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STUDIES ON THE GLYCOSIDASES OF SEMEN

FURTHER PURIFICATION AND CHARACTERIZATION OF TWO HEXOSAMINIDASES FROM BULL SEMINAL PLASMA

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Summary

Two isozymes of β-*N*-acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxy glucohydrolase, EC 3.2.1.30) (A and B) from bull seminal plasma were purified to homogeneity by isoelectric focusing having p*I* values of 5.31 and 6.78. The two proteins were glycoproteins with very similar amino acid composition but isozyme A contained more sialic acid than isozyme B. The molecular weights of isozyme A and B were estimated at 200 000 and 190 000 by gel filtration. Two identical subunits corresponding to molecular weights of 53 000 and 13 400 were obtained from hexosaminidase A and B when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Similar results were obtained when dissociation of the isozymes was effected with mercaptoethanol, guanidine hydrochloride and urea in presence of sodium dodecyl sulphate and the subunits separated by acrylamide gel electrophoresis.

The two isozymes were more stable in frozen conditions than at the refrigerated temperature. Of the divalent ion tested, glucosaminidase and galactosaminidase activities of isozymes A and B were strongly inhibited by Hg^{2+} and Ag^{+} thus suggesting the presence of thiol groups in the two proteins. The two isozymes were active on natural substrates; isozyme B being more active than isozyme A.

Introduction

Bull seminal plasma contains two hexosaminidase A and B (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) which were

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purifed 13- and 84-fold by ion-exchange chromatography. Each isozyme hydrolysed *p*-nitrophenyl β-2-acetamido-2-deoxy-D-glucopyranoside and the correspinding galactopyranoside. The K_m, V and the dissociation of the enzyme/inhibitor complex (K_i) using acetate, glucosamine, galactosamine and their *N*-acetyl derivatives, 2-acetamido-2-deoxy-D-gluconolactone and the corresponding galactonolactone as inhibitors, pointed to the different nature of two isozymes. That the glucosaminidase and galactosaminidase activities of each isozyme were associated with a single protein was supported by pH and heat stability profiles and a constant ratio obtained throughout the purification procedure. The mixed substrate analysis also revealed that the two activities were catalysed from the same site but the kinetic studies with a numbers of inhibitors did not support this finding [1]. The two isozymes have been purified to homogeneity using isoelectric focusing and this paper describes these details and the physiochemical properties of the two hexosaminidases.

Materials and Methods

Chemicals

Sodium dodecyl sulphate (SDS) 2-mercaptoethanol and guanidine hydrochloride of high purity were purchased from Fluka, Switzerland. Urea was a product of BDH. Thiobarbituric acid, Coomassie Brilliant Blue were purchased from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals were the same as those in the accompanying paper [2].

Enzyme assays

N-Acetyl β-D-glucosaminidase/N-Acetyl β-D-galactosaminidase. *p*-Nitrophenyl β-2-acetamido-2-deoxy-D-glucopyranoside (0.5 μmol) was incubated in a total volume of 1 ml containing 70 μmol of citrate/phosphate buffer, pH 4.5, 100 μg of bovine serum albumin and 0.1—1.0 unit of enzyme. After 30 min, the reaction was terminated by the addition of 5 ml of 0.1 M glycine/NaOH buffer, pH 10.4. The intensity of released *p*-nitrophenol was measured at 420 nm. *N*-Acetyl D-galactosaminidase was assayed similarly except that the substrate was the corresponding galactosaminide and the buffer was of pH 3.8.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. The SDS-polyacrylamide electrophoresis was carried out according to Fairbanks et al. [3]. Protein samples (0.1—0.2 mg/ml) were prepared by adding the following (to the stated final concentrations): 1% SDS, 10% sucrose, 10 mM Tris · HCl (pH 8.0), 1 mM EDTA and 5 μg/ml of Bromophenol Blue. The clear sample solutions were incubated at 37° C for 30 min either in the presence of 10 mM 2-mercaptoethanol or 4 mM guanidine hydrochloride or 8 M urea. Molecular weight markers were also treated in the same manner. Electrophoresis was carried out using a current of 8 mA/gel for 2 h and the gels were stained with Coomassie Brilliant Blue. Destaining was done in 7.5% acetic acid containing 5% methanol.

Analytical methods. Protein was determined by the method of Lowry et al. [4] or according to Warburg and Christian [5]. For sialic acid estimation, the samples were hydrolysed with 0.1 N H_2SO_4 at 80° C for 1 h and the amount was estimated by the thiobarbituric acid method of Warren [6]. The hexosamines were determined according to the method of Reissig et al. [7]. The

sugars present in the protein hydrolysate were separated by paper chromatography on Whatman paper No. 1, using the solvent system propan-1-ol/ethyl acetate/water (7 : 1 : 2 by vol) [8]. The spots were located by silver nitrate/alkali method for reducing sugars and by acetylacetone/*p*-dimethylaminobenzaldehyde for amino sugars [9]. Glycoprotein staining was done according to Fairbanks et al. [3] using periodic acid and Schiff's reagent.

Results

Isoelectric focusing of isozymes A and B

Isoelectric focusing was conducted by the method of Haglund [10] using an LKB 8100-10 column of 110 ml capacity. The protein samples (Fraction VI, ref. 1), 1% Ampholine carrier ampholytes (pH 3.5—10) and the sucrose solution were mixed by means of a gradient mixer and the column was filled with the help of a peristaltio pump at a speed of 1.5 ml/min, so as to achieve a sucrose gradient. The central electrode chamber was filled with dense electrode solution containing sucrose and sulphuric acid (anode). After the density gradient was made, it was layered by the light electrode solution containing sodium hydroxide (cathode) so as to dip the top electrode. Electrofocusing was carried out at 4°C for 40 h at a constant voltage of 300 V. At the end of experiment, the current had dropped to 0.3 mA. After electrofocusing, the central electrode chamber valve was closed and the contents of the column were drained out by force of gravity. Fractions of 2.5 ml were collected using an LKB fraction collector 7000-3/4. The protein pattern was seen with the help of an LKB Uvicord II-8300. The fractions were tested for enzyme activity and the pH was measured with an expanded scale pH meter (Radiometer, Copenhagen). The fractions having high specific activity of the enzyme were pooled (Fig. 1) and

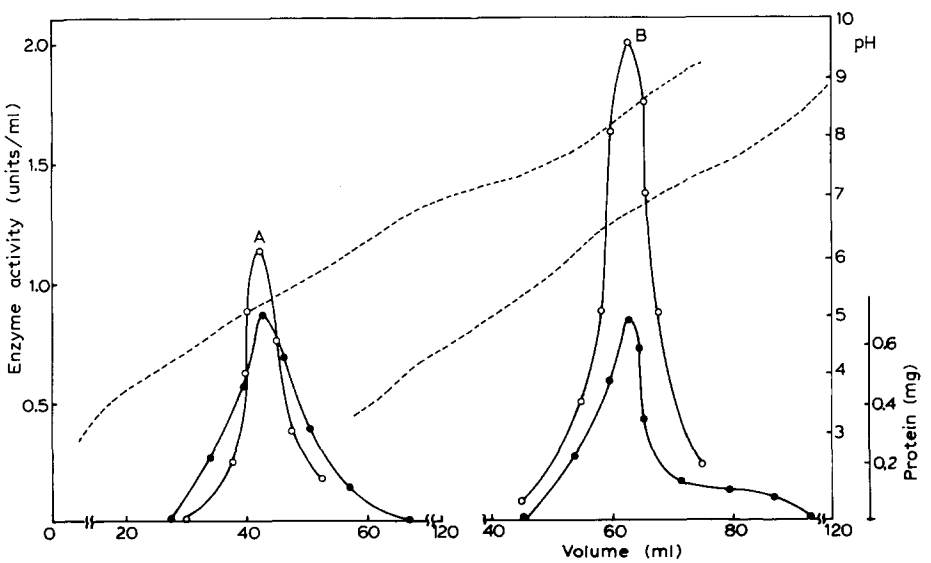

Fig. 1. Isoelectric focusing patterns of hexosaminidase A and B. pH (- - - - -), glucosaminidase activity (○), protein (●).

dialysed against 0.05 M KPO_4 buffer, pH 6.0 for 48 h to remove sucrose and carrier ampholytes.

Enzyme fractionation and purity

The final purified preparations of hexosaminidase A and B were purified 120- and 436-fold over the crude extract with a recovery of 2% and 15%. Isozyme A had a specific activity of 120 units/mg protein and isozyme B 436 units/mg protein. The two proteins appeared to be homogenous because on isoelectric focusing, only one peak with absorbance at 280 nm was observed for each isozyme (Fig. 1). The isoelectric pH's of isozyme A (p*I* 5.31) and B (p*I* 6.78) were lower than the p*I* of 7.96 determined for the sperm *N*-acetylglucosaminidase [2]. However, these were within the range of p*I* 5.85—7.15 reported for seven glucosaminidase activities in ram epididymis and testes [11]. Further evidence of their purity was obtained when isozymes A and B were examined in 7.5% polyacrylamide gels, pH 8.5 [12]. A single protein band was detected for each isozyme on staining the gels with amido black.

Molecular weight and subunit structure

The molecular weight of isozyme A and B was determined by Sephadex G-200 gel filtration as described earlier [2]. Isozyme A had a molecular weight of 200 000 while isozyme B had 190 000. When subjected to SDS-acrylamide electrophoresis in Tris · HCl buffer (pH 8.0), two bands were obtained. Their migration on SDS-acrylamide gels remained unaltered when the enzymes A and B, before electrophoresis, were incubated with 10 mM mercaptoethanol, 4 mM guanidine hydrochloride or 8 M urea. The two subunits had different molecular size, the bigger subunit had a molecular weight of 53 000 whereas the smaller with 13 400 (Fig. 2). From these observations, the occurrence of at least two types of subunits in seminal isozymes is inferred.

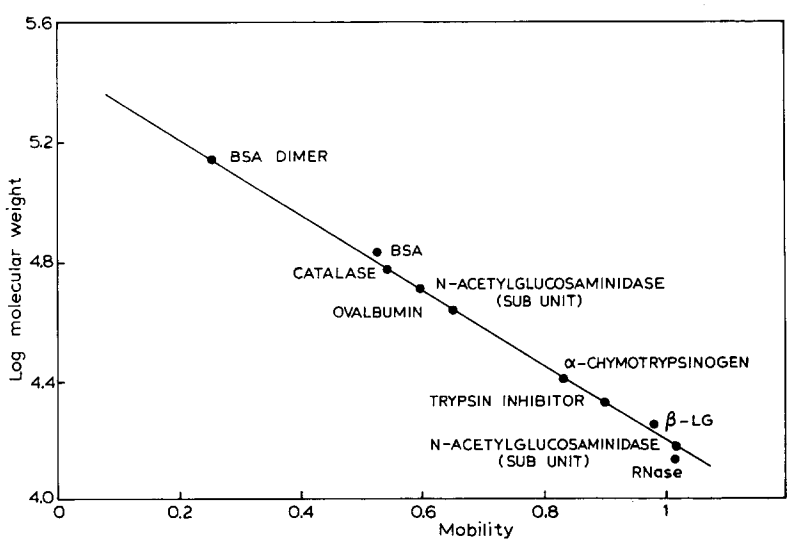

Fig. 2. Standard curve for the determination of molecular weight by SDS-polyacrylamide gel electrophoresis at pH 8.0. BSA, bovine serum albumin; B-LG, β-lactoglobulin.

TABLE I

AMINO ACID AND CARBOHYDRATE COMPOSITION (mg/100 mg PROTEIN) OF SEMINAL ISOZYME A AND B

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

Amino/acid carbohydrate	Isozyme A	Isozyme B
Lysine	7.0	6.3
Histidine	6.3	5.0
Arginine	5.2	4.2
Aspartic acid	7.6	8.0
Threonine	3.5	3.8
Serine	5.6	5.2
Glutamic acid	10.0	9.0
Proline	6.3	5.4
Glycine	3.8	5.0
Alanine	7.8	6.2
Cystine	2.1	7.1
Valine	5.5	7.4
Methionine	2.0	2.4
Isoleucine	4.2	3.6
Leucine	6.3	6.1
Tyrosine	6.4	5.8
Phenylalanine	3.4	4.4
Sialic acid	2.4	1.3
Glucosamine	2.5	2.9
Galactose	— *	— *

* Not determined.

Glycoprotein nature of isozyme A and B

The two isozymes are glycoproteins was revealed by positive staining with periodic acid/Schiff's reagent. The enzyme proteins were hydrolysed with HCl and the hydrolysate was examined by paper chromatography [8]. Two spots having mobilities corresponding to authentic glucosamine and galactose were detected. In addition, sialic acid was also present. The quantitative amounts of these carbohydrates are given in Table I.

Amino acid composition

The amino acid composition of the two enzymes (Table I) resemble very closely as 27% of proteins in two isozymes has amino acids with non-polar side chain and about an equivalent amount with polar side chain. Isozyme B had much higher concentration of cystine than the isozyme A but the inhibition of both the enzymes with Ag^{+}, Hg^{2+} and chloromercuribenzoate strongly suggest the presence of thiol groups.

Effect of storage on enzyme activities

Isozyme A and B in 0.05 M sodium citrate buffer, pH 5.0 were stored at 10° C and —10° C for a period of six months. At different intervals, the two proteins were assayed for glucosaminidase and galactosaminidase activities. At refrigerated temperatures, the enzymes were inactivated during 2.5 months while in frozen conditions a 40% loss was observed in six months. Freezing and thawing had no effect on enzyme activities.

TABLE II

EFFECT OF DIFFERENT INHIBITORS ON SEMINAL GLUCOSAMINIDASE AND GALACTOSAMINIDASE ACTIVITIES

Inhibitor	Concentration (mM)	% inhibition			
		Isozyme A		Isozyme B	
		GlcNAc	GalNAc	GlcNAc	GalNAc
$AgNO_3$	1.0	100	100	100	100
$AgNO_3$ + cysteine	1.0, 1.0	48	63	27	74
$HgCl_2$	0.1	100	100	100	100
$HgCl_2$ + cysteine	0.1, 1.0	33	37	16	31
$FeSO_3$	1.0	26	0	51	55
$CuSO_4$	10.0	72	52	43	75
Ascorbic acid	1.0	64	67	68	77
$CuSO_4$ + ascorbic acid	10.0, 1.0	72	61	51	50
$CoCl_2$	10.0	50	22	10	100
Iodoacetamide	100.0	100	82	80	96
N-Ethylamleimide	200.0	30	25	8	2
Cysteine	1.0	72	88	9	2
$K_4Fe(CN)_6$	1.0	22	12	16	14

Effect of inhibitors

$AgNO_3$ and $HgCl_2$ inhibited the glucosaminidase and galactosaminidase activities of isozymes A and B completely at a concentration of 1 mM. The inhibitions were partially reversed when cysteine was added to the assay system. *p*-chloromercuribenzoate also inhibited the two activities completely but iodoacetamide required higher concentrations for inhibition to the same extent. L-Ascorbic acid was reported to enhance the cleavage of glycosides [13] and the rates of the reaction are faster in the presence of Cu^{2+}. The cleavage of both glucosaminide and galactosaminide was inhibited by L-ascorbic acid and Cu^{2+}. Inhibitions by other compounds are summarized in Table II.

TABLE III

HYDROLYSIS OF NATURAL SUBSTRATES BY SEMINAL ISOZYME A AND B

Substrate *	Incubation time (h)	Isozyme A		Isozyme B	
		Enzyme units	NADG *** released (%)	Enzyme units	NADG released (%)
Chitin disaccharide	10	4.1	35.0	14	43.0
Chitin trisaccharide	10	4.1	21.0	14	26.4
Chitin tetrasaccharide	10	4.1	3.8	14	4.7
α_1-Acid glycoprotein **	24	8.2	4.2	38	7.4
Ovomucoid glycopeptide **	48	8.2	4.5	38	8.7
κ-Casein glycopeptides	24	8.2	5.3	38	14.5
Ovalbumin glycopeptide	48	8.2	8.7	38	17.3

* The substrates were prepared as described by Khar and Anand [2].

** Stalic acid and galactose residues were removed by treatment with neurominidase (*Cl. perfringens*) and β-galactosidase (*E. coli*).

*** NADG, *N*-acetyl deoxyglucose.

Substrate specificity

The ability of hexosaminidase A and B to hydrolyse natural substrate was evaluated by their action on chitin disaccharide, chitin trisaccharide, chitin tetrasaccharide, κ-caseinglycopeptides, ovalbumin glycopeptides, α_1-acid glycoprotein and ovomucoid glycopeptides. Each substrate was incubated under standard assay conditions with enzyme units and period of incubation indicated in Table III. Toluene was added to prevent bacterial growth. The reaction was terminated by boiling the incubation mixture in water bath for 3 min and the contents were estimated for *N*-acetylglucosamine. Chitin oligosaccharides were better substrates than the glycopeptides which were hydrolysed to varying degrees by the two isozymes. Isozyme B was more active of the two hexosaminidases on the natural substrates.

Discussion

Hexosaminidase A and B from bull seminal plasma were shown to be glycoproteins; isozyme A contained more sialic acid than the isozyme B. This is consistent with the position in other tissues where isozyme A had more neuraminic acid residues than isozyme B [14—16]. The final homogeneous preparations of isozyme A and B were found to be more stable in frozen state than at the refrigerated temperatures. The glucosaminidase/galactosaminidase activities of each isozyme were retained for six months in frozen conditions whereas, at refrigerated temperatures, these were inactivated in three months. The two hexosaminidases have very similar properties yet they have different nature was revealed through kinetic studies [1]. This is further supported the results of the present study. The amino acid composition of isozyme A and B was strikingly similar. Likewise, the molecular weights of the two isozymes were very close (200 000 for isozyme A and 190 000 for isozyme B). However, the isoelectric pH values were different; 5.31 for isozyme A and 6.78 for isozyme B.

The relationship amongst the multiple components of β-*N*-acetylhexosaminidases has been investigated in two studies. It was proposed that hexosaminidase A is converted to hexosaminidase B like activity by the action of viral neuraminidase and thus may serve as a precursor of isozyme B [14,17]. This hypothesis seemed to be in agreement with the reported occurrence of hexosaminidases in all the mammalian sources investigated so far and the ratio of isozyme A/B was found to be high. But in recent studies [16,18,19], this relationship has not been substantiated. In the present study, the prepondrance of isozyme B over A (4 : 1 ratio) in seminal plasma makes it less likely that isozyme B is formed from isozyme A by loss of sialic acid. On the contrary, the reverse may be more probable if a specific sialotransferase is detected in semen. Nevertheless, another possibility and the more likely one, is that these enzymes have some common subunit structure. The two seminal isozymes had identical molecular weights but were shown to have subunit structures. One of the subunits had a molecular weight of 53 000 while the other was smaller with 13 400. Besides the difference in polysaccharide chains of the two isozymes, they may differ from one-another in the manner of the association of two subunits to yield two different functional enzyme molecules with very similar properties. Similar subunit structures were obtained from β-*N*-acetylglucosaminidase of human placenta [16,18].

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References

1 Khar, A. and Anand, S.R. (1974) Indian. J. Biochem. Biophys. 11, 292—298
2 Khar, A. and Anand, S.R. (1977) Biochim. Biophys. Acta, in the press
3 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
4 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
5 Warburg, O.H. and Christian, W. (1941) Biochem. Z. 310, 384—421
6 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
7 Ressig, J.L., Strominger, J.L. and Leloir, L.F. (1965) J. Biol. Chem. 217, 959—966
8 Glegg, R.E. and Eidinger, D. (1954) Anal. Chem. 26, 1365—1367
9 Partridge, S.M. (1948) Biochem. J. 42, 238—250
10 Haglund, H. (1971) Methods Biochem. Anal. 19, 1—104
11 Bullock, S. and Winchester, B.G. (1973) Biochem. J. 133, 593—599
12 Tombs, M.P. and Akroyd, P. (1967) Shandon Instrument Application No. 18
13 Caygill, J.C. (1968) Biochim. Biophys. Acta 170, 1—5
14 Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321—327
15 Murphy, J.V. and Craig, L. (1972) Clin. Chim. Acta 42, 267—272
16 Tallman, J.F., Brady, R.O., Quirk, J.M., Villalba, M. and Gal, A.E. (1974) J. Biol. Chem. 249, 3489—3499
17 Goldstone, A., Konecny, P. and Koenig, H. (1971) FEBS Lett. 13, 68—70
18 Srivastava, S.K., Yoshida, A., Awasthi, Y.C. and Beutler, E. (1974) J. Biol. Chem. 249, 2049—2053
19 Carrol, M. and Robinson, D. (1974) Biochem. J. 137, 217—221