Biochimica et Biophysica Acta, 364 (1974) 88–96

BBA 67294

PHYSICAL PROPERTIES AND BIOLOGICAL ACTIVITIES OF TWO FORMS OF α-*N*-ACETYLGLUCOSAMINIDASE FROM BOVINE SPLEEN

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(Received April 8th, 1974)

SUMMARY

α-*N*-Acetylglucosaminidase (2-acetamido-2-deoxy-α-D-glucoside acetamido-deoxyglucohydrolase, EC 3.2.1.50) was purified 108-fold from bovine spleen homogenates and could be separated into two interconvertible forms (I and II) by gel filtration and disc electrophoresis, whereas separation was not possible by DEAE-cellulose chromatography or isoelectric focusing. The two forms have a molecular weight ratio of 2:1 and display identical enzyme kinetics and the same biological activity in the correction of the altered heparan sulfate catabolism of cultured human Sanfilippo B fibroblasts. The lower corrective activity of the bovine α-*N*-acetylglucosaminidase as compared with that of the human urinary enzyme is caused by a lower rate of uptake into the fibroblasts.

INTRODUCTION

α-*N*-Acetylglucosaminidase (2-acetamido-2-deoxy-α-D-glucoside acetamido-deoxyglucohydrolase, EC 3.2.1.50) has been demonstrated in various tissues and body fluids with synthetic aryl 2-acetamido-2-deoxy-α-D-glucosides as substrates [1–3]. Purified enzyme preparations were reported to exist in multiple forms, the human urinary α-*N*-acetylglucosaminidase being separable into two subfractions [4]. Bannister and Phizackerley [5] have described an α,β-*N*-acetylglucosaminidase and α, β-*N*-acetylglucosaminidase from the limpet which contain the same two types of subunits in the ratios 2:1 and 1:1, respectively.

Sanfilippo B disease (mucopolysaccharidosis III B) which is caused by a deficiency of α-*N*-acetylglucosaminidase [4, 6] is characterized by abnormal heparan sulfate storage. Accordingly, heparan sulfate and its partial degradation products were found to be natural substrates of α-*N*-acetylglucosaminidase (von Figura, K. and Kresse, H., unpublished).

In this paper two forms of bovine spleen α-*N*-acetylglucosaminidase are described, and their physical properties and ability to correct the altered heparan sulfate catabolism of Sanfilippo B fibroblasts characterized.

MATERIALS AND METHODS

Chemicals and reagents. Phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside was synthesized according to Weissmann [7]. It was prepared in crystalline form and was free of the β-anomer as shown by a test with β-*N*-acetylhexosaminidase (EC 3.2.1.52) highly purified from bovine spleen. Phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was a kind gift from Dr E. Werries. *o*-Nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside was obtained from Koch–Light Laboratories (Colnbrook, England) and Folin–Ciocalteus phenol reagent from E. Merck (Darmstadt, Germany). A set of highly purified proteins for molecular weight determination by gel filtration was purchased from Serva (Heidelberg, Germany). Pre-swollen microgranular DEAE-cellulose anion exchanger (DE 52) was supplied by Whatman Biochemicals (Maidstone, England), Sephadex G-150 by Pharmacia (Uppsala, Sweden) and isoelectric focusing equipment and Ampholine pH 4–6 by LKB (Bromma, Sweden). All other reagents used were of reagent grade.

The following buffers were routinely used: 0.1 M citrate buffer, pH 6.0, containing 0.15 M NaCl, and 0.02% NaN_3 unless otherwise stated. 0.005 M Tris–HCl, pH 8.0, containing 0.01 M NaCl and 0.02% NaN_3 was used in ion-exchange chromatography. The pH optima and pH stabilities of the enzymes were determined in 0.1 M citrate buffers (pH 3–6), 0.1 M phosphate buffers (pH 5–8), and 0.1 M glycine–NaOH buffers (pH 8–11.5).

Assays. α-*N*-Acetylglucosaminidase activity was assayed by estimation of the phenol liberated from phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside according to the method of Pugh et al. [8]. The standard assay contained up to 0.4 ml of enzyme solution and 2 mM of the substrate in a final volume of 0.5 ml citrate buffer of pH 4.7. The mixture was incubated at 37 °C up to 4 h. Enzyme action was stopped by the addition of 1 ml Folin–Ciocalteus phenol reagent (1:5 diluted). The incubation mixture was centrifuged and 1.5 ml of 12% Na_2CO_3 solution were added to 1 ml of the clear supernatant. The colour developed after 20 min at 37 °C was read at 650 nm. 1 unit of enzyme activity was defined as the amount of enzyme required to catalyze the splitting of 1 μmole of substrate per min at 37 °C.

Purification procedure. Freshly collected bovine spleen was passed through a meat mincer and stored frozen until required. 500 ml of citrate buffer were added to 250 g of the minced tissue and the mixture was homogenized with cooling in an Ultraturrax (Janke and Kunkel) for about 20 min. The homogenate was twice frozen and thawed, 250 ml of citrate buffer were added and the mixture stirred for 3 h at room temperature before being centrifuged (30 min, 70 000 × *g*). The supernatant was brought to 41% saturation by addition of solid $(NH_4)_2SO_4$ and centrifuged. The precipitate was dissolved and dialysed against citrate buffer and the material precipitating between 4 and 31% $(NH_4)_2SO_4$ saturation was collected by centrifugation. This fraction was again dissolved and dialysed against citrate buffer and once more precipitated by addition of the amount of solid $(NH_4)_2SO_4$ to yield a 25.5% saturated solution. The precipitate isolated by centrifugation was dissolved in Tris–HCl buffer and dialysed against the same buffer for 24 h. The dialysed solution was centrifuged at 23 000 × *g* and loaded onto a DEAE-cellulose column (4 cm × 40 cm, 6 mg protein/ml column bed) equilibrated with Tris–HCl buffer. The proteins were eluted with 1–2 column volumes of starting buffer and then with 6 column volumes of Tris–

HCl buffer with a NaCl gradient 0.01–0.4 M. The α-*N*-acetylglucosaminidase activity was eluted from the column in a single peak (Fig. 1, inset). The active fractions were collected, concentrated at 37 °C in a rotatory evaporator, dialysed against starting buffer and rechromatographed under the same conditions.

Gel filtration. Analytical and preparative gel filtrations were performed on 1.5 cm × 150 cm and 3.6 cm × 190 cm cooled columns of Sephadex G-150 equilibrated with citrate buffer, respectively. Samples of up to 2 and 20 ml, respectively, were applied to the columns. The ratio of activity of the two separated forms was calculated from the activity of the appropriate fractions. For molecular weight determinations a Sephadex G-150 column was calibrated with dextran blue and the following pure proteins (molecular weights in parentheses): trypsin (23 700), ovalbumin (40 000), bovine serum albumin (monomer and dimer, 65 400 and 130 800).

Isoelectric focusing experiments. Isoelectric focusing was performed in a 110-ml column (LKB) with a gradient from pH 4 to 6 (1 % Ampholine, LKB) stabilized with sucrose. The enzyme solution, previously dialysed against a 1 % glycine solution, was applied to the column and the focusing performed at 500 V for 48 h, after which fractions of about 4 % of the column volume were collected.

Disc gel electrophoresis. Disc electrophoresis was performed in the polyacrylamide gel system described by Ornstein [9] and Davis [10]. The running distance was 5 cm, in routine experiments the gel concentration was 7 %. For the molecular weight determinations gel concentrations of 4, 5.6 and 7 % were chosen. Each sample was run twice. One gel was stained with amido black, the second one was cut into 4-mm thick slices, which were extracted with citrate buffer, and assayed for α-*N*-acetylglucosaminidase activity.

Cell culture. Sanfilippo B fibroblasts derived from skin biopsies of patients affected with mucopolysaccharidosis III B were maintained in culture as previously described [11, 12]. Enzymes to be incubated with the fibroblasts were dialysed against 10 mM sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl prior to sterilisation by passage through Millipore filters.

Determination of the biological activity against Sanfilippo B fibroblasts. 10 milliunits α-*N*-acetylglucosaminidase in 1 ml 10 mM sodium phosphate buffer (pH 6.0, 0.15 M NaCl) were added to confluent cultures grown in 20 ml of medium. Determination of the enzyme activity taken up into the cells within 16 h [13] was modified by replacing UDP-*N*-acetylglucosamine by 6 mM phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside as substrate. Corrective factor activity was determined as previously described [14]. Human urinary α-*N*-acetylglucosaminidase was purified according to von Figura et al. [13].

RESULTS

Heterogeneity of α-N-acetylglucosaminidase

α-*N*-Acetylglucosaminidase was purified 108-fold with a 5 % yield from bovine spleen (Table I). The most purified preparation still contained several contaminant proteins as shown by disc electrophoresis, and had a β-*N*-acetylhexosaminidase activity of 9.3 milliunits/mg. Unless otherwise stated the experiments were done with this preparation.

Gel filtration on Sephadex G-150 (Fig. 1) of α-*N*-acetylglucosaminidase as well

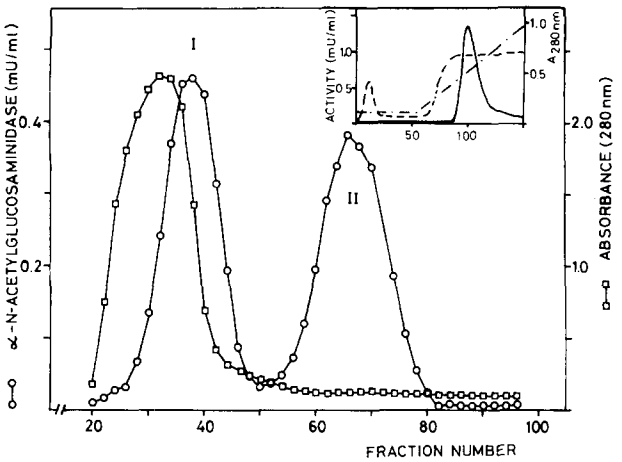

Fig. 1. Elution pattern of bovine spleen α-*N*-acetylglucosaminidase. (A) Gel filtration (Sephadex G-150, column 3.6 cm × 190 cm, fraction volume 9.8 ml, eluted with citrate buffer). ○—○, α-*N*-acetylglucosaminidase activity (milliunits/ml); □—□, absorbance at 280 nm. (B) (Inset): Ion-exchange chromatography (DEAE-cellulose, column 4 cm × 40 cm, 6 mg protein/ml bed volume, fraction volume 18.5 ml, eluted with 0.005 M Tris–HCl buffer, pH 8.0, 0.02% NaN_3, 0.01 M NaCl, gradient: 0.01–0.4 M NaCl). ———, α-*N*-acetylglucosaminidase activity (milliunits/ml); ---------, absorbance at 280 nm; -·-·-·, NaCl concentration (start: 0.01 M, final: 0.4 M).

as disc electrophoresis yielded a higher and a lower molecular size fraction of activity (Form I and II, respectively) whereas this α-*N*-acetylglucosaminidase appeared to be homogeneous on anion-exchange chromatography (DEAE-cellulose, Fig. 1, inset) and isoelectric focusing. The ratio of Forms I to II depended on the conditions of preparation of α-*N*-acetylglucosaminidase. While in aqueous extracts of fresh bovine spleen only Form I could be detected the mean activity ratio of Form I to Form II

TABLE I

PURIFICATION OF α-*N*-ACETYLGLUCOSAMINIDASE

1000 g of fresh bovine spleen were used for this preparation.

Procedure	Total activity (milliunits)	Specific activity (milliunits/mg)	Yield (%)	Purification
Homogenate	10 863	0.0692	100	1
$(NH_4)_2 SO_4$ precipitate (41% saturation)	7 116	0.108	65	1.6
$(NH_4)_2 SO_4$ precipitate (4–31% saturation)	4 782	0.216	44	3.1
$(NH_4)_2 SO_4$ precipitate (25.5% saturation)	3 411	0.374	31	5.4
Change of buffer and centrifugation	2 112	0.513	19	7.4
First DE 52 chromatography	1 252	3.173	12	45.9
Second DE 52 chromatography	542	7.494	5	108.3

obtained from $(NH_4)_2SO_4$ fractions was 1:0.8. Similar ratios were also found at further stages of purification.

Addition of 4 M urea to the enzyme preparation as well as its storage at 37 °C for 4 days lead to the complete dissociation of Form I to Form II without loss of activity, whereas pretreatment of α-*N*-acetylglucosaminidase with 1% sodium dodecylsulfate (24 h, 4 °C), 2 M NaCl (48 h, 4 °C), or neuraminidase *(Vibrio cholerae)* and subsequent gel filtration on Sephadex G-150 with citrate buffer as eluant did not affect the ratio of the two fractions. Gel electrophoresis in presence of sodium dodecylsulfate [20] lead to an entire inactivation of the enzyme.

Isolation and characterization of Forms I and II

Form I could be separated from Form II by gel filtration on Sephadex G-150 and subsequent vacuum filtration of the pooled fractions. The same procedure with the fractions containing Form II lead to a partial conversion of Form II into Form I. Form II could be isolated free of Form I by addition of 4 M urea to the mixture of both forms for 4 days and subsequent removal of urea by dialysis (Fig. 2A). Form II

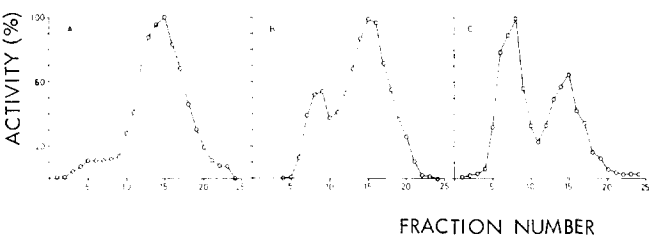

Fig. 2. Ratio of Forms I and II after 4 days treatment with 4 M urea and subsequent removal of urea by dialysis. (A) Immediately after removal of urea (zero time). (B) 1 day after removal of urea. (C) 5 days after removal of urea.

could not be stored without urea as conversion into a mixture of Forms I and II took place within some days (Fig. 2A–2C).

The molecular weights of both forms were determined to be 127 500 and 64 500 for Forms I and II, respectively, by gel filtration on a calibrated Sephadex G-150 column. The ratio of the molecular weights was calculated from disc electrophoresis experiments in gels of various concentrations. Two straight lines were obtained in a plot of the logarithm of the distance covered by the two enzyme forms versus gel concentration (Ferguson plot, Fig. 3). The slopes of these straight lines were calculated by a least squares method to be in the ratio of 2.1:1.

The K_m of Forms I and II were computed as $0.32 \cdot 10^{-3}$ and $0.29 \cdot 10^{-3}$ M for phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside as substrate and $0.67 \cdot 10^{-4}$ and $0.43 \cdot 10^{-4}$ M for *o*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside, respectively. Both peaks were equally stable when stored at 37 °C in 0.1 M acetate buffer (pH 5.5). An increase in activity was observed after 24 h followed by a gradually decrease to about 50% of the starting activity within 5 weeks under the same conditions. When the enzyme was heated for 30 min at various temperatures in citrate buffer, pH 6.0, an identical pattern of inactivation was obtained for both forms.

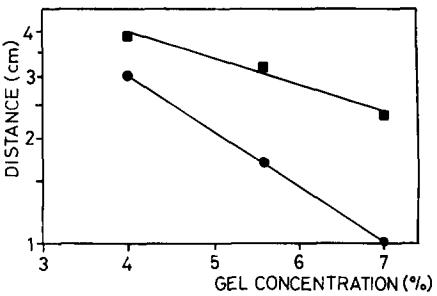

Fig. 3. Disc electrophoresis of Form I (●) and Form II (■) in gels of different concentrations. Logarithm of running distance is plotted versus gel concentration.

Heating to 50 °C did not affect the catalytic activity, whereas heating at 70 °C lead to 90% inactivation.

The isoelectric points of the two forms were determined by isoelectric focusing. They were between pH 4.7 and 4.9 for both forms in several runs. No alteration of the isoelectric points could be observed after treatment with neuraminidase for 20 h in acetate buffer of pH 5.5 at 37 °C. Both forms had a pH optimum of 4.7 and showed the same shape of the pH activity curve (Fig. 4). The stability of both forms was almost constant between pH 5.0 and 10.5 but sharply decreased beyond this region.

Dialysis of Forms I and II against 10^{-4} M EDTA as well as addition of 10^{-2} M $MgCl_2$ and $ZnSO_4$ did not affect the activity. Complete inactivation occurred on addition of $2 \cdot 10^{-4}$ M $HgCl_2$.

Biological activity

Both forms of bovine spleen α-*N*-acetylglucosaminidase exhibited corrective activity if administered to Sanfilippo B fibroblasts (Table II). The biological activity of the bovine spleen enzyme forms, however, was only 1/24 to 1/35 of that of human urinary α-*N*-acetylglucosaminidase, when equal amounts of catalytic activity against phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside of the various enzymes were added. Table III indicates that this difference is mainly caused by a different rate of uptake of the enzymes into the cells. If the different forms of bovine spleen α-*N*-acetylglucos-

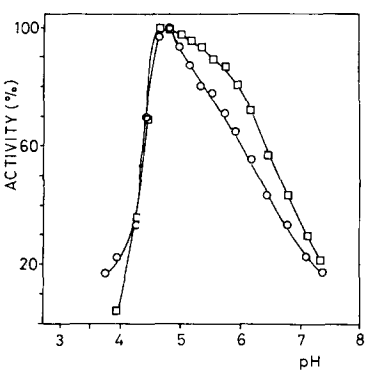

Fig. 4. pH-activity curves of Form I (○) and Form II (□).

TABLE II

COMPARISON OF CORRECTIVE ACTIVITY OF α-*N*-ACETYLGLUCOSAMINIDASE FROM BOVINE SPLEEN AND HUMAN URINE IN SANFILIPPO B FIBROBLASTS

Units of corrective activity are calculated from the equation 1/unit = (maximal correction/correction) − 1. Correction is defined as reduction of ^{35}S-sulfated mucopolysaccharide accumulation brought about by the sample. Addition of 10 milliunits of human urinary α-*N*-acetylglucosaminidase causes maximal correction and reduces the accumulated activity by 17 800 cpm/mg cell protein to the normal level of about 10 000 cpm/mg.

α-*N*-Acetylglucosaminidase added		Correction of ^{35}S-sulfated mucopolysaccharide accumulation (cpm/mg cell protein)	Units corrective activity milliunit α-*N*-acetylglucosaminidase
Source	Amount (milliunit)		
Bovine spleen Forms I + II	1	6470	0.57
Bovine spleen Form I	1	8180	0.85
Bovine spleen Form II	1	7910	0.80
Human urine	0.1	11870	20

TABLE III

PINOCYTOSIS OF BOVINE SPLEEN AND HUMAN URINARY α-*N*-ACETYLGLUCOSAMINIDASE BY SANFILIPPO B FIBROBLASTS

5 milliunits of each enzyme preparation were added to the medium. The amount of pinocytosed enzyme was determined after 16 h.

Enzyme	α-*N*-acetylglucosaminidase pinocytosed (milliunit/mg cell protein)
Bovine spleen Forms I + II	0.0124
Bovine spleen Form I	0.0140
Bovine spleen Form II	0.0120
Human urine	0.265

aminidase were added to confluent cultures of Sanfilippo B cells, 0.24–0.30% of the applied activity were internalized per mg cell protein within 16 h, whereas the uptake of the urinary enzyme was 18–22 times higher under the same conditions. Both the rate of pinocytosis and the corrective activity were the same, within the margin of experimental error, for the two separated forms of bovine spleen α-*N*-acetylglucosaminidase and of an unseparated mixture of them.

DISCUSSION

Bovine spleen was known to contain an appreciable α-*N*-acetylglucosaminidase activity [3], but the possible heterogeneity of the enzyme from this source had not been studied. The detection of two forms of this enzyme can be explained by the difference in their molecular weight. The very similar physical properties of the two forms of α-*N*-acetylglucosaminidase from bovine spleen, especially their identical behaviour in separatory systems which differentiate proteins of different charge and

hence composition, and the ratio of 2:1 for the molecular weights of Form I to Form II obtained by two independent methods, make it improbable that the two forms represent the free enzyme (Form II) and the enzyme associated with a non-active protein (Form I) but rather suggest a model of Form I being an aggregate of two molecules of Form II.

The values obtained for the Michaelis constant were lower than the one formerly given for this enzyme [3]. pH optimum and pH stability were in agreement with those of the bovine liver and spleen α-*N*-acetylglucosaminidases [2, 3]. The asymmetry of the pH activity curve may be caused by inactivation of the enzyme during incubation below pH 5.0. The reason for an increase in activity on storage of the enzyme at 37 °C, which was also observed for human serum α-*N*-acetylglucosaminidase is not known (von Figura, K., Lögering, M. and Kresse, H., unpublished).

The biological function of glycosidases is indicated by their capacity to be taken up into cultured human skin fibroblasts as well as into fibroblasts deficient of a distinct enzyme (refs 13, 15–17, and von Figura, K., Lögering, M. and Kresse, H., unpublished, and Cantz, M., personal communication), in the latter case the disturbed catabolism is corrected upon uptake of the replacement enzyme. A more detailed study of this process revealed that human urinary α-*N*-acetylglucosaminidase is taken up by adsorptive pinocytosis, which requires recognition sites both of the enzyme molecule and the cell surface [13]. The involvement of carbohydrates for pinocytosis has been shown for β-*N*-acetylhexosaminidase, the uptake of this enzyme was prevented when oxidized by periodate under conditions not affecting its catalytic properties [16]. From these results the determination of the rate of pinocytosis seems to be a sensitive indicator for structural differences in the carbohydrate moieties of different α-*N*-acetylglucosaminidases.

Although no evidence was supplied from our results for the glycoprotein nature of bovine spleen α-*N*-acetylglucosaminidase, the two forms were taken up by cultured Sanfilippo fibroblasts and exhibited intracellular corrective activity, both normalizing the deranged heparan sulfate catabolism to the same extent. Whether the marked difference in biological activity between bovine spleen and human urinary α-*N*-acetylglucosaminidase is caused by differences in their carbohydrate moieties remains to be investigated. The low rate of uptake of the bovine spleen enzyme compared with that of the human urinary α-*N*-acetylglucosaminidase is not necessarily due to its heterologous nature since similar differences in pinocytosis and corrective activity are observed between human urinary and serum α-*N*-acetylglucosaminidases (von Figura, K., Lögering, M. and Kresse, H., unpublished). Other authors have also described the uptake and corrective activity of bovine β-glucuronidase [18] and even of plant α-galactosidase [19] in cultured human cells deficient of these enzymes.

The results of the pinocytosis experiments support the thesis that Forms I and II have similar or identical structures and show that the aggregation and dissociation of the two forms do not influence their ability to be pinocytosed.

ACKNOWLEDGEMENTS

We are indebted to Dr B. Ashton for critical reading of the manuscript and to Mrs G. Scheidgen for skilful technical assistance. These experiments were supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 104).

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