Purification, biochemical properties and active sites of *N*-acetyl- β -D-hexosaminidases from human seminal plasma

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Two isoenzymes of *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) (Hex A and Hex B) from human seminal plasma were purified to homogeneity with specific activities of 26 and 60 units/mg of protein respectively. *N*-Acetyl- β -D-glucosaminidase activity was inseparable from *N*-acetyl- β -D-galactosaminidase activity in both Hex A and Hex B by various conventional chromatographic procedures. Although K_m values of *N*-acetyl- β -glucosaminidase activity of Hex A and Hex B were similar (1.33 mM), those of *N*-acetyl- β -galactosaminidase activity were 0.14 mM for Hex A and 0.40 mM for Hex B. However, pH optima and temperature optima were identical for *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities of both isoenzymes; Hex A was far more heat-sensitive than Hex B. Thiol-reactive compounds such as silver salts, mercuric salts, *p*-chloromercuribenzoate and thimerosal strongly inhibited the *N*-acetyl- β -glucosaminidase activities of both isoenzymes. GSH protected the enzyme activities from inactivation caused by these reagents, confirming the presence of thiol groups at the active centres. Inhibitions of *N*-acetyl- β -glucosaminidase activities of both isoenzymes by metal salts and organic anions were comparable; acetate and arsenite were effective inhibitors for both isoenzymes. In contrast, inhibitions of *N*-acetyl- β -glucosaminidase activities of the two isoenzymes by iodoacetic acid, iodoacetamide and ethylmaleimide were not comparable; Hex B was more susceptible to inhibition by these agents at 20 mM concentration. The *N*-acetyl- β -glucosaminidase activities of both isoenzymes are strongly inhibited, in decreasing order, by *N*-acetylglucosamine, mannosamine, disaccharic acid lactone, *N*-acetylglucosamine and gluconolactone. The K_i values of the *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities for *N*-acetylhexosamines and results from mixed-substrate kinetics indicated that the activities for the two substrates are located at different sites in Hex A and at the same site in Hex B. The M_r values of Hex A and Hex B were determined to be 195000 and 210000 respectively by gel filtration through Sephadex G-200. SDS/polyacrylamide-gel electrophoresis revealed that Hex A and Hex B are each composed of four subunits corresponding to M_r about 50000 each. No further polypeptide chain was obtained after reduction and alkylation of Hex A and Hex B with 10 mM-dithiothreitol and 10 mM-iodoacetamide.

INTRODUCTION

The occurrence of *N*-acetyl- β -D-hexosaminidase in mammalian testes, semen and epididymis is well known (Allison & Hartree, 1970; Woolen *et al.*, 1961; Leaback & Walker, 1967; Majumdar & Turkington, 1974). These glycosidase activities in the acrosome are known to play a vital role in the penetration of sperm and interaction with the egg (Allison & Hartree, 1970; McRorie & Williams, 1974). *N*-Acetyl- β -D-glucosaminidase has been purified from bull epididymis and semen (Pokorony & Glaudemans, 1975; Khar & Anand, 1977), rabbit sperm cytoplasmic droplets and seminal plasma (Farooqui & Srivastava, 1979, 1980) and mouse testes (Gupta & Kapur, 1981). Though *N*-acetyl- β -hexosaminidases have been studied extensively, no attention has been given to the characterization of two forms of the enzyme from human semen (Gupta & Agarwal, 1982; Gupta & Kapur, 1983). The present paper describes the isolation of two *N*-acetyl- β -hexosaminidase isoenzymes from human seminal plasma and a study of their comparative kinetic properties. Immunological specificity and identity of seminal-plasma *N*-acetyl- β -glucosaminidase with acrosomal *N*-acetyl- β -hexosaminidase have been reported previously (Kapur & Gupta, 1985).

MATERIALS AND METHODS

Enzyme assay

N-Acetyl- β -D-glucosaminidase (EC 3.2.1.30) activity was assayed with *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as substrate (Tarentio & Maley, 1972). One unit of enzyme is that amount of protein which liberates 1 μ mol of *p*-nitrophenol/min at 37 °C. Hyaluronidase and β -glucuronidase activities were measured as reported previously (Gupta & Goldberg, 1981), and protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Purification of Hex A and Hex B

Step 1. Human semen obtained from healthy donors was centrifuged at 3000 *g* for 20 min, and the supernatant was re-centrifuged at 11000 *g* for 30 min to obtain clear seminal plasma. Seminal plasma free from spermatozoa was saturated at 40% with solid (NH₄)₂SO₄ at 4 °C and centrifuged at 11000 *g* for 40 min. The supernatant, saturated to 80% with (NH₄)₂SO₄, was left at 4 °C overnight and spun at 11000 *g*. The residue was dissolved and dialysed against 20 mM-Tris/HCl buffer, pH 7.3.

Abbreviations used: Hex A and Hex B, isoenzymes of *N*-acetyl- β -D-hexosaminidase.

Fig. 1. Elution profile of seminal-plasma Hex A and Hex B from a DEAE-cellulose column

N-acetyl- β -hexosaminidase was obtained from $(\text{NH}_4)_2\text{SO}_4$ -precipitated proteins (step 1) and applied to a DEAE-cellulose column. Hex B activity was eluted with 20 mM-Tris/HCl buffer, pH 7.3, and Hex A activity was eluted with a linear 0–0.5 M-NaCl gradient in Tris buffer (----). ●, A_{280} (protein); ○, A_{405} (*N*-acetyl- β -glucosaminidase activity).

Step 2. The dialysis residue from Step 1 was applied to a DEAE-cellulose column (2.5 cm $\times$ 20 cm) pre-equilibrated with 20 mM-Tris/HCl buffer, pH 7.3, at 4 °C. The column was eluted with the same buffer and subsequently by applying a gradient of 0–0.5 M-NaCl in Tris buffer at a flow rate of 20 ml/h. Two enzyme peaks, designated Hex A and Hex B (Fig. 1) were pooled separately, concentrated and dialysed against phosphate-buffered saline (0.9% NaCl in 20 mM-phosphate buffer, pH 6.0).

Step 3. The two forms of the enzyme from step 2 were applied to separate concanavalin A-agarose columns (1.0 cm $\times$ 10 cm) pre-equilibrated with phosphate-buffered saline and eluted with 10% α -methyl D-mannoside in phosphate-buffered saline. The active fractions of the A and the B forms of the enzyme were concentrated and dialysed against 20 mM-phosphate buffer, pH 6.0, and the B form was concentrated and dialysed against 10 mM-citrate buffer, pH 5.0.

Step 4. The A form of the enzyme after step 3 was re-chromatographed on a DEAE-cellulose column (2.5 cm $\times$ 15 cm) to remove any contamination by Hex B. The column was pre-equilibrated with 20 mM-sodium phosphate buffer, pH 6.0, and eluted with a 0–0.2 M-NaCl gradient. Active fractions of Hex A were concentrated and dialysed against 50 mM-phosphate buffer, pH 6.0.

Step 5. The B form of the enzyme from step 3 was applied to a CM-cellulose column (2.5 cm $\times$ 15 cm) pre-equilibrated with 1 mM-citrate buffer, pH 5.0, and eluted with a 0.05–0.9 M-NaCl gradient. Active fractions were dialysed and concentrated against 50 mM-phosphate buffer, pH 6.0.

Fig. 2. Elution profiles of seminal-plasma Hex A (a) and Hex B (b) from a Sephadex G-200 column

Hex A and Hex B were obtained from Sepharose 6B columns (step 6) and applied to Sephadex G-200 columns. *N*-Acetyl- β -hexosaminidase activity was eluted with 50 mM-phosphate buffer, pH 6.0. ●, A_{280} (protein); ○, A_{405} (*N*-acetyl- β -D-glucosaminidase activity).

Step 6. The purified fractions with *N*-acetyl- β -glucosaminidase activity from steps 4 and 5 were passed through a Sepharose 6B column (2.2 cm $\times$ 70 cm) pre-equilibrated with 50 mM-phosphate buffer, pH 6.0, and eluted. Fractions containing Hex A and Hex B were each pooled and freeze-dried.

Step 7. The purified enzyme fractions from Step 6 were each fractionated on a Sephadex G-200 column (1.8 cm $\times$ 80 cm) pre-equilibrated with phosphate-buffered saline and eluted with the same buffer. Active fractions of Hex A and Hex B were each pooled, dialysed against water and freeze-dried (Figs. 2a and 2b).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out on 7.5% gels at pH 8.3 (Davis, 1964). Gels were stained for proteins with Coomassie Blue R-250, and enzyme bands were stained by the method of Hayashi (1965) with Naphthol AS-BI β -D-glucopyranoside as substrate.

Determination of subunit M_r

SDS/polyacrylamide-gel electrophoresis was carried out according to the method of Weber & Osborn (1969). The subunit M_r was determined from a standard curve by plotting electrophoretic mobility (R_F) versus $\log M_r$ of standard proteins. R_F was calculated as follows:

$$R_F = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye front}} \times \frac{\text{Gel length before staining}}{\text{Gel length after staining}}$$

Table 1. Summary of purification of *N*-acetyl- β -hexosaminidases from human seminal plasma

Step	Protein (mg)	Activity (units)	Sp. activity (units/mg of protein)	Yield (%)	Purification (fold)
1. Seminal plasma	1800	310.0	0.17	100	1
2. 80% -satn.-(NH ₄) ₂ SO ₄ ppt.	850	264.0	0.31	85	2
3. DEAE-cellulose chromatography					
Hex A	132	62.4	0.47	50*	7*
Hex B	256	118.4	0.47	63*	5*
Hex A					
4. Concanavalin A-agarose chromatography	10.5	42.0	0.0	33*	60*
5. DEAE-cellulose chromatography	4.2	28.0	6.7	23*	100*
6. Sepharose 6B chromatography	1.4	23.5	17.0	19*	250*
7. Sephadex G-200 chromatography	0.8	20.5	26.0	10.5*	380*
Hex B					
8. Concanavalin A-agarose chromatography	16.0	103.7	6.5	56*	64*
9. CM-cellulose chromatography	3.2	73.0	23.0	40*	226*
10. Sepharose 6B chromatography	1.7	60.0	35.3	32*	346*
11. Sephadex G-200 chromatography	0.85	52.0	60.0	27*	590*

* Percentage yield and fold purification are calculated on the basis that Hex A is 40% and Hex B is 60%.

Fig. 3. Polyacrylamide-gel electrophoresis of seminal-plasma Hex A (gels c-h) and Hex B (gels i-n) after various steps of purification

The gels h and n were stained for enzyme activity, and the other gels were stained for protein with Coomassie Blue R-250. Gel a, seminal plasma; gel b, 80% -satn.-(NH₄)₂SO₄ ppt.; gels c and i, DEAE-cellulose fractions; gels d and j, concanavalin A-agarose fractions; gel e, DEAE-cellulose fraction; gel k, CM-cellulose fraction; gels f and l, Sepharose fractions; gels g and m, Sephadex G-200 fractions; gels h and n, enzyme activity in purified preparations. D represents band due to dye front.

RESULTS

Purification of Hex A and Hex B

Purified seminal-plasma Hex A and Hex B showed 380-fold and 590-fold purification respectively as compared with activities in seminal plasma (Table 1). Polyacrylamide-gel electrophoresis of each isoenzyme showed one protein band corresponding to the enzyme activity (Fig. 3). Moreover, it has been demonstrated that

these preparations are also immunologically homogeneous (Kapur & Gupta, 1985). Antisera raised against these purified preparations showed single precipitation arcs with most human tissue extracts in Ouchterlony plates.

Purified Hex A and Hex B were free of hyaluronidase and β -glucuronidase activities and did not show any release of *p*-nitrophenol when tested with *p*-nitrophenyl substrates of various glycosidases except for *p*-nitrophenyl β -fucosidase activity.

Biochemical properties of Hex A and Hex B

Under standard assay procedure seminal-plasma Hex A and Hex B showed identical optimum temperature (60 °C) and pH (4.5) values. After being heated at 50 °C for 20 min and cooled for 2 min at 4 °C Hex A lost 80% of its activity, whereas Hex B retained 75–80% of its activity after being heated at 50 °C for 60 min. K_m and V_{max} values of Hex A and Hex B were compared with *p*-nitrophenyl *N*-acetyl- β -glucosaminide (0.2–1.2 mM) and *p*-nitrophenyl *N*-acetyl- β -galactosaminide (0.1–1.2 mM) as substrates. The K_m values of the *N*-acetyl- β -glucosaminidase activities in Hex A and Hex B were identical (1.33 mM). On the other hand, the K_m of the *N*-acetyl- β -galactosaminidase activity of Hex A was 0.14 mM and was in contrast with the Hex B K_m value of 0.40 mM (Table 5). Results suggest that the substrates protect both isoenzymes from thermal denaturation.

Effect of salts and thiol-reactive compounds

The activities of the Hex A and Hex B isoenzymes remained unchanged in the presence of BaCl₂, NiCl₂, MnCl₂ and CoCl₂ and sodium salts of citrate, succinate and maleate at 10 mM for 30 min before the addition of substrate. On the other hand, ZnCl₂ (10 mM), arsenite (20 mM) and acetate (20 mM) inhibited both isoenzymes by up to 30–50%. *N*-Acetyl- β -hexosaminidases from colon mucosa are known to be unaffected by arsenite, whereas acetate is a competitive inhibitor (Mian *et al.*, 1978).

Table 2. Effects of thiol-reactive agents on *N*-acetyl- β -glucosaminidase activity

Values are expressed as percentages of controls in which agent has not been added. Abbreviation: PCMB, *p*-chloromercuribenzoate.

Inhibitor	Activity (%)	
	In Hex A	In Hex B
HgCl ₂ (0.02 mM)	0	0
AgNO ₃ (0.02 mM)	0	0
PCMB (0.02 mM)	0	0
Thimerosal (5.0 mM)	56	32
HgCl ₂ (0.02 mM)+GSH (10.0 mM)	80	90
AgNO ₃ (0.02 mM)+GSH (10.0 mM)	95	94
PCMB (0.02 mM)+GSH (10.0 mM)	93	83
Thimerosal (5.0 mM)+GSH (10.0 mM)	70	57

Table 3. Effects of various sugars and their derivatives on *N*-acetyl- β -glucosaminidase activity

Values are expressed as percentages of controls in which agent has not been added.

Inhibitor	Concn. (mM)	Activity (%)	
		In Hex A	In Hex B
<i>N</i> -Acetylglucosamine	20	35	40
	50	28	23
<i>N</i> -Acetylgalactosamine	20	8	8
	50	3	3
Mannosamine	20	16	12
	50	9	5
Gluconolactone	20	48	51
	50	22	33
Disaccharic acid 1,4-lactone	20	30	40
	50	13	10

Iodoacetic acid, iodoacetamide and *N*-ethylmaleimide at 5 mM concentration did not show any significant effect on Hex A or Hex B, whereas HgCl₂, AgNO₃, *p*-chloromercuribenzoate and thimerosal were potent inhibitors for both isoenzymes (Table 2). Addition of 10 mM-GSH along with silver and mercury compounds reversed the inactivation of both Hex A and Hex B. Whether GSH displaces the inhibitor from the binding site or binds to the inhibitor during protection of the isoenzymes remains unascertained.

Effects of sugars and their derivatives

Mannose, glucose, fructose, sucrose, maltose, methyl α -D-glucoside and galactonolactone (50 mM) did not produce any effect on the enzyme activity of either Hex A or Hex B. However, in order of sensitivity *N*-acetyl- β -galactosamine, mannosamine, disaccharic acid 1,4-lactone, *N*-acetyl- β -glucosamine, gluconolactone and glucosamine were effective inhibitors of both isoenzymes (Table 3). α -Methyl D-mannoside and glucosamine were effective inhibitors only at 50 mM. Moreover, the magnitudes of inhibition of two isoenzymes by a single compound were more or less identical.

Dixon plots for various concentrations of *N*-acetyl- β -hexosamines and glucosamine suggested that hexos-

amines and their acetylated derivatives are competitive inhibitors of seminal-plasma Hex A and Hex B. From the K_i values of Hex A and Hex B for hexosamines (Table 4) it is apparent that *N*-acetyl- β -galactosamine is a more effective inhibitor than *N*-acetyl- β -glucosamine, and that *N*-acetyl- β -glucosamine is more effective than glucosamine with both *N*-acetyl- β -glucosaminide and *N*-acetyl- β -galactosaminide substrates for the two isoenzymes. However, when data for two different substrates are compared for the same isoenzyme, it is seen that the K_i value for each of these inhibitors with respect to *p*-nitrophenyl *N*-acetyl- β -glucosaminide is lower than the value with *p*-nitrophenyl *N*-acetyl- β -galactosaminide as substrate. This indicates that these inhibitors have stronger binding affinity at the active centre of the *N*-acetyl- β -glucosaminidase activity than at the active centre of the *N*-acetyl- β -galactosaminidase activity of the two isoenzymes. At the same time it is clear from the K_i values that Hex B has greater binding affinity than Hex A for hexosamines and acetylated hexosamines.

Kinetics of mixed-substrate reaction

Kinetics of the mixed-substrate reaction were monitored in accordance with Dixon & Webb (1964) and Mian *et al.* (1978) for the appearance of *p*-nitrophenol in the

Table 4. Inhibition constants of *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities of Hex A and Hex B in the presence of glucosamine and *N*-acetylhexosamines

The values were determined from Dixon plots, by using a regression best-fit curve ($y = mx + c$) with correlation coefficient $r = 0.98$, $P < 0.01$ and degree of freedom $(n - 2) = 7$.

Inhibitor	K_i (mM)			
	<i>N</i> -Acetyl- β -glucosaminidase		<i>N</i> -Acetyl- β -galactosaminidase	
	Hex A	Hex B	Hex A	Hex B
<i>N</i> -Acetylgalactosamine	0.75	0.45	2.60	2.0
<i>N</i> -Acetylglucosamine	3.50	2.90	8.70	5.6
Glucosamine hydrochloride	20.0	8.0	—	—

Table 5. Kinetic parameters for Hex A and Hex B in the presence of various ratios of two substrates present simultaneously

The values are means for three experiments, calculated by using a regression curve ($y = mx + c$) with correlation coefficient $(r) = 0.95-0.99$, $P < 0.01$ and degree of freedom $(n - 2) = 5$. K_a , K_b , V_a , V_b , V_{a+b} and μ are defined in the text.

	K_a (mM)	K_b (mM)	V_a	V_b	μ	V_{a+b} (units mg of protein)	
						Observed	Calculated
Hex A	1.37	0.14	17	2.1	1	13	3.50
					5	27	7.00
					0.2	4.9	2.40
Hex B	1.37	0.40	35	4.37	1	11.1	11.3
					5	22.6	23.2
					0.2	6.0	6.1

presence of different proportions of two substrates (*p*-nitrophenyl *N*-acetyl- β -glucosaminide and *p*-nitrophenyl *N*-acetyl- β -galactosaminide). K_m and V_{max} values for each substrate alone and in various proportions in two-substrate mixtures were determined by Lineweaver-Burk plots. V_{max} based on Lineweaver-Burk plots was compared with maximum velocity (V_{a+b}) calculated by the Dixon & Webb equation:

$$\frac{K_a}{\mu \cdot K_b} = \frac{V_a - V_{a+b}}{V_{a+b} - V_b} \quad (1)$$

where K_a and V_a denote K_m and V_{max} values of the enzyme for *p*-nitrophenyl β -*N*-acetylglucosaminide as substrate, and K_b and V_b are the corresponding values for *p*-nitrophenyl β -*N*-acetylgalactosaminide as substrate. V_{a+b} is the maximum velocity observed when the glucosaminide and galactosaminide substrate derivatives are present in the reaction mixture in the ratio $a/b = \mu$. V_{a+b} (i.e. V_{max}) values from Lineweaver-Burk plots and the Dixon & Webb equation for Hex A are given in Table 5. This shows that V_{a+b} values based on the Dixon & Webb equation are lower than the values from Lineweaver-Burk plots for Hex A. On the other hand, calculated values for V_{a+b} of Hex B are identical with those shown by Lineweaver-Burk plots.

M_r values of Hex A and Hex B and their subunits

The M_r of native Hex A determined on a Sephadex G-200 column (1.8 cm $\times$ 80 cm) ($V_t = 204$ ml, $V_0 = 71$ ml) was 19 500, whereas Hex B showed slightly

Fig. 4. Analytical gel filtration of seminal-plasma *N*-acetyl- β -hexosaminidases on Sephadex G-200

The column was calibrated with standard proteins, namely catalase, alcohol dehydrogenase (ADH), bovine serum albumin (BSA), pepsin and cytochrome *c* (Cyt. *c*).

faster migration with an M_r of 210 000 (Fig. 4). The M_r values of subunits of Hex A and Hex B were obtained by SDS/polyacrylamide-gel electrophoresis under two conditions: for separation of subunits that are not covalently linked the isoenzymes were treated with SDS only, whereas for separation of individual polypeptide chains they were reduced with 10 mM-dithiothreitol and alkylated with 10 mM-iodoacetamide to avoid re-aggregation. Results on SDS/polyacrylamide-gel electro-

Fig. 5. SDS/polyacrylamide-gel electrophoresis of seminal-plasma Hex A (gel f) and Hex B (gel g) in the presence of 10 mM-dithiothreitol

Standard proteins are shown in gels a–e: gel a, bovine serum albumin; gel b, egg albumin; gel c, pepsin; gel d, trypsinogen; gel e, lysozyme.

phoresis (Fig. 5) show that non-reduced isoenzymes migrate as a single band with an M_r of about 50000, suggesting that Hex A and Hex B are each composed of four subunits. Addition of reducing agents did not dissociate this protein into smaller polypeptide chains. A single band of M_r about 50000 was observed with either isoenzyme in the presence of reducing agent.

DISCUSSION

Human seminal plasma appears to contain two forms of *N*-acetyl- β -hexosaminidase, corresponding to the acidic form Hex A and the basic form Hex B from different tissues (Robinson & Stirling, 1968). The identical kinetics for the *N*-acetyl- β -glucosaminidase activity, such as K_m , optimum pH, optimum temperature, inhibition pattern by various salts, by heavy-metal ions, by thiol-reactive agents and by sugars and their derivatives, amino acid composition (Verpoorte, 1972) and immunological reactions (Srivastava *et al.*, 1974), are consistent with the view that Hex A and Hex B from human seminal fluid might represent different conformational stages for the same protein. But this view is invalidated when percentage inhibition of the two forms of *N*-acetyl- β -hexosaminidase by iodoacetate, ethyl-maleimide and thimerosal is examined, and when K_i values of the *N*-acetyl- β -glucosaminidase activity are evaluated in the presence of hexosamines and *N*-acetylhexosamines. Moreover, the K_m values of the *N*-acetyl- β -glucosaminidase activity indicate that Hex A has greater affinity from Hex B for *p*-nitrophenyl *N*-acetyl- β -galactosaminide. If the two isoenzymes had identical binding centres, the pattern of inhibition and binding affinities would have been identical under identical conditions. Thus it is evident that the two forms of *N*-acetyl- β -hexosaminidase from human seminal

plasma do not represent the same protein in different conformational states. This supports our earlier suggestion that the antigenic structures of Hex A and Hex B at the catalytic sites are not identical. This was based on the lack of enzymic activity in immune complexes formed between Hex A and anti-(Hex B) serum and between Hex B and anti-(Hex A) serum, though immune complexes formed between Hex A and anti-(Hex A) serum and between Hex B and anti-(Hex B) serum did retain appreciable activity (Kapur & Gupta, 1985). In spite of this, seminal-plasma *N*-acetyl- β -glucosaminidase activity appears to bear many kinetic properties identical with those of *N*-acetyl- β -hexosaminidases of other tissues (Robinson & Stirling, 1968; Mian *et al.*, 1978; Lo & Kritchevsky, 1979).

It is still controversial whether the *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities in *N*-acetyl- β -hexosaminidase are associated with two different glycoproteins or belong to a glycoprotein that has two kinds of active centres, each specific for the hydrolysis of one kind of substrate (Aruna & Basu, 1975; Brattain *et al.*, 1977; Mian *et al.*, 1978; Verpoorte, 1974). The present studies suggest that *N*-acetyl- β -galactosaminidase activity is inseparable from the *N*-acetyl- β -glucosaminidase activity by several purification steps and the ratio of two activities during purification remained constant. K_m and V_{max} values of both Hex A and Hex B for *p*-nitrophenyl *N*-acetyl- β -glucosaminide are higher than those observed for *p*-nitrophenyl *N*-acetyl- β -galactosaminide, and K_i values in the presence of hexosamines vary with the two different substrates. Therefore it is not possible to explain these kinetics on the basis of competition for a common active site. The data on reaction rates with two substrates present simultaneously do not yield results comparable with the rates of reactions obtained by the Dixon & Webb equation for a single active site in Hex A catalysing two reactions simultaneously, although with Hex B the results were comparable for a single active site. Thus these results favour the view that *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activities are located at different sites in Hex A and at the same site in Hex B, and can be explained on the hypothesis of heterogeneity of the Hex A peak, which might contain two or more enzymic sites, i.e. one site for subunit α and one site for the subunit β , each site hydrolysing either substrate with a slightly different kinetic constant. Active site(s) for Hex B could be explained on the reaction scheme proposed by Walker *et al.* (1961), which seems to be compatible with the present observations: two specific sites with cross-inhibition and single site with displacement.

The M_r values obtained for Hex A and Hex B are in accordance with values reported by Khar & Anand (1977) for the bull semen isoenzymes and higher than the 100000–130000 values obtained by gel filtration in other studies (Tallman *et al.*, 1974; Srivastava *et al.*, 1974; Geiger & Arnon, 1976). Probably the carbohydrate moiety as well as lack of spherical shape of glycoproteins may lead to differences in the migration as well as elution pattern in gel filtration (Andrews, 1970). Seminal-plasma Hex A and Hex B were each dissociated into four subunits of M_r about 50000. These subunits could not be further dissociated after reduction and alkylation. Probably this non-dissociation is due to the low concentration of reducing agents compared with the concentrations used by Mahuran & Lowden (1980). The most probable subunit structure of *N*-acetylhexos-

aminidases has been given as $\alpha_2\beta_2$ for Hex A and $\beta_2\beta_2$ for Hex B (Geiger & Arnon, 1976). Srivastava *et al.* (1974) believe that a 50000- M_r dimer is the result of hydrophobic interactions, whereas Geiger & Arnon (1976) claim it as a result of incomplete disulphide-bond cleavage. To rule out the possibility of hydrophobic interactions, Mahuran & Lowden (1980) determined the M_r values of subunits to be 50000 and 25000 in Hex A and 25000 in Hex B by gel filtration in the presence of 6 M-guanidinium chloride and by SDS/polyacrylamide-gel electrophoresis under rigorous conditions after reduction and alkylation of enzyme preparations to ensure complete breakdown of disulphide bonds, and concluded that either the α -chain in *N*-acetyl- β -hexosaminidase has an M_r of 50000 with one α -chain per α -subunit, or two 25000- M_r chains are united by a non-disulphide cross-links. Since the difference in M_r of native enzyme from semen and from other organs is very clear, this therefore suggests that semen *N*-acetyl- β -hexosaminidases must possess some additional polypeptide chains that are not present in other tissue *N*-acetyl- β -hexosaminidase. The exact number of polypeptide chains in intact molecules of the seminal-plasma isoenzymes may vary from four to eight as reported for other *N*-acetyl- β -hexosaminidases (Beutler & Srivastava, 1973; Tallman *et al.*, 1974; Beutler *et al.*, 1975). The phenomenon of non-dissociation of seminal-plasma *N*-acetyl- β -hexosaminidases into constituent polypeptide chains after reduction could be due to aging of preparations of enzymes, as observed by Mahuran & Lowden (1980).

The role of hexosaminidase in reproduction has been suggested earlier (McRorie & Williams, 1974). Our earlier studies indicated that antibodies to seminal-fluid *N*-acetyl- β -hexosaminidases cross-react with spermatozoal *N*-acetyl- β -hexosaminidases (Gupta & Kapur, 1983) and vice versa. Since the acrosome has been considered to be specialized lysosome, it is therefore presumed that acrosomal hydrolases, namely hyaluronidase, β -glucuronidase and hexosaminidase, might be playing similar role in the penetration of spermatozoa through the cumulus oophorus for fertilization (Zaneveld *et al.*, 1973) by degrading the sulphated glycosaminoglycans and sulphoglycolipids present in the ovum (Hayashi, 1977, 1978). Alternatively, the lethal accumulation of gangliosides in tissues during Tay-Sachs disease and Sandhoff disease (Okada & O'Brien, 1968; Sandhoff, 1969) suggests that these enzymes might be responsible for the degradation of gangliosides present in the ovum.

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