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STUDIES ON THE GLYCOSIDASES OF SEMEN

PURIFICATION AND PROPERTIES OF β-*N*-ACETYLGLUCOSAMINIDASE FROM BULL SPERM

ASHOK KHAR * and S.R. ANAND **

National Dairy Research Institute, Karnal-132001 (India)

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Summary

β-*N*-Acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamido-deoxyhydrolase, EC 3.2.1.30) was purified from bull sperm acrosomal extracts by ion-exchange chromatography and isoelctric focusing. The final preparation was fractionated 135-fold with a 38% recovery of enzyme activity and had a specific activity of 19 units/mg protein. Isoelectric focusing (p*I* 7.96) and disc electrophoresis gave single protein bands corresponding to enzyme activity. Additional proof of homogeneity was provided by acrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The molecular weight of the enzyme, determined by gel filtration, was 190 000 and the amino acid composition is reported.

β-*N*-Acetylglucosaminidase from bull sperm has optimum activity at pH 4.5 while β-*N*-acetylgalactosaminidase activity which is intrinsic to this protein, has pH optimum of 3.4. The K_m and V values for hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside at 37° C was 0.52 mM and 0.38 μmol/min per mg protein and for the corresponding galactopyranoside 0.27 mM and 0.019 μmol/min per mg protein. Glycopeptides with terminal *N*-acetylglucosamine/*N*-acetylgalactosamine residues were degraded slowly but rates were faster with chitin saccharides. The two activities were inhibited by a number of compounds; the most potent being Hg^{2+} and lactones.

* Present address: C.N.R.S. Laboratorie des Hormones Polypeptidiques, 91191 Gif-Sur-Yvette, France.

** To whom all the correspondance may be addressed.

Introduction

Acrosome is a specialised lysosome which is evolved during the development of spermatozoa. Interest in this sperm organelle has been stimulated in recent years because of the role it plays in the process of fertilization [1—3]. The hydrolases contained in the acrosome are emptied at the site of fertilization which then attack specific linkages in glycoproteins of cumulus oophorus and the zona pellucida to facilitate the entry of sperm into the ovum [1—3]. Metz et al. [4,5] observed that species-specific antibodies produced against purified acrosomal hydrolases could be used to prevent gamete interaction. This provided stimulus for the purification and characterization of hydrolases from different species. The purification of sperm hyaluronidase has thus been reported from more than one species [6—8]. Acrosin, another hydrolase having trypsin-like specificity, was studied extensively and fractionation has yielded electrophoretically pure enzyme preparations [9—12]. β-*N*-Acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) occurs in high concentrations in sperm acrosome [12,13] and partial purification of this enzyme was reported from rat sperm [14]. We have purified β-*N*-acetylglucosaminidase from bull sperm to homogeneity and this article describes these details and the properties of the enzyme.

Materials and Methods

Chemicals

CM-Sephadex, Sephadex G-200 were the products of Pharmacia, Sweden. DEAE-cellulose, CM-cellulose were from W & R Balson Ltd., England. Ampholine carrier ampholytes were the products of LKB, Sweden. Cyanogum-41, Tris, Hyamine 2389 and Triton X-100 were obtained from BDH, England. 2-Acetamido-2-deoxy-D-gluconolactone and the corresponding galactonolactone, ribonuclease, trypsin inhibitor from soyabean and *p*-chloromercuribenzoate were purchased from Koch-Light Laboratories, Colnbrook, U.K. Ammonium persulfate, *N-N-N'-N'*-tetramethyl ethylenediamine, pronase and Amido Black were the products of E. Merck, Darmstadt, Germany. Bovine serum albumin, acrylamide, *N-N'*-methylenebisacrylamide, *N*-ethylmaleimide, iodoacetamide and 2-mercaptoethanol were obtained from Fluka, Switzerland. β-Lactoglobulin was a product of Nutritional Biochemicals, U.S.A. α_1-Acid glycoprotein was a gift from the American National Red Cross, Maryland, U.S.A.

p-Nitrophenyl-β-2-acetamido-2-deoxy-D-glucopyranoside and the corresponding galactopyranoside, *p*-nitrophenyl derivatives of α- and β-mannosides, α and β-galactosides, α and β-fucosides and α and β-glucosides, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosaminehydrochloride, galactosaminehydrochloride, phenolphthalein glucuronide, hyaluronic acid, chitin, ovalbumin, ovomucoid, catalase, γ-globulin, β-galactosidase and neuraminidase were purchased from Sigma Chemical Co., St. Louis, U.S.A. All other reagents used were of analytical grade.

Enzyme assays

N-Acetyl β-D-glucosaminidase. The standard incubation mixture contained

the following in a total volume of 1 ml: citrate/phosphate buffer (pH 5.5) 70 μmol; *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 0.5 μmol and enzyme 0.1—1.0 unit. After 30 min incubation at 37°C, 5 ml of 0.1 M glycine/NaOH buffer, pH 10.4 was added. The intensity of released *p*-nitrophenol was measured at 420 nm.

N-Acetyl β-D-galactosaminidase. This enzyme was assayed under the conditions described for glucosaminidase except that the substrate was the corresponding galactosaminide and the citrate/phosphate buffer was of pH 3.4. One unit of glucosaminidase or galactosaminidase is defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside or the correspinding galactopyranoside in 30 min at 37°C under the standard assay conditions. The specific activity is expressed as units/mg protein.

Other glycosidases and hyaluronidase. β-Glucuronidase was assayed according to Fishman et al. [15] with phenolphthalein glucuronide as the substrate. Hyaluronidase, α and β-mannosidases, α and β-galactosidases, α and β-fucosidases and α and β-glucosidases were assayed as described earlier [16].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done in 7.5% gel according to the procedure described in Shandon Instrument Applications [17]. The protein sample (50—100 μg) in 8% sucrose solution contining bromophenol blue was applied to the gel and the electrophoresis was performed at 100 V for 3 h using a continuous Tris · HCl buffer, pH 8.5. The gels were extruded and stained with 1% Amido Black solution in 7.5% acetic acid and then destained by repeating washings with 5% acetic acid. For enzyme activity, the gels were sliced with a razor blade slicer into 1-mm sections and were incubated in 0.5 ml of 0.7 M citrate/phosphate buffer, pH 4.5 at 37°C for 30 min, and an aliquot was assayed for glucosaminidase activity.

Amino acid analysis

Amino acid analysis was performed according to the method of Spackman et al. [18] with a TSM-Technicon Sequential Amino Acid Autoanalyzer having double column assembly, with norleucine as the standard amino acid.

Molecular weight determination

A Sephadex G-200 column (2 × 90 cm) was equilibrated with 0.05 M KPO_4 buffer, pH 6.0 containing 0.01% NaCl. The column was calibrated with the following standards: ovalbumin, catalase (bovine liver), bovine serum albumin, β-galactosidase (*Escherichia coli*). Standards (1 mg/ml) or β-*N*-acetylglucosaminidase (2—3 mg/ml) were dissolved in 0.5 ml of the column buffer. The void volume was determined using Dextran Blue. Following application of the sample of the column, fractions of 5 ml were collected with a flow rate of 12 ml/h, and absorbance at 280 nm was measured. The molecular weight of the acrosomal *N*-acetylglucosaminidase was determined from the curve obtained with standards.

Preparation of natural substrates

Chitin oligosaccharides were prepared by the method of Rupley [19] on

charcoal/celite column as described by Barker et al. [20]. Ovalbumin glycopeptides were prepared by pronase treatment according to Huang et al. [21]. Glycopeptides from ovomucoid were prepared by pronase treatment as described by Kanamori and Kawabata [22]. κ-Casein from goat milk was prepared by the salt fractionation method of Zittle and Custer [23] which was then digested with rennin. The glycopeptide released was fractionated according to Wheelock and Sinkinson [24].

Analytical methods

Protein was determined by the method of Lowry et al. [25] with bovine serum albumin (1 mg/ml) as the reference protein or spectrophotometrically according to Warburg and Christian [26]. The release of hexosamine from chitin oligosaccharides and glycopeptides was estimated by the method of Ressig et al. [27].

Results

Purification of β-N-acetylglucosaminidase

Step 1: Preparation of acrosome from spermatozoa. Freshly ejaculated bull semen (3 ml) was mixed with 9 ml of 0.05 M Tris · HCl buffer, pH 7.4 and left at room temperature for 5 min. The hypotonic buffer immobilizes the spermatozoa and also increases the fragility of cytoplasmic droplets. The diluted semen was centrifuged at 1100 × *g* for 10 min and the supernatant was discarded. The pellet containing packed spermatozoa was resuspended in 10 ml of the same buffer and centrifuged at 900 × *g* for 8 min. The supernatant was decanted and the pellet was washed three times more with this buffer at 900 × *g* for 8, 6 and 5 min, respectively. The fourth washing was tested for acid phosphatase and *N*-acetylglucosaminidase; the absence of these enzyme activities indicated the removal of seminal plasma. The sperm pellet thus obtained was suspended in 3 ml of 0.05% hyamine 2389 and 0.05% Triton X-100 in Tris/maleate buffer, pH 6.1 and cooled in ice for 5 min [1]. The suspension was then incubated at 37°C for 90 min, chilled and centrifuged at 900 × *g*. The supernatant was retained and another detergent treatment was given to the pellet. The supernatants were pooled and constituted the acromsomal preparation. This was dialyzed atainst 0.05 M potassium phosphate buffer, pH 6.0 for 7—8 h to remove hyamine and Triton X-100.

Step 2: Ammonium sulphate precipitation. To the dialyzed acrosomal preparation (150 ml), solid ammonium sulphate was added to a saturation of 60%. The contents were stirred for 2 h and the precipitated proteins were harvested by centrifugation at 10 000 × *g* for 15 min. The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 6.0 and dialyzed for 6 h against the same buffer (Fraction II).

Step 3: DEAE-cellulose chromatography. A glass column (2.5 × 35 cm) was packed to a height of 28 cm with washed DEAE-cellulose [28] equilibrated with 0.05 M potassium phosphate buffer, pH 6.0. Fraction II (10 ml) was applied to the resin and the column was washed with 100 ml of the same buffer. The exchanged proteins were eluted with 200 ml of a linear gradient of NaCl (0.1—1.0 M). 5-ml fractions were collected at a flow rate of 1 ml/min. Only one

isozyme of hexosaminidase, i.e. the less acidic hexosaminidase-B, came off the column with the wash buffer. The tubes with high specific activtiy were pooled as shown by horizontal bar (Fig. 1a). The pooled fraction was concentrated by ultrafilitration (Fraction III).

Step 4: CM-Sephadex chromatography. A CM-Sephadex column (2.5 × 40 cm) was equilibrated with 0.05 M potassium phosphate buffer, pH 6.0. Fraction III (7 ml) was applied to the column and the gel was washed with 50 ml of the same buffer. The protein was eluted with 200 ml of a linear NaCl gradient from 0.1—1.0 M concentration. 5-ml fractions were collected at a flow rate of 1.5 ml/min. The fractions were tested for enzyme activity and protein and pooled as shown by the bar (Fig. 1b). The pooled enzyme was dialyzed against 0.05 M sodium citrate buffer, pH 5.0, for 4 h and concentrated by ultrafilitration (Fraction IV).

Step 5: CM-cellulose chromatrogaphy. A CM-cellulose column (1.2 × 20 cm) was equilibrated with 0.05 M sodium citrate buffer, pH 5.0. This was loaded with 6 ml of Fraction IV and washed with 50 ml of the same buffer. Protein

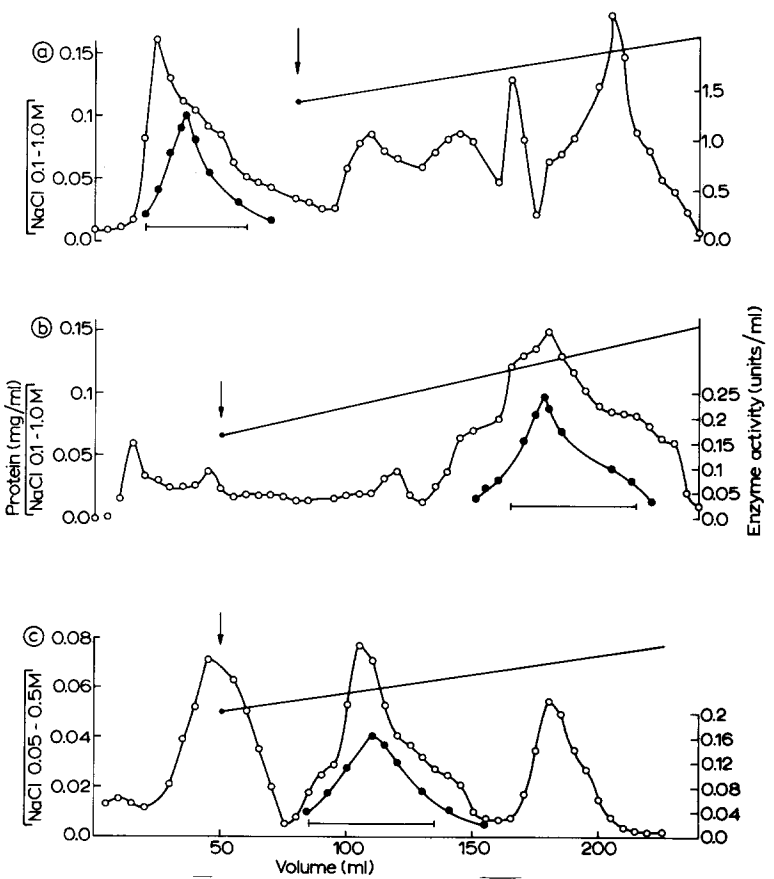

Fig. 1 (a) DEAE-cellulose chromatography of Fraction II; (b) CM-Sephadex chromatography of Fraction III; (c) CM-cellulose chromatography of Fraction IV. Protein (•); glucosaminidase activity (○). Arrow indicates the start of NaCl gradient. For other details, see the text.

was eluted with a linear gradient of NaCl from 0.05 to 0.5 M concentration in 200 ml of the sodium citrate buffer. 5-ml fractions were collected at a rate of 2 ml/min and the fractions were tested for enzyme activity and protein. The tubes with high specific activity of the enzyme were pooled (Fig. 1c) and dialyzed against deionized water for 4—5 h and concentrated by ultrafiltration (Fraction V).

Step 6: Isoelectric focusing. Fraction V was subjected to isoelectric focusing as described by us [29]. A 48-h run was given and the column was drained by collecting 3 ml portions at a speed of 3 ml/5 min under the force of gravity. The fractions were assayed for enzyme activity, protein and pH. The tubes with high specific activity were pooled (Fig. 2) and dialyzed against sodium citrate buffer, pH 5.0 for 48 h (Fraction VI).

Properties of N-acetylglucosaminidase

Purity of enzyme preparation. In Table I, the purification data is summarized which shows that bull sperm *N*-acetylglucosaminidase was purified 135-fold from the acrosomal extract with a 38% recovery of enzyme activity. The final purified enzyme preparation was free of all other glycosidases except the *N*-acetylgalactosaminidase which was fractionated simultaneously during the purification steps (Table I). On polyacrylamide gel electrophoresis. Fraction VI gave only one sharp protein band corresponding to enzyme activity. The protein load on the gels of enzyme preparations purified at different intervals was between 100—200 μg. In those instances, when the gels were overloaded (concentration greater than 200 μg), no evidence of heterogeneity was observed. Additional proof about the homogeneity of sperm *N*-acetylglucosaminidase was proveded by isoelectric focusing and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

Isoelectric focusing gave a single protein band which migrated in the same region as the enzyme activity (Fig. 2). Electrophoresis in sodium dodecyl sul-

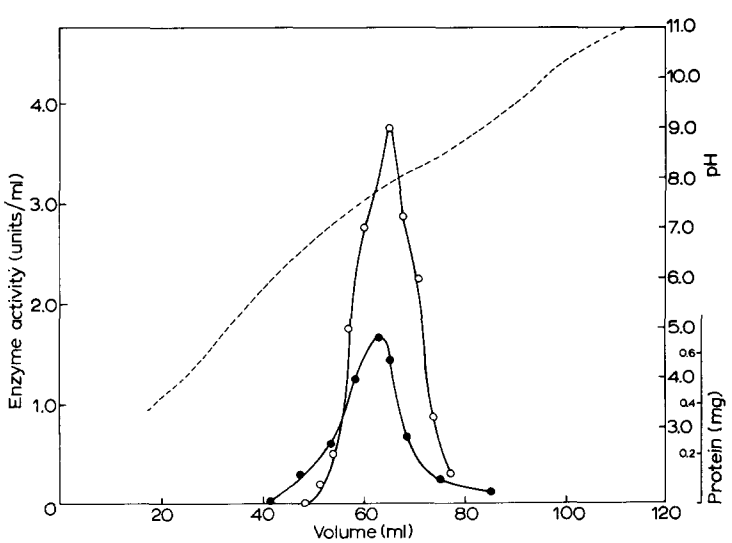

Fig. 2. Isoelectric focusing of Fraction V. Protein (•), glucosaminidase activity (○), pH (- - - - -).

TABLE I

PURIFICATION OF β-*N*-ACETYLGLUCOSAMINIDASE FROM BULL SPERM ACROSOME

Fraction and procedure	Volume (ml)	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Glu/Gal
I Acrosomal preparation	150	1.52	31.5	0.138	100	4.0
II Ammonium sulphate	10	9.0	28.0	0.311	91	3.1
III DEAE-cellulose	33	1.50	22.5	0.450	72	3.6
IV CM-Sephadex	50	0.25	16.5	1.32	54	3.3
V CM-Cellulose	74	0.04	15.5	5.7	50	3.4
VI Isoelectric focusing	10	0.06	12.2	18.8	38	3.3

phate/acrylamide gels in Tris · HCl buffer, pH 8.0 [30] gave two bands. These bands were also obtained when protein samples were preincubated with 10 mM 2-mercaptoethanol, 4 mM guanidine hydrochloride or 8 mM urea, thereby showing that sperm enzyme has a subunit structure. The two subunits corresponding to the molecular weights of 53 000 and 13 400 were identical to those obtained for seminal isozymes A and B [29].

Molecular weight and amino acid composition. The molecular weight of sperm *N*-acetylclucosaminidase estimated from Sephadex G-200 chromatography was 190 000 (Fig. 3). The amino acid composition of the enzyme is given in Table I which shows that 37% of the protein contain amino acids with charged polar side chain and 32% of the non polar amino acids, glycine, alanine, valine, leucine and isoleucine. The glycoprotein nature of the enzyme was revealed through positive staining with periodic acid and Schiff's reagent

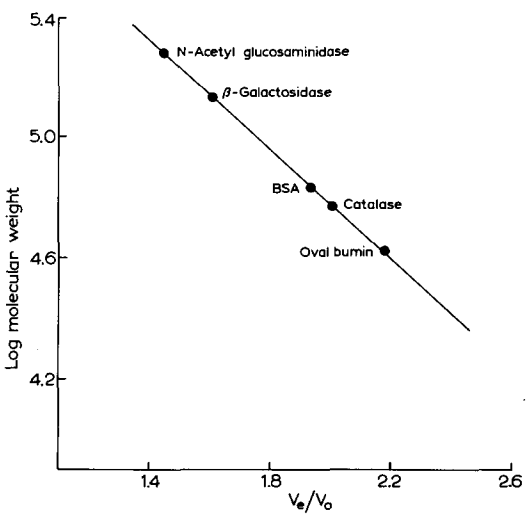

Fig. 3. Standard curve for the determination of molecular weight by Sephadex G-200 gel chromatography.

TABLE II

AMINO ACID COMPOSITION OF BULL SPERM β-*N*-ACETYLGLUCOSAMINIDASE

Amino acid	mg/100 mg protein *	Amino acid	mg/100 mg protein *
Lysine	5.8	Alanine	7.9
Histidine	4.5	Cystine	2.4
Arginine	8.0	Valine	5.6
Aspartic acid	7.4	Methionine	2.9
Threonine	4.0	Isoleucine	4.2
Serine	5.8	Leucine	9.3
Glutamic acid	11.8	Tyrosine	5.2
Proline	6.3	Phenylalanine	4.5
Glycine	5.2		

* Values represent the average of determinations at 24- and 72-h hydrolysis.

but the carbohydrate composition was not determined because of the lack of sufficient material.

Enzymatic properties. The enzyme showed a linear release of *p*-nitrophenol from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside at pH 4.5 and 37°C for 60 min and with varying amounts of enzyme concentrations. The enzyme could also hydrolyze the corresponding galactopyranoside. The optimum pH for glucosaminidase and galactosaminidase activities were determined by using 0.1 M citrate/phosphate, 0.1 M potassium phosphate and 0.1 M Tris · HCl buffers between the pH range 3—9. The hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was observed to be maximum at pH 4.5 while that of the corresponding galactosaminide at pH 3.4. Unlike seminal plasma hexosaminidases [16], sperm enzyme activity was not affected by bovine serum albumin.

The effect of substrate concentration on the glucosaminidase/galactosaminidase activities was seen by incubating them with different substrate concentrations (0.0625—1.0 mM). The affinity of the two enzyme activities for their substrates and the *V* value of hydrolysis were evaluated from the double reciprocal plots (Fig. 4) [31]. The average K_m and *V* values, based on 9 experiments, were 0.52 mM and 0.088 μmol/min per mg protein for glucosaminidase and 0.27 and 0.019 μmol/min per mg protein for galactosaminidase.

Inhibition studies. The effect of various inhibitors on the glucosaminidase and galactosaminidase activities were examined by incubating the enzyme and the inhibitor at 25°C for 30 min. The reaction was initiated by the addition of substrate and the enzyme activities were assayed according to standard assay conditions. The results are summarized in Table III. Hg^{2+} is a more potent inhibitor of both the activities as compared to silver. Cysteine helps to prevent inhibition by Hg^{2+} when added simultaneously but not completely. Iodoacetamide and *p*-chloromercuribenzoate were inhibitory but glucosaminidase activity was affected more than galactosaminidase. This could mean that the two activities associated with the same enzyme protein are catalysed from two active sites and are being differently affected by the inhibitors.

Action on natural substrates. Sperm *N*-acetylglucosaminidase was active on all the natural substrates tested (Table IV). For chitinsaccharides, the rate of hydrolysis decreased with increase in chain length. Amongst the glycopeptides,

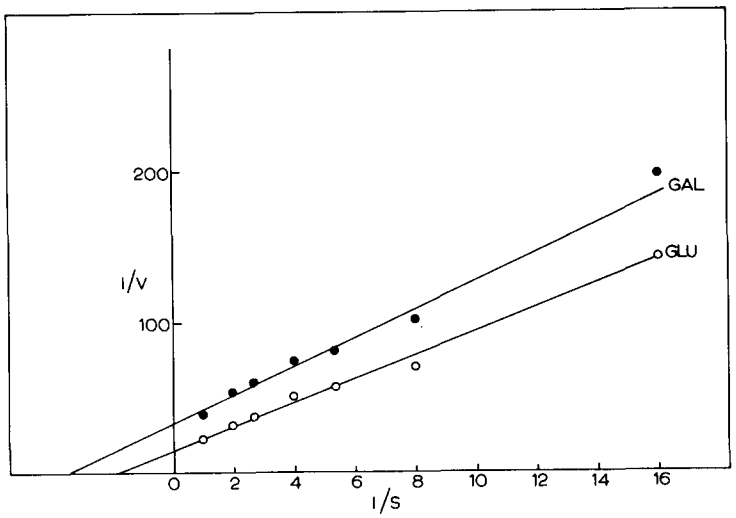

Fig. 4. Double reciprocal plot for the glucosaminidase (○) and galactosaminidase (●) activities of bull sperm hexosaminidase.

TABLE III

EFFECT OF DIFFERENT INHIBITORS ON BULL SPERM GLUCOSAMINIDASE AND GALACTOSMINIDASE

Inhibitor	Concentration (mM)	% Inhibition	
		Glucosaminidase	Galactosaminidase
$AgNO_3$	1.0	34.0	60.0
$HgCl_2$	0.1	93.0	75.0
$HgCl_2$ + cysteine	0.1, 1.0	27.0	40.0
Cysteine	1.0	7.0	30.0
PCMB *	1.0	70.0	25.0
N-Ethylmaleimide	20.0	10.0	10.0
Iodoacetamide	20.0	83.0	70.0
Acetate	10.0	0.0	15.0
Glucosmine	10.0	0.0	10.0
N-Acetylglucosamine	20.0	20.0	25.0
Galactosamine	10.0	0.0	70.0
N-Acetylgalactosamine	0.5	60.0	40.0
Gluconolactone	0.01	61.0	55.0
Galactonolactone	0.01	60.0	45.0

* *p*-chloromercuribenzoate.

TABLE IV

HYDROLYSIS OF NATURAL SUBSTRATES BY SPERM β-*N*-ACETYLGLUCOSAMINIDASE

Substrate	Incubation time (h)	Enzyme units	NADG ** released (%)
Chitin disaccharide	10	2.0	3.6
Chitin trisaccharide	10	2.0	2.3
Chitin tetrasaccharide	10	2.0	0.7
-Acid glycoprotein *	24	4.5	2.9
Ovomucoid glycopeptide *	48	4.5	1.2
κ-Casein glycopeptide	24	4.5	1.7
Ovalbumin glycopeptide	48	4.5	3.5

* Sialic acid and galactose residues were removed by treatment with neuraminidase (*Clostridium perfingens*) and β-galactosidase (*E. coli*).

** NADG, *N*-acetyl deoxyglucose.

the release of *N*-acetylglucosamine was maximum with obalbumin followed by α_1-acid glycoprotein, κ-casein glycopeptides (*N*-acetylgalactosamine) and ovomucoid glycopeptides.

Discussion

We have described a procedure for obtaining a homogeneous protein possessing *N*-acetylglucosaminidase activity from acrosomal extracts of bull spermatozoa. Like other enzymes of this class, the ability of this protein to hydrolyze *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside is intrinsic. Bull sperm enzyme has properties strikingly similar to isozymes A and B isolated from bull seminal plasma [16,29], yet there were differences which show that the sperm hexosaminidase is distinct from the seminal hexosaminidases. The molecular weight of acrosomal *N*-acetylglucosaminidase (190 000) was identical to seminal isozymes but they had different isoelectric points: 7.96 for sperm glucosaminidase and 6.78 for isozyme B and 5.31 for isozyme A. Amino acid composition, though very similar, revealed sperm enzyme to contain higher arginine and leucine content than the seminal isozymes A and B.

Differences were also apparent in the kinetic properties of seminal plasma and sperm hexosaminidases. The pH dependence of glucosaminidase activities of three enzymes (pH optimum 4.5) was the same but the sperm galactosaminidase consistently had a lower pH optimum of 3.4, against 3.8 observed for seminal isozyme A and B [16]. Bovine serum albumin, when added to assay mixture, enhanced both glucosaminidase and galactosaminidase activities of seminal isozymes but had no effect on the acrosomal enzyme. The affinity of sperm enzyme for the two substrate was different; the K_m for glucosaminidase was 0.52 mM and for galactosaminidase 0.27 mM. For partially purified glucosaminidase from rat spermatozoa, a higher K_m value (0.68 mM) was reported [14]. The maximum velocity for the glucosaminidase was 4—5 times greater than that of galactosaminidase and a comparison with the V values observed for isozymes A and B [16] revealed that the bull sperm enzyme functions at a much lower level on the synthetic substrates. Natural substrates were hydrolyzed also at slower rates than those observed for seminal hexosaminidases [29].

The physiological role of acrosomal hydrolases is to facilitate the penetration of spermatozoon into the female egg. Prior to penetration, the acrosome reaction takes place during which outer acrosomal membrane overlying plasma membrane are lost, and the inner acrosomal membrane becomes the limiting membrane over the entire acrosomal region apart from its distal region, the equatorial segment [32—34]. In studies involving the detachment of acrosome from ram spermatozoa [35,36], acrosin was found largely, and hyaluronidase partly, associated with inner acrosomal membrane. In subsequent studies on goat and buffalo spermatozoa, acrosin, hyaluronidase and β-*N*-acetylglucosaminidase were found partly in the solubilized and partly in the bound form associated with the inner acrosomal membrane [37]. The loss in density of acrosomal contents during the acrosome reaction may be accounted for by the availability of soluble enzymes and this may explain the accepted role of hyaluronidase to disperse cumulus oophorus cells [38] and the involvement of *N*-acetylglucosaminidase in capacitation [39]. The bound enzymes lying on the

inner acrosomal membrane may have an additional role of concerted attack on the zona pellucida to make a passage for the spermatozoon to get through.

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References

1 Allison, A.C. and Hartree, E.F. (1970) J. Reprod. Fertil. 21, 501—515
2 Stambaugh, R. and Buckley, J. (1969) J. Reprod. Fertil. 19, 423—432
3 Hartree, E.F. (1971) Lysosomes and Fertilization, in Of Microbes and Life, p. 271. (Monod, J. and Borek, E., eds.) Columbia Univ. Press, New York,
4 Metz, C.B., Seiguer, A.C. and Castro, A.E. (1971) Biol. Bull. 141, 373—374
5 Metz, C.B. (1972) Biol. Reprod. 6, 358—383
6 Zaneveld, L.J.D., Polakoski, K.L. and Schumacher, G.F.B. (1973) J. Biol. Chem. 248, 564—570
7 Yang, C.H. and Srivastava, P.N. (1974) J. Reprod. Fertil. 37, 17—25
8 Yang, C.H. and Srivastava, P.N. (1975) J. Biol. Chem. 250, 79—83
9 Schleuning, W.D., Schiessler, H. and Fritz, H. (1973) Hoppe-Seyler's Z. Physiol. Chem. 356, 550—554
10 Polakoski, K.L., McRorie, R.A. and Williams, W.L. (1973) J. Biol. Chem. 248, 8178—8182
11 Stambaugh, R. and Smith, M. (1974) Science 186, 745—746
12 Conchie, J. and Mann, T. (1957) Nature 179, 1190—1191
13 Khar, A. and Anad, S.R. (1974) Indian. J. Biochem. Biophys. 11, 88—89
14 Majumdar, G.C. and Turkington, R.W. (1974) Biochemistry 13, 2857—2864
15 Fishman, W.H., Springer, B. and Brunetti, R. (1948) J. Biol. Chem. 173, 449—456
16 Khar, A. and Anad, S.R. (1974) Indian. J. Biochem. Biophys. 11, 292—298
17 Tombs, M.P. and Akroyd, P. (1967) Shandon Instrum. Appl. No. 18
18 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
19 Rupley, J.A. (1964) Biochim. Biophys. Acta 83, 245—255
20 Barker, S.A., Foster, A.B., Stacey, M. and Weber, J.M. (1958) J. Chem. Soc. 2218—2227
21 Huang, C.C., Mayer, Jr., H.E. and Montogomery, R. (1970) Carbohydr. Res. 13, 127—137
22 Kanomori, M. and Kawabata, M. (1969) Agric. Biol. Chem. 33, 220—224
23 Zittle, C.A. and Custer, J.H. (1963) J. Dairy Sci. 46, 1183—1188
24 Wheelock, J.V. and Sinkinson, G. (1973) J. Dairy Res. 40, 413—420
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
26 Warburg, O.H. and Christian, W. (1941) Biochem. Z. 310, 384—421
27 Ressig, J.L., Strominger, J.L. and Leloir, L.F. (1965) J. Biol. Chem. 217, 959—966
28 Peterson, E.A. and Sober, H.A. (1957) Methods Enzymol. 5, 3—26
29 Khar, A. and Anand, S.R. (1977) Biochim. Biophys. Acta, in the press
30 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
31 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658
32 Bedford, J.M. (1970) Biol. Reprod. Suppl. 2, 128
33 Befored, J.M. (1971) Am. J. Anat. 133, 213
34 Piko, L. (1969) Gamete Structure and Sperm Entry in Mammals; in Fertilization (Metz, C.B. and Monroy, A., eds.), Vol. II, p. 325, Academic Press, New York
35 Brown, C.R. and Hartree E.F. (1974) J. Reprod. Fert. 36, 195—198
36 Brown, C.R. (1975) J. Reprod. Fert. 45, 537—539
37 Anand, S.R., Kaur, S.P. and Chaudhry, P.S. (1977) Hoppe-Seyler's Z. Physiol. Chem., in the press
38 McRorie, R.A. and Williams, W.L. (1974) Annu. Rev. Biochem. 43, 777—803
39 Gwatkin, R.B.L. and Anderson, O.F. (1973) J. Reprod. Fert. 35, 565—567