Biochimica et Biophysica Acta, 659 (1981) 255—266
Elsevier/North-Holland Biomedical Press

BBA 69278

ISOLATION AND FURTHER CHARACTERIZATION OF BOVINE BRAIN HEXOSAMINIDASE C

BERNARD OVERDIJK, WILLY M.J. VAN DER KROEF, GÉ J. VAN STEIJN and JAN J.W. LISMAN

Department of Medical Chemistry, Vrije Universiteit, Van Der Boechorststraat 7, P.O. Box 7161, 1007 MC Amsterdam, (The Netherlands)

(Received October 3rd, 1980)

Key words: Hexosaminidase C; (Bovine brain)

Summary

Hexosaminidase C (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) was partially purified from bovine brain tissue. The resulting preparation, free of its lysosomal counterparts, was used for the characterization of the enzyme and for further purification (lectin affinity chromatography, hydrophobic interaction chromatography, substrate-ligand affinity chromatography, ion-exchange chromatography, chromatography on activated thiol-Sepharose 4B).

Only ion-exchange chromatography on DEAE-Sephacel appeared to improve the purity. The Michaelis constant was 0.46 mM for the substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside. The enzyme was not inhibited by acetate or *N*-acetylgalactosamine. Inhibition by *N*-acetylglucosamine was competitive, with a K_i value of 8.0 mM. Inhibition by divalent metal ions increased in the order Fe < Zn < Cu. Dithiothreitol and β-mercaptoethanol, at an optimum concentration of about 10 mM, stimulated the activity. The enzyme is apparently not a glycoprotein since it did not bind to various lectins, nor did sialidase change its isoelectric point.

Introduction

β-Hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) exists as multiple forms. Robinson and Stirling [1] were the first to describe an A and a B type in human spleen, which could be related to some forms of lysosomal storage diseases (for review, see Ref. 2). Hexosaminidase C, a third form of the enzyme, was discovered by Hooghwinkel et al. [3]. The enzyme was distinguished from the lysosomal

hexosaminidases A and B by its greater anodal mobility and its specificity for artificial *N*-acetylglucosaminide substrates. Hexosaminidase C appeared to be present in human and bovine brain, but could not be detected in brain tissue from patients with Tay-Sachs disease or in normal human liver [3]. The following characteristics of hexosaminidase C have been elucidated.

The molecular weight of hexosaminidase C in human and bovine brain was higher than that of hexosaminidase A and B [4—6]. However, the use of Sephadex G-200 and Bio-Gel P-200 in these studies prevented correct estimation of the molecular size of the enzyme, since hexosaminidase C was eluted in the void volume of the column. Penton et al. [7] found a molecular weight of 190 000 for the hexosaminidase C of human placenta, whereas Swallow et al. [8] estimated the molecular weight of the human brain enzyme at 430 000. The optimum activity of hexosaminidase C in bovine and human brain and in human placenta was between pH 6—7 [5,7—11]. In neonatal human brain, the optimum activity appeared to be pH 5.5—6.0 [4]. The substrate specificity of hexosaminidase C for artificial glucosaminide substrates [3] was confirmed by other authors [4,7,8]. Hexosaminidase C was localized in the cytoplasm [7]. The isoelectric point of hexosaminidase C varied with the tissue from which it was derived: pH 4.5 in adult human brain [8]; pH 4.77 in neonatal human brain [4] and pH 5.7 in human placenta [7]. The enzyme showed no immunological cross-reactivity with antisera against either hexosaminidase A or B [11,12], which indicates that there is no structural relation between hexosaminidase C and the lysosomal hexosaminidases. Hexosaminidase C appeared to be present in a wide variety of normal human tissues [8,11,12] and in tissues of patients with Tay-Sachs disease [11,13,14]. The residual hexosaminidase activity in tissues of patients with Sandhoff's disease was composed of hexosaminidase S and hexosaminidase C [15,16]. The purification of hexosaminidase C from human placenta resulted in an enzyme preparation which was 18-times more active than the 800 × *g* supernatant of a tissue homogenate [7]. The enzyme showed no affinity for concanavalin A [8]. Hexosaminidase C could not be recovered from DEAE-cellulose or DEAE-Sephadex [5—7,11].

In this report we describe attempts to purify hexosaminidase C from bovine brain by various chromatographic procedures such as affinity chromatography using different immobilized lectins or substrate as affinity ligand; hydrophobic interaction and ion-exchange chromatography. Furthermore, some kinetic data and the influence of thiol compounds, metal ions and inhibitors on the enzymatic activity are presented.

Materials and Methods

Materials

Bovine brain, obtained from the local slaughterhouse, was stored at 4°C (within 2 h after death) until use. The following materials were purchased: Sephacryl S-200, Sepharose 6B, CH-Sepharose 4B, activated thiol-Sepharose 4B, octyl-Sepharose CL-4B, phenyl-Sepharose CL-4B, Con A-Sepharose, DEAE-Sephacel, CM-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden); the lectins from *Ricinus communis* type I and type IIA, and from *Ulex*

europaeus, immobilized on agarose, and the lectin from wheat germ (*Triticum vulgaris*) attached to Sepharose 6B, *N*-acetyl-D-glucosamine, L-cysteine, and D,L-dithiothreitol (Sigma Chem. Co., St. Louis, MO, U.S.A.); *N*-acetyl-D-galactosamine, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.); *p*-aminophenyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside (Bachem Fine Chemicals, Torrance, CA, U.S.A.); Cellogel (Chemetron, Milan, Italy); *Vibrio cholerae* sialidase (Behringwerke A.G., Marburg, F.R.G.); Ampholine PAG plates no. 1804—101, ampholines (LKB-Produkter AB, Bromma, Sweden); calibration proteins, Combithek (Boehringer, Mannheim, F.R.G.). All other reagents were of analytical grade or of the best grade available.

Methods

The purification procedure and all column chromatographic experiments were carried out at 2—4°C.

Purification of hexosaminidase C. A homogenate (25%, w/v) of fresh brain tissue (280 g) in 0.32 M sucrose was centrifuged for 1 h at 100 000 × *g* in a Beckman Ti 45 rotor using a Sorvall OTD-2 ultracentrifuge. The supernatant was brought to 33% saturation with $(NH_4)_2SO_4$. After stirring overnight at 0°C, the precipitate was collected by centrifugation for 20 min at 27 500 × *g* in a GSA rotor in a Sorvall RC-5 centrifuge. The precipitate was dissolved in 200 ml of a 0.05 M Tris-HCl/0.1 M KCl solution, pH 7.0. Insoluble material was removed by centrifugation for 20 min at 27 500 × *g*. The supernatant was loaded on a Sephacryl S-200 or Sepharose 6B column (95 × 11 cm diameter, upward flow). This resulted in two peaks of hexosaminidase activity, the one with the higher molecular weight containing hexosaminidase C. The fractions of this peak were pooled and dialyzed against a 0.02 M Tris-HCl/1.0 M NaCl solution, pH 7.4. Then the enzyme preparation was loaded on a Con A-Sepharose column (7.5 × 5 cm diameter) equilibrated in the above Tris/NaCl solution. Hexosaminidase C does not bind to this lectin [8]. The unretained fraction was concentrated 10—20-fold by ultrafiltration in an Amicon cell fitted with a PM-10 membrane.

Enzyme suspensions, prepared in this way, were dialyzed against the appropriate buffer and were then subjected to the following procedures.

Lectin affinity chromatography. Pasteur pipettes were filled with 1 ml immobilized lectin (*Ricinus communis* lectin type I and type IIA, wheat germ agglutinin, and *Ulex europaeus* lectin), and equilibrated with a 0.05 M Tris-HCl solution, pH 7.0. On each of these columns 0.5 ml of a hexosaminidase C solution (50—100 mU) was applied. After an equilibration period of 1 h at 4°C, the unretained material was eluted with 5 ml of the starting solution. Bound material was eluted with 0.5 M solutions of the appropriate sugar in the starting buffer: *N*-acetylgalactosamine for *R. communis* lectin type I, galactose for *R. communis* lectin type IIA, *N*-acetylglucosamine for wheat germ agglutinin, and fucose for *U. europaeus* lectin.

Hydrophobic interaction chromatography. Phenyl- and octyl-Sepharose columns (6 × 1.5 cm diameter) were equilibrated with a 0.1 M sodium phosphate buffer/4 M NaCl, pH 7.4. To each of these columns 250—500 mU hexos-

aminidase C (2 ml) were applied. Unretained material was washed off with the starting buffer (30 ml). The elution was continued with 60 ml of a linear gradient of NaCl from 4 to 0 in 0.1 M sodium phosphate buffer, pH 7.4, followed by 60 ml of a linear gradient of ethylene glycol (0—50%, v/v) in the same sodium phosphate buffer, or of Triton X-100 (0—1%, v/v) in that buffer.

Substrate-ligand affinity chromatography. *p*-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was reduced to the corresponding *p*-aminophenyl derivative. This product and *p*-aminophenyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside were each coupled to CH-Sepharose. We have already described the reduction and coupling procedures [17]. The resulting gels were designated GlcNAc-Sepharose and thio-GlcNAc-Sepharose, respectively. Columns with the derivatized CH-Sepharose gels (6 × 1.5 cm diameter) were equilibrated with 0.05 M sodium phosphate buffer, pH 7.4. After the sample of hexosaminidase C (250—500 mU in 2 ml) had been loaded, the unretained material was washed off with the starting phosphate buffer (30 ml). Subsequently, the columns were eluted with 50 ml of a linear gradient of *N*-acetylglucosamine (0—100 mg/ml) in the phosphate buffer, followed by a linear gradient of NaCl (0—0.5 M) in the phosphate buffer (50 ml). The chromatography on the thio-GlcNAc-Sepharose column was then continued with the phosphate buffer, containing 40 mM β-mercaptoethanol (30 ml).

Ion-exchange chromatography. CM-Sephadex C-50 chromatography was carried out in 0.05 M sodium phosphate buffer at various pH values between 4.5 and 6.0. Hexosaminidase C (100—200 mU in 1 ml) was loaded on columns (5 × 1.5 cm diam.) equilibrated with the above buffers. Unretained material was washed off with 30 ml of the starting buffer, and the elution was completed with 50 ml of a linear gradient of NaCl (0—0.4 M) in the same buffer. DEAE-Sephacel chromatography was performed in 0.05 M sodium phosphate buffer, pH 5.5. Hexosaminidase C (100—200 mU in 1 ml) was applied to the column (5 × 1.5 cm diam.). After removal of unretained material with 30 ml of the starting buffer, the elution was continued with a linear gradient of NaCl (0—0.4 M) in the phosphate buffer (50 ml), and completed with a 0.4 M solution of NaCl in the same buffer (25 ml).

Chromatography on activated thiol-Sepharose 4B. To an enzyme sample (100 mU in 0.5 ml), dialyzed against a 0.05 M Tris-HCl/0.1 M NaCl solution, pH 7.0, dithiothreitol was added to a final concentration of 10 mM. After incubation for 24 h at 4°C, the dithiothreitol was removed by gel filtration on Sephadex G-25 equilibrated in the above Tris/NaCl solution. The enzyme preparation was then applied to a column (5 × 1.5 cm diam.) of activated thiol-Sepharose 4B, equilibrated in the Tris/NaCl solution. All the buffers were deaerated immediately before use to avoid oxidation of the free thiol groups. After elution of the unretained material with 25 ml of the Tris/NaCl solution, a linear gradient of L-cysteine (0—10 mM) in the starting solution was applied (50 ml).

Sialidase treatment. Hexosaminidase C (1.5 U in 5 ml) was treated with 0.5 U of *Vibrio cholerae* sialidase in 0.05 M sodium acetate buffer/1.5 M NaCl/9 mM $CaCl_2$, pH 5.5. The mixture was incubated for 2.5 h at 37°C. After dialysis against distilled water at 4°C, the incubation mixture was used for an electrofocusing experiment in a 110 ml LKB-column.

Electrofocusing experiments. Electrofocusing experiments were performed with the LKB-8100 equipment (110 ml column) using ampholines in the pH range 3.5—6.0, and with the LKB 2117 Multiphor, using thin-layer polyacrylamide gels (PAG plates) with pH range 3.5—9.5. The manufacturer's instructions were followed. The amounts of enzyme used were: 1.5 U (5 ml) for the 110 ml column (both native enzyme, and enzyme treated with sialidase), and 2 mU (20 μl) for the PAG-plates. On completion, the content of the column was collected in 50 fractions, in which the pH and the hexosaminidase C activity were determined. In the polyacrylamide gel the pH was measured with a surface electrode. The enzyme bands were made visible by covering (30 min at 37°C) the gel with filter paper soaked in 0.1 M citric acid/0.2 M sodium phosphate buffer, pH 6.5, saturated with 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside. Protein bands were visualized in a solution of Coomassie brillant blue R-250 (0.25%, w/v) in methanol/acetic acid/water (4 : 1 : 5, v/v).

Gel chromatography. The molecular weight of hexosaminidase C was estimated with the aid of Sephacryl S-200 and Sepharose 6B columns (90 × 5 cm diameter), equilibrated with a 0.05 M Tris-HCl/0.1 M KCl solution, pH 7.0. Enzyme samples containing 1—2 U in 10 ml were loaded on these columns. The following proteins were used as calibration proteins: ferritin, catalase from beef liver, aldolase from rabbit muscle, albumin from bovine serum, albumin from hen egg, chymotrypsinogen A and cytochrome *c*. The molecular weight of hexosaminidase C was estimated from the linear relationship between the square roots of the molecular weights of the proteins and the cube roots of their distribution coefficients [18].

Enzyme kinetics. To determine the Michaelis constant (K_m) of hexosaminidase C the following incubation mixtures were prepared: 50 μl of the enzyme preparation in 0.05 M Tris-HCl/0.1 M KCl, pH 7.0; 150 μl of a 0.2 M citric acid/0.4 M sodium phosphate buffer, pH 6.5, and 100 μl of a substrate solution in water. The final substrate concentration in the incubation mixture varied between 0 and 3.33 mM. In three parallel incubation series the influence of acetate, *N*-acetylgalactosamine and *N*-acetylglucosamine was studied. In each series the concentration of the additive was varied between 0 and 6.67 mM, whereas the substrate concentration was kept constant (1 mM). The Michaelis constant was calculated with the method of Hofstee [19]. The enzyme inhibitor constant K_i was determined as described by Dixon [20].

Influence of thiol compounds and metal ions. Three sets of experiments were performed. In the first one a preparation of hexosaminidase C in a 0.05 M Tris-HCl/0.1 M KCl solution, pH 7.0, was preincubated for 0 and 20 h at 4°C in the absence of any additive, and in the presence of 6 mM $FeSO_4$, $ZnCl_2$ or $CuCl_2$. In a second series of preincubations, dithiothreitol or β-mercaptoethanol were added to the enzyme suspension with a final concentration of 10 and 40 mM. The third series contained 6 mM of one of the metal ions together with 10 or 40 mM of thiol compound. After preincubation the usual enzyme assay was carried out.

Electrophoresis. Cellogel electrophoresis was performed as described by Poenaru and Dreyfus [21].

Assays. The activity of hexosaminidase C was determined as described by

Overdijk et al. [6] with the artificial substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside. The buffer used for the incubation was 0.1 M citric acid/0.2 M sodium phosphate, pH 6.5. 1 unit enzyme activity liberates 1 μmol 4-methylumbelliferone per min at 37°C. Protein was determined by the method of Lowry et al. [22]. Crystalline human serum albumin was used as a standard. Unless otherwise stated each experiment was performed at least three times, whereas the determinations of the enzymatic activity were done in duplicate.

Results

Purification of hexosaminidase C. The results of a typical purification procedure for hexosaminidase C are given in Table I. The enzymatic activity was measured at pH 6.5 and 4.4 (data not shown in table). The ratio of the specific activities at both pH values, has been taken as a measure for the contamination of the hexosaminidase C preparation by the lysosomal hexosaminidases A and B. It can be concluded from this ratio that after step 2 and 3 hexosaminidase A and B still contribute appreciably to the enzymatic activity measured at pH 6.5. The most effective steps in this procedure are the separation on Sepharose 6B and on Con A-Sepharose, as is clearly demonstrated by the sharp increase in the above ratio. The absence of the hexosaminidases A and B was confirmed by Cellogel electrophoresis. The purification factor varied between 25 and 40.

Chromatographic procedures. The results of the lectin affinity chromatography, the hydrophobic interaction chromatography, the affinity chromatography with substrate as ligand, and the activated thiol-Sepharose 4B chromatography are summarized in Table II. Only with the chromatography on thio-GlcNAc-Sepharose was a 10-fold purification achieved. However, after one chromatographic run the column had lost its binding capacity for hexosaminidase C, which makes it unsuitable for routine use. None of the other techniques appeared to improve the purification of the hexosaminidase C preparation. Nevertheless, the results reveal some characteristics of the enzyme which will be dealt with in the Discussion.

Chromatography on CM-Sephadex showed binding of hexosaminidase C below pH 5.0, but with a very low recovery of the enzymatic activity. Addition

TABLE I

PURIFICATION OF BOVINE BRAIN HEXOSAMINIDASE C

The figures in this table are the results of a typical purification procedure. Activity ratio = activity at pH 6.5/activity at pH 4.4.

Purification step	Total protein (mg)	Total activity (Units)	Specific activity (mUnits/mg protein)	Purification factor	Yield (%)	Activity ratio
1. Homogenate	19360	17.4	0.9	1	100	0.5
2. Supernatant	3220	11.4	3.5	4	66	0.6
3. $(NH_4)_2SO_4$ precipitate	810	10.2	12.5	14	58	0.9
4. Sepharose 6B	162	3.1	19.6	22	18	12
5. Con A-Sepharose	107	3.0	28.1	31	17.5	40

TABLE II

RESULTS OF VARIOUS CHROMATOGRAPHIC EXPERIMENTS WITH HEXOSAMINIDASE C OF BOVINE BRAIN

Medium	Results
1. Lectin affinity chromatography a. *R. communis* lectin type I (GalNAc) b. *R. communis* lectin type IIA (Gal) c. Wheat germ agglutinin (GlcNAc) d. *U. europaeus* lectin (Fuc)	1. a—d. No binding of hexosaminidase C to any of these lectins.
2. Hydrophobic interaction chromatography a. Phenyl-Sepharose CL-4B b. Octyl-Sepharose CL-4B	2. a, b. Binding of hexosaminidase C. Elution with a Triton X-100 gradient. Recovery 80—90%. No increase in specific activity.
3. Substrate-ligand affinity chromatography a. GlcNAc-Sepharose	3. a. Nonspecific binding of hexosaminidase C. Elution with NaCl, but not with *N*-acetylglucosamine.
b. Thio-GlcNAc-Sepharose	b. Binding of hexosaminidase C. No elution with NaCl or *N*-acetylglucosamine. Elution with β-mercaptoethanol, with 40% recovery and a 10-fold purification. The column lost its binding capacity for hexosaminidase C after the above procedure.
4. Activated thiol-Sepharose 4B	4. Binding of hexosaminidase C. Elution with L-cysteine, with 65% recovery. No increase in specific activity.

of dithiothreitol or β-mercaptoethanol did not improve these results. Hexosaminidase C was bound to DEAE-Sephacel above pH 5.5, and could be eluted with a NaCl gradient. The specific activity of the enzyme increased by a factor 3, and the recovery was 75—80% (Fig. 1).

The apparent molecular weight of bovine brain hexosaminidase C was estimated with the aid of Sephacryl S-200 and Sepharose 6B gel chromatography. With both media it was found to be 240 000 ± 20 000.

Sialidase treatment and electrofocusing. We were unable to establish the exact isoelectric point of hexosaminidase C. On the column the observed p*I* of the main peak varied between pH 5.0 and 5.4. There was also a minor peak with a p*I* 0.3—0.4 pH units below this value. Electrofocusing on polyacrylamide gels revealed one band at pH 4.8—5.2. Protein staining of the gels showed the presence of many other bands. After treatment of the native enzyme with sialidase the electrofocusing pattern of hexosaminidase C did not change. When hexosaminidase C was pretreated with 10 mM dithiothreitol (16 h at 4°C) there was a shift of 0.2—0.4 pH units in the isoelectric points of both the major and the minor peak towards lower pH.

Enzyme kinetics. The results of the kinetic experiments are shown in Fig. 2. Hexosaminidase C exhibited normal Michaelis-Menten kinetics. The kinetic parameter K_m was determined with the aid of a Hofstee plot (inset of Fig. 2). The K_m value, calculated from the slope of the straight line in the Hofstee plot, was 0.46 mM. Acetate and *N*-acetylgalactosamine have no influence on the kinetics of hexosaminidase C. On the contrary, *N*-acetylglucosamine appeared

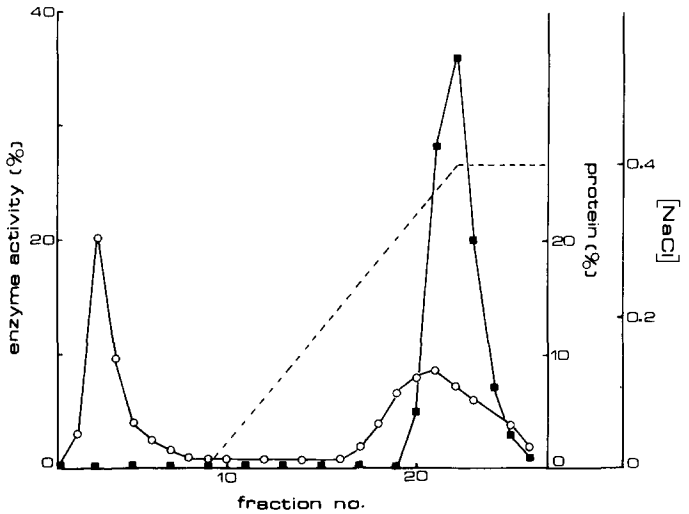

Fig. 1. DEAE-Sephacel anion exchange chromatography of bovine brain hexosaminidase C. The enzymatic activity (■——■) and protein content (○——○) of each fraction are given as the percentage of the total amounts recovered. The broken line - - - - - - represents the NaCl concentration (M).

to be a competitive inhibitor of the enzymatic activity. The K_i value for *N*-acetylglucosamine was determined as described by Dixon [20], and proved to be 8.0 mM (Fig. 3).

Influence of thiol compounds and metal ions. The effect of divalent metal

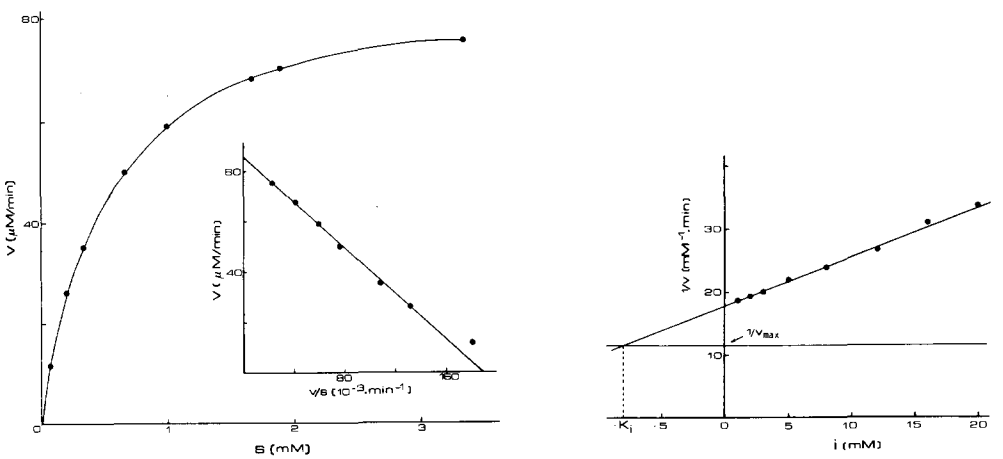

Fig. 2. Substrate kinetics of bovine brain hexosaminidase C. The ordinate shows the reaction velocity v (μmol/min), and the abscissa the substrate concentration s (mM). Inset: The inset shows the linear transformation of the v-s curve to a Hofstee plot [19]. On the ordinate the reaction velocity is plotted (μmol/min); on the abscissa v/s is given ($10^{-3} \cdot \text{min}^{-1}$).

Fig. 3. Determination of K_i of the competitive inhibitor *N*-acetylglucosamine of the hexosaminidase C activity. The ordinate shows the reciprocal value of the reaction velocity, $1/v$ ($\text{mmol}^{-1} \cdot \text{min}$); on the abscissa the concentration i of the inhibitor *N*-acetylglucosamine is given (mM). K_i lies at the intersection point of the line, drawn horizontally through the value $1/V$, and the inhibitor line [20].

TABLE III

INFLUENCE OF DITHIOTHREITOL AND DIVALENT METAL IONS ON THE ACTIVITY OF BOVINE BRAIN HEXOSAMINIDASE C

The enzymatic activities were measured immediately after addition of dithiothreitol and/or the metal ions (a), or after a preincubation period of 20 h at 2—4°C after the addition of these substances (b). The enzymatic activities are expressed as percentages of the activity measured in the absence of any additive and without preincubation. DTT, dithiothreitol.

Additions	0 mM DTT	10 mM DTT	40 mM DTT
a. No preincubation			
no metal	100	159	121
Fe^{2+}	56	97	103
Zn^{2+}	60	66	77
Cu^{2+}	9	0	0
b. 20 h preincubation at 2—4°C			
no metal	67	96	84
Fe^{2+}	36	62	51
Zn^{2+}	7	34	15
Cu^{2+}	0	0	0

ions and dithiothreitol on the hexosaminidase C activity is shown in Table III. In the absence of metal ions the thiol compound stimulated the enzymatic activity maximally at 10 mM. The metal ions inhibited the enzymatic activity in the order Fe < Zn < Cu. This inhibition could be partially overcome by adding dithiothreitol. The same influence of dithiothreitol and metal ions was observed after 20 h preincubation of the enzyme preparation with these additives. The effect of β-mercaptoethanol is qualitatively the same as that of dithiothreitol (data not shown in Table III).

Discussion

We have purified hexosaminidase C from bovine brain 25—40-fold and have obtained an ultimate specific activity of 28 mU/mg protein. The purification factor is in fact higher, since in all steps before the Con A-Sepharose chromatography, the lysosomal β-hexosaminidases contribute to the measured activity at pH 6.5. Penton et al. [7] specifically determined the hexosaminidase C activity of human placenta after precipitation of the lysosomal hexosaminidases A and B with antibodies against these enzymes. They obtained a purification factor of about 18 with respect to the starting material (a 800 × *g* supernatant), and a specific activity of 7 mU/mg protein. After the Con A-Sepharose step the hexosaminidase C preparation was free of other β-hexosaminidases as can be judged from the high ratio of the specific activities measured at pH 6.5 and 4.4 (Table I), and from the results of Cellogel electrophoresis.

Hexosaminidase C showed no affinity for various lectins with different sugar specificity. The negative reaction with concanavalin A has already been described by Swallow et al. [8], and we have now confirmed this finding. However, Fiddler et al. [23] reported the binding of hexosaminidase C from human liver to several lectins: to concanavalin A, wheat germ agglutinin and *Bandierea simplicifolia* lectin. These apparently conflicting results may be

explained on closer examination of the enzyme suspension which was used by these authors. They used a supernatant (26 600 × *g*) of a liver homogenate, without any separation between the solubilized lysosomal hexosaminidases A and B and hexosaminidase C. Moreover, the activity of hexosaminidase C in human liver is very low when compared with that in other tissues [3,8]. Thus, the measured activity at pH 7.0, supposedly hexosaminidase C, is presumably largely due to the rest activity of the acid hydrolases at this pH.

The isoelectric point of hexosaminidase C from bovine brain did not change after sialidase treatment. Similarly hexosaminidase C from human brain appeared to be insensitive to sialidase treatment [4,8]. Our negative results with regard to the binding of hexosaminidase C to different lectins, together with the ineffectiveness of sialidase in changing its isoelectric point, lead us to the tentative conclusion that hexosaminidase C is not a glycoprotein.

Of all purification steps tried, the DEAE-Sephacel chromatography proved to be the most useful one, resulting in a specific activity of about 100 mU/mg protein. We cannot explain the results with this anion exchanger, since the use of DEAE-Sephadex and DEAE-cellulose always led to a complete loss of hexosaminidase C activity [5—7,11], whereas DEAE-Sephacel is nothing but a beaded form of DEAE-cellulose.

In an earlier study [17] we found no binding of hexosaminidase C to GalNAc-Sepharose, as might be expected as a consequence of the substrate specificity of hexosaminidase C for artificial *N*-acetylglucosaminides. We therefore tried GlcNAc-Sepharose with the sugar linked either *O*- or *S*-glycosidically to *p*-aminophenol. Hexosaminidase C was bound to GlcNAc-Sepharose in 0.05 M sodium phosphate buffer at pH 7.4. However, this binding was non-specific, since elution was accomplished by NaCl, but not by the competitive sugar *N*-acetylglucosamine. These results appeared to be independent of pH values between 5.0 and 7.5.

At first glance the results with the thio-GlcNAc-Sepharose are surprising, but they may be explained by considering the results with the thiol compounds dithiothreitol and β-mercaptoethanol.

Addition of thiol compounds during the purification stabilized the enzyme (unpublished data). From these data it can be concluded that hexosaminidase C contains free SH-groups. Oxidation of these groups may be the cause of the instability of the enzyme, as was also found by Besley and Broadhead [4] for hexosaminidase C of human brain. The binding of hexosaminidase C to activated thiol-Sepharose 4B, and the subsequent elution of the enzyme by L-cysteine, support our conclusion concerning the presence of free SH-groups.

The chromatographic behaviour of the enzyme on thio-GlcNAc-Sepharose is similar to that on activated thiol-Sepharose. However, we did not investigate the nature of the binding between the enzyme and the thio-GlcNAc ligand. The enzyme could be eluted with β-mercaptoethanol but not with the various other eluents that have been mentioned under Materials and Methods. This suggests a covalent binding between enzyme and ligand, similar to the binding between activated thiol-Sepharose 4B and proteins containing free SH-groups. It is not known whether the *N*-acetylglucosamine moiety was still present at the time that the enzyme was bound to the column, or was eliminated by a non-hydrolytic reaction mechanism. Simple hydrolysis of the ligand is highly improbable,

since *O*-glycosidic substrates like *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside do not show hydrolysis under the chromatographic conditions employed.

Although hexosaminidase C showed binding to both thiol-Sepharose 4B, octyl- and phenyl-Sepharose, we did not obtain a specific elution.

The action of the divalent metal ions may result from chelate formation between these ions and the amino acids histidine and cysteine of the enzyme [24]. Such chelate formation may cause a disturbance in the native conformation of the enzyme and yield an enzymatically inactive form. The stability of the metal ion-ligand complex increases in the order Fe < Zn < Cu [24], which is reflected by an increasing inhibition of the enzyme activity. Addition of thiol compounds then has a double effect. On the one hand the enzyme is stabilized, resulting in an activation, on the other hand, more SH-groups become available with a concomitant higher degree of chelate formation (see Table III). There is a discrepancy between the results of Swallow et al. [8] who reported a molecular weight of 430 000 ± 48 000 for human brain hexosaminidase C, and ours (apparent molecular weight 240 000 ± 20 000). A possible explanation for this fact is: (1) the different source of the enzyme, and (2) Swallow et al. [8] stored their enzyme sample after tissue extraction at −20°C for some time before use.

Two K_m values of hexosaminidase C have been reported: 0.05 mM for the enzyme from human neonatal brain [4], and 0.83 mM for the enzyme from human placenta [7]. The K_m value of the bovine brain enzyme lies between those two figures, 0.46 mM. The kinetic experiments with some inhibitors once more show the difference between hexosaminidase C and the lysosomal hexosaminidases A and B. Acetate inhibits the hexosaminidase A and B activity [10,25], whereas it has no influence on the hexosaminidase C activity. The competitive inhibition of hexosaminidase C by *N*-acetylglucosamine but not by *N*-acetylgalactosamine is in agreement with its substrate specificity.

Future research on hexosaminidase C will be concerned with the elucidation of the biological function of the enzyme.

Acknowledgement

We would like to thank Dr. D.O.E. Gebhardt for improving the standard of English in our manuscript.

References

1 Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321—327
2 Sandhoff, K. and Christomanou, H. (1979) Hum. Genet. 50, 107—143
3 Hooghwinkel, G.J.M., Veltkamp, W.A., Overdijk, B. and Lisman, J.J.W. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 839—841
4 Besley, G.T.N. and Broadhead, D.M. (1976) Biochem. J. 155, 205—208
5 Braidman, I., Carroll, M., Dance, N. and Robinson, D. (1974) Biochem. J. 140, 295—301
6 Overdijk, B., Van der Kroef, W.M.J., Veltkamp, W.A. and Hooghwinkel, G.J.M. (1975) Biochem. J. 151, 257—261
7 Penton, E., Poenaru, L. and Dreyfus, J.C. (1975) Biochim. Biophys. Acta 391, 162—169
8 Swallow, D.M., Evans, L., Saha, N. and Harris, H. (1976) Ann. Hum. Genet. Lond. 40, 55—66
9 Overdijk, B., Veltkamp, W.A. and Hooghwinkel, G.J.M. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1236

10 Overdijk, B. (1975) M.D. Thesis, Vrije Universiteit, Amsterdam
11 Braidman, I., Carroll, M., Dance, N., Robinson, D., Poenaru, L., Weber, A., Dreyfus, J.C., Overdijk, B. and Hooghwinkel, G.J.M. (1974) FEBS Lett. 41, 181—184
12 Poenaru, L., Weber, A., Vibert, M. and Dreyfus, J.C. (1973) Biomedicine 19, 538—540
13 Galjaard, H., Hoogeveen, A., De Wit-Verbeek, H.A., Reuser, A.J.J., Keijzer, W., Westerveld, A. and Bootsma, D. (1974) Exp. Cell Res. 87, 444—448
14 Reuser, A.J.J. and Galjaard, H. (1976) FEBS Lett. 71, 1—5
15 Beutler, E. and Kuhl, W. (1977) Ann. Hum. Genet. Lond. 41, 163—167
16 Ikonne, J.U., Rattazzi, M.C. and Desnick, R.J. (1975) Am. J. Hum. Genet. 27, 639—650
17 Lisman, J.J.W. and Overdijk, B. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 1019—1022
18 Andrews, P. (1964) Biochem. J. 91, 222—233
19 Hofstee, B.H.J. (1952) Science 116, 329—331
20 Dixon, M. (1953) Biochem. J. 55, 170—171
21 Poenaru, L. and Dreyfus, J.C. (1973) Clin. Chim. Acta 43, 439—442
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
23 Fiddler, M.B., Ben-Yoseph, Y. and Nadler, H.L. (1979) Biochem. J. 177, 175—180
24 Martin, R.B. (1964) in Introduction to Biophysical Chemistry, pp. 335—340, McGraw-Hill Book Co., New York
25 Frohwein, Y.Z. and Gatt, S. (1967) Biochemistry 6, 2775—2782