pooled semen was centrifuged at 900g for

20 min at 22°C to sediment spermatozoa and the supernatant containing the seminal plasma was stored at -20°C until used.

Enzyme assay

The enzyme was assayed spectrophotometrically by measuring the production of *p*-nitrophenol from *p*-nitrophenyl β -*N*-acetyl-D-glucosaminide. The reaction mixture consisted of 10 μ mol of sodium citrate buffer, pH 4.0, 4.8 μ mol of *p*-nitrophenyl β -*N*-acetyl-D-glucosaminide and up to 10 μ g of the enzyme protein in a total volume of 0.5 ml. After 10 min at 37°C, the reaction was terminated by the addition of 2.5 ml of 1 M-NaOH and the yellow colour emanating from liberated *p*-nitrophenol was read at 405 nm. One unit of the enzyme is defined as the amount liberating 1 μ mol of *p*-nitrophenol per min in the above conditions. The specific activity is expressed as units per mg of protein. The protein was determined by the method of Bradford (1976) with crystalline bovine serum albumin as the standard. The elution of protein from the chromatographic columns was monitored at A_{280} .

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel disc electrophoresis was performed at room temperature as described by Brewer & Ashworth (1969) on 7.5 and 10% gels in 0.03 M-Tris/HCl, pH 7.6, and Tris/glycine, pH 8.6, buffers at a current of 2 mA/tube. Bromophenol Blue was used as the tracking dye. The gels were stained with Coomassie Brilliant Blue G-250 in HClO₄ and destained with water (Reisner *et al.*, 1975). For activity determination, unstained gels were cut into 0.5 cm segments and incubated in the enzyme assay system for 2 h. For activity localization the gels were also stained by the method of Yoshikawa *et al.* (1972), using naphthol AS-B1 *N*-acetyl- β -glycosaminide (naphthol AS-B1 is 7-bromo-3-hydroxy-2-naphthol-*o*-aniside) and Fast Violet B instead of Fast Red Violet LB salt. The activity staining gave a red-violet band in the gel against the light-yellow background, which corresponded to the protein band obtained by the method of Reisner *et al.* (1975).

Treatment of rabbit ova with β -*N*-acetylhexosaminidase

Freshly superovulated ova were obtained by flushing the oviducts with 0.15 M-NaCl as described previously (Srivastava & Farooqui, 1979; Srivastava *et al.*, 1979). The cumulus clots were dispersed by hyaluronidase and the corona radiata was removed by suspending the ova in 2 ml of 0.1 M-NaHCO₃, pH 9.0, and vortex-shaking for 1 min (Srivastava *et al.*, 1979). The ova were washed three times with 2 ml of 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.15 M-NaCl (this is the buffer

used for all experiments in this section). Finally, the ova were transferred into small beakers containing 0.3 ml of the buffer. Each beaker had at least five ova. Various amounts of β -*N*-acetylhexosaminidase and rabbit testicular arylsulphatase A (Farooqui & Srivastava, 1979b) exhaustively dialysed against the above buffer were added to these beakers. The total volume of the incubation mixture was 0.5 ml. Five ova kept in 0.5 ml of the buffer served as controls. The beakers were incubated at 37°C and the ova were periodically examined under an Olympus inverted microscope.

Purification of β -*N*-acetylhexosaminidase from the rabbit seminal plasma by hydroxyapatite column chromatography

The seminal plasma, thawed at 4°C overnight, was centrifuged at 27000g for 30 min. The supernatant was dialysed for 18 h against 0.025 M-sodium phosphate buffer, pH 6.0. The precipitate that appeared during dialysis was removed and the clear supernatant was applied to a column (0.7 cm $\times$ 45 cm) of hydroxyapatite equilibrated in 0.025 M-sodium phosphate buffer, pH 6.0, at room temperature. The column was developed with the same buffer at 16 ml/h and 5 ml fractions were collected until the A_{280} reached zero. None of these fractions showed the enzyme activity. The column was then washed with 1 M-NaCl in 0.025 M-sodium phosphate buffer, pH 6.0. The enzyme appeared as a minor peak. The active fractions (peak I) were pooled and concentrated by ultrafiltration on a PM 10 Amicon membrane. No loss of activity was observed during the concentration. The elution of this column was continued with a linear gradient of 200 ml of 0.025 M-sodium phosphate buffer, pH 6.0, containing 1 M-NaCl and 200 ml of 0.5 M-sodium phosphate in the same buffer (pH 6.0). The major peak of enzyme activity (peak II) appeared after 100 ml of the gradient had passed through the column. The active fractions were pooled and concentrated as above. Both the peaks were dialysed against 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl.

Sephadex G-200 gel filtration

Peaks I and II were separately applied to a Sephadex G-200 column (4 cm $\times$ 90 cm) equilibrated with 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl. The column was washed with the same buffer in 2.5 ml fractions at a flow rate of 10 ml/h (Fig. 2). The fractions containing enzyme were pooled and concentrated by Amicon ultrafiltration as described above. Both preparations were then dialysed against 0.02 M-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl, 1 mM-MnCl₂ and 1 mM-CaCl₂.

Concanavalin A–Sephadex chromatography

The above preparations were separately applied at room temperature to a concanavalin A–Sephadex column (1.5 cm $\times$ 13 cm) equilibrated with 0.02 M-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl, 1 mM-MnCl₂ and 1 mM-CaCl₂ and eluted with the same buffer at a flow rate of 16 ml/h. After 150 ml of the buffer had passed through the column, the elution was continued with a linear gradient of 100 ml of the starting buffer and 100 ml of 0.5 M-methyl α -D-mannoside in the same buffer. The active fractions were pooled and concentrated to 3 ml by ultra-filtration.

Results

The purification scheme reported in the present paper (Table 1) gave an electrophoretically homogeneous preparation of β -N-acetylhexosaminidase with a specific activity of 56 μ mol/min per mg of protein. The yield of the enzyme was 54% from a 226-fold purification. The enzyme appeared to be tightly bound to the hydroxyapatite column as in pilot experiments the enzymically active fractions appeared only when the NaCl concentration in the eluting buffer reached 1 M. The elution profile of the enzyme through the hydroxyapatite column is shown in Fig. 1. This procedure results in separation of two forms of β -N-acetylhexosaminidase, one minor and the other major, designated as peaks I and II. Peak I is eluted with 1 M-NaCl and represents about 10% of the total β -N-acetylhexosaminidase applied to the column. Peak II, which represents about 70% activity of this enzyme, is eluted with 0 to 0.5 M-sodium phosphate gradient. The elution profiles of peaks I and II through Sephadex G-200 are shown in Fig. 2. The enzyme appears as a sharp peak that does not coincide with the main protein peak. The elution profile of peak II through the

concanavalin A–Sephadex column shows that 90% of the protein does not bind to the column but the enzyme is completely retained (Fig. 3). The enzyme appeared as a broad peak when the column was eluted with a gradient of methyl α -D-mannoside. A similar elution profile was obtained for peak I of β -N-acetylhexosaminidase. Since the enzyme activity of peak I was very low, it was not studied in detail. The purity of the final preparation (peak II) was judged by using polyacrylamide-gel disc electrophoresis at pH 7.6 and 8.6 on 7.5 and 10% polyacrylamide gels. The enzyme moved as a single band at these pH values (Fig. 4). The enzyme activity on unstained gel corresponded to the protein band of the stained gel by both methods of the activity localization (see the Materials and methods section). The kinetic data reported in the present paper were obtained by using peak II enzyme.

Fig. 1. Elution profile of rabbit seminal-plasma β -N-acetylhexosaminidase through the hydroxyapatite column

The experimental conditions and analysis of fractions for enzyme activity and protein are described in the Materials and methods section. The A and B arrows indicate the start of the 1 M-NaCl and 0.5 M-sodium phosphate gradients respectively. Symbols: $\bullet$, A_{280} ; $\blacksquare$, enzyme activity.

Table 1. Purification of β -N-acetylhexosaminidase from the rabbit seminal plasma

One unit of enzyme activity is defined as the amount liberating 1 μ mol of *p*-nitrophenol/min under conditions described in the text.

	Total protein (mg)	Total units	Specific activity (units/mg of protein)	Yield (%)
Dialysed seminal plasma	517	131	0.25	100
Hydroxyapatite				
Peak I	15.6	15	0.96	
Peak II	52.0	90	1.73	80
Sephadex G-200				
Peak I	3.4	9	2.64	
Peak II	11.0	75	6.81	64
Concanavalin A–Sephadex				
Peak I	0.25	3.5	14.0	
Peak II	1.20	68.0	56.6	54

Fig. 2. *Sephadex G-200 chromatography of rabbit seminal-plasma β -N-acetylhexosaminidase (peaks I and II)*

The experimental conditions and analysis of fractions for enzyme activity and protein are described in the Materials and methods section. Symbols: ●, A_{280} ; ■, enzyme activity. (a) Peak I; (b) peak II.

Fig. 3. *Concanavalin A-Sepharose chromatography of rabbit seminal-plasma β -N-acetylhexosaminidase (peak II)*

The experimental conditions and analysis of fractions for enzyme activity and protein are described in the Materials and methods section. The arrow indicates the start of the methyl α -D-mannoside gradient, —, A_{280} ; ----, enzyme activity.

Kinetic properties of β -N-acetylhexosaminidase

The homogeneous preparation of β -N-acetylhexosaminidase showed optimal activity at pH 4.0. The K_m value and V_{max} for p-nitrophenyl β -N-acetyl-D-glucosaminide were 1.4 mM and 56.6 μ mol/min per mg of protein respectively. The metal ions Fe^{3+} , Zn^{2+} , Ca^{2+} and Mn^{2+} had no effect on the activity of β -N-acetylhexosaminidase (Table 2). Ag^+ and Hg^{2+} caused a strong inhibition of the enzyme activity. Thus $AgCl$ (1 and 2 mM) produced 70 and 92% inhibition of enzyme activity. Similarly, $HgCl_2$ (0.05 and 0.1 mM) caused 45 and 70% of β -N-acetylhexosaminidase activity. p-Chloro-

Fig. 4. *Polyacrylamide-gel electrophoresis of purified rabbit seminal-plasma β -N-acetylhexosaminidase (peak II)*

The experimental conditions are described in the Materials and methods section. (a) 10% polyacrylamide gel at pH 8.6; (b) 7.5% polyacrylamide gel at pH 7.6. The lower band on the gels is due to tracking dye; 15 and 45 μ g of enzyme was used on (a) and (b) respectively.

mercuribenzoate at 0.025 and 0.05 mM produced 70 and 83% inhibition of the enzyme activity. Glutathione (1 mM) alone had no effect on β -N-acetylhexosaminidase activity but its addition to p-chloromercuribenzoate-treated enzyme led to a moderate stimulation (Table 2).

A comparison of heat stability of peak I and peak II enzyme in 0.04 M-sodium citrate, pH 4.0, at 55°C indicated that peak I enzyme did not lose any activity when subjected to 20 min treatment under above conditions, whereas peak II enzyme lost 85% of its activity in 20 min at 55°C.

Effect of β -N-acetylhexosaminidase on the zona pellucida of the rabbit ova

The separate treatment of the rabbit ova with 4 units of arylsulphatase A and 1 unit of β -N-

Table 2. *Effect of metal ions and p-chloromercuribenzoate on β -N-acetylhexosaminidase (peak II)*

The enzyme (5.0 μ g) was pre-incubated with indicated concentrations of metal ions and *p*-chloromercuribenzoate for 30 min at 37°C. The reaction was started by the addition of substrate and terminated after 10 min with 2.5 ml of 1 M-NaOH as described in the text.

Metal ion	Concn. (mM)	Activity (%)
Control	—	100
Fe ³⁺	2	100
Zn ²⁺	2	104
Mg ²⁺	2	100
Ca ²⁺	2	103
Mn ²⁺	2	102
Ag ⁺	0.5	57
	1.0	31
	2.0	8
Hg ²⁺	0.05	55
	0.10	30
	0.20	0.0
<i>p</i> -Chloromercuribenzoate	0.025	30
	0.05	17
<i>p</i> -Chloromercuribenzoate + reduced glutathione	0.025 + 1	67
Reduced glutathione	1	105

acetylhexosaminidase respectively at 37°C for 24 h or more did not produce any change in the morphology of the zona pellucida (Figs. 5a and 5b). However, a combination of 1 unit of β -*N*-acetylhexosaminidase and 4 units of arylsulphatase A caused a considerable swelling of the zona pellucida in 2 h (Fig. 5c). After 20 h the zona were further swollen but remained intact. The mode of action of these enzymes was determined in the following manner. The ova, having intact zona pellucida, were divided into three groups. The first group received 4 units of arylsulphatase A, the second group received 1 unit of β -*N*-acetylhexosaminidase and the third served as a control. After 20 h at 37°C there was no change in any group. The ova were then washed with 2 ml of 0.15 M-NaCl twice by transferring from one beaker to another to remove the respective enzymes. At this point, the washed ova from the first group (i.e. arylsulphatase A-treated) received 1 unit of β -*N*-acetylhexosaminidase; the second (i.e. β -*N*-acetylhexosaminidase-treated) received 4 units of arylsulphatase A and the control received an equivalent amount of the buffer. The arylsulphatase A-pretreated ova underwent swelling comparable with that shown in Fig. 5(c), in 30 min at 37°C after the addition of β -*N*-acetylhexosaminidase. The β -*N*-acetylhexosaminidase-pretreated ova did not show any swelling even 20 h after the addition of arylsulphatase A and were indistinguishable from the control (Fig. 5d). Once again the swollen zona remained intact (Fig. 5c). When the ova with swollen zona were placed in 5 M-NaCl for 20 h there was no evidence of any shrinkage indicating, thereby, that the swelling event was

enzymic, irreversible and that it was not a consequence of the osmotic shock. The size of vitellus remained approximately the same in all ova, except a combined treatment with arylsulphatase A and β -*N*-acetylhexosaminidase, which caused a considerable swelling of the zona pellucida (Fig. 5c), also gave an appearance of slight shrinkage of the vitellus.

Discussion

The results reported in the present paper indicate that the rabbit seminal plasma contains two forms of β -*N*-acetylhexosaminidase that can be separated on a hydroxyapatite column. The most efficient purification steps comprise chromatography on hydroxyapatite and concanavalin A-Sepharose. The retention of the rabbit seminal plasma β -*N*-acetylhexosaminidase on the concanavalin A-Sepharose column and its elution by methyl α -D-mannoside indicate that the binding of this enzyme with concanavalin A-Sepharose is through the carbohydrate moiety. Similar results were obtained by Banerjee & Basu (1975), Brattain *et al.* (1977) and Wiktorowicz *et al.* (1977). The specific activity of the purified enzyme (peak II) was 90 times higher than that reported for the homogeneous preparations of the bull epididymis (Pokorny & Glaudemans, 1975) and bull semen (Khar & Anand, 1977) β -*N*-acetylhexosaminidase. The reported specific activities for the homogeneous preparations of human kidney and beef spleen β -*N*-acetylhexosaminidase with *p*-nitrophenyl *N*-acetylglucosaminide as a substrate are 85 and 21 μ mol/min per

Fig. 5. Effect of β -N-acetylhexosaminidase and arylsulphatase A on the zona pellucida of the rabbit ova. The experimental conditions are described in the Materials and methods section. (a) Arylsulphatase A (4 units); (b) β -N-acetylhexosaminidase (4 units); (c) arylsulphatase A (4 units) + β -N-acetylhexosaminidase (1 unit) for 2 h; (d) control. Abbreviations used: Z, zona pellucida; V, vitellus. Magnification $\times 1000$.

mg respectively (Verpoorte, 1972; Marinkovic & Marinkovic, 1977). β -N-Acetylhexosaminidase activity of various tissues can be separated into two major molecular forms, A and B, by chromatographic and electrophoretic procedures (Robinson & Stirling, 1968; Price & Dance, 1972; Swallow *et al.*, 1976). Heating at pH 4.4 and 50°C destroys the activity of β -N-acetylhexosaminidase A, whereas the B form of this enzyme is quite stable under the above conditions (Okada & O'Brien, 1969; O'Brien *et al.*, 1970; Lo & Kritchevsky, 1979). Peak I of the rabbit seminal plasma β -N-acetylhexosaminidase did not lose any activity at pH 4.0 and 55°C for 20 min, whereas peak II lost 85% of its activity in 20 min at 55°C. This observation suggests that peaks I and II of the rabbit seminal plasma are similar to β -N-acetylhexosaminidases B and A activities respectively of other tissues. The kinetic properties of the homogeneous preparation of the rabbit seminal β -N-acetylhexosaminidase are comparable with the bull seminal-plasma enzyme (Khar & Anand, 1977).

The newly ovulated mammalian ova are embedded in a mass of follicular cells, namely the cumulus oophorus, the corona radiata and acellular zona pellucida. At present nothing is known about the chemical composition of these layers, but hyaluronic acid is considered to be the main constituent of the cumulus oophorus (Piko, 1969). Previous work from this laboratory (Farooqui & Srivastava, 1979b) has indicated that homogeneous preparations of the rabbit testicular arylsulphatase A were able to disperse the cumulus oophorus of the rabbit ova. It was also reported that arylsulphatase A had no effect on the corona radiata and the zona pellucida of the rabbit ova. In the present study it was observed that β -N-acetylhexosaminidase or arylsulphatase A alone had no effect on the zona pellucida. However, when ova were treated with a mixture of β -N-acetylhexosaminidase and arylsulphatase A, the zona pellucida underwent considerable swelling, a remarkable morphological change that is important in relation to the proposed role of β -N-acetylhexosaminidase and arylsulphatase A in the degradation of sulphated glycoproteins, sulphated glycosaminoglycans and sulphoglycolipids (Davidson, 1970; Hayashi, 1977, 1978). The zona pellucida of the mammalian ova has been reported to be a relatively thick acellular layer rich in glycoproteins and sulphated glycoproteins and sulphated glycosaminoglycans (Braden, 1952; Gothie, 1958; Seshachar & Bagga, 1963).

Although our work does not provide any quantitative evidence that sulphated glycoproteins and glycosaminoglycans of the zona pellucida are the substrates for β -N-acetylhexosaminidase and arylsulphatase A, a recent paper by Dunbar *et al.* (1980) suggests that the zona pellucida is indeed a glycoprotein layer containing sulphate and phosphate

groups. Therefore, the effective penetration of this layer by spermatozoa must involve the use of arylsulphatase A, and β -N-acetylhexosaminidase, and possibly phosphohydrolases, before the proteolytic digestion by acrosin. The results reported in the present paper indicate, for the first time, that the effect of these enzymes on the zona pellucida is a sequential process. For inducing the morphological changes in the zona it was necessary to treat the ova first with arylsulphatase A and then with β -N-acetylhexosaminidase. No change was observed in those ova that were first treated with β -N-acetylhexosaminidase and then with arylsulphatase A. This is consistent with the results of Hayashi (1978), who reported that β -N-acetylhexosaminidase does not act on sulphated galactosaminyl bonds of the sulphated oligosaccharides. The treatment of these oligosaccharides with sulphatase is a prerequisite for the catabolism of these substances. It has been reported (Akruk *et al.*, 1979) that during capacitation and acrosomal reaction the vesiculation of the plasma and outer acrosomal membranes releases arylsulphatase A and β -N-acetylhexosaminidase. The activity of these enzymes on the glycosyl side-chain of the zona glycoproteins and glycosaminoglycans may expose certain receptors necessary for sperm-egg interaction, and may, consequently, facilitate the subsequent proteolytic digestion by acrosin (Gwatkin & Williams, 1978; Gwatkin, 1978; Max, 1978; Peterson *et al.*, 1980).

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