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Serum α -N-acetylglucosaminidase: Determination, Characterization, and Corrective Activity in *Sanfilippo* B Fibroblasts

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Summary: Assays for the determination of serum α -N-acetylglucosaminidase (EC 3.2.1.50) activity are described employing *p*-nitrophenyl-N-acetyl- α -D-glucosaminide, phenyl-N-acetyl- α -D-glucosaminide, and UDP-N-acetylglucosamine as substrates. A log normal distribution of the serum enzyme activity was found. The determination of serum α -N-acetylglucosaminidase activity proved to be a valuable tool for the recognition of homozygous and heterozygous carriers of the *Sanfilippo* B gene.

The enzyme was characterized with regard to its pH optimum, K_m , isoelectric point, and the influence of several bivalent cations was studied.

Serum α -N-acetylglucosaminidase was added to *Sanfilippo* B fibroblast cultures, which are deficient in this enzyme. This resulted in an intracellular enzyme level, which was one twelfth of that found after addition of an equal amount of enzyme derived from human urine. The pinocytosed serum enzyme, however, exhibited the same corrective activity towards the accumulated heparan sulfate as the urinary enzyme.

α -N-Acetylglucosaminidase: Bestimmung, Charakterisierung und aufgenommene Aktivität in Sanfilippo B-Fibroblasten

Zusammenfassung: Die Aktivität der α -N-Acetylglucosaminidase (EC 3.2.1.50) im Serum wurde mit Hilfe von *p*-Nitrophenyl-N-acetyl- α -D-glucosamin, Phenyl-N-acetyl- α -D-glucosamin und UDP-N-acetylglucosamin als Substrat bestimmt. Die Häufigkeitsverteilung der Enzymaktivität entspricht in einer Normalpopulation einer lognormalen Verteilung. Die Bestimmung dieses Enzyms im Serum, dessen Inaktivität die *Sanfilippo* B-Erkrankung (Mucopolysaccharidose III B) verursacht, ermöglicht die Erkennung von homozygoten und heterozygoten *Sanfilippo* B-Genträgern.

Das pH-Optimum, die K_m und der isoelektrische Punkt des Enzyms sowie der Einfluß verschiedener zweiwertiger Kationen auf die Enzymaktivität wurde bestimmt.

Nach Zusatz von Serum α -N-Acetylglucosaminidase zu *Sanfilippo* B-Fibroblastenkulturen betragen die intrazellulären Enzymaktivitäten nur 1/12 der Aktivität, die nach Zusatz einer gleichen Enzymmenge aus menschlichem Urin erreicht werden. Das aufgenommene Serumenzym besitzt die gleiche katalytische Aktivität gegenüber dem gespeicherten Heparansulfat wie das Urinenzym.

Introduction

The inactivity of α -N-acetylglucosaminidase (EC 3.2.1.50) has been shown to be the basic defect in mucopolysaccharidosis III B (*Sanfilippo* B disease), an inherited disorder of heparan sulfate catabolism (1, 2). The enzyme deficiency has been demonstrated so far in liver, kidney, fibroblasts, serum and urine. The determination of serum α -N-acetylglucosaminidase activities has made possible the detection not only of *Sanfilippo* B patients but also of heterozygous carriers of the *Sanfilippo* B gene (3). In the present paper the physical and kinetic properties of normal serum α -N-acetylglucosaminidase are described. As there are insufficient experimental data for a critical evaluation of enzyme replacement therapy

of the mucopolysaccharidoses by serum infusions, the pinocytotic uptake and the biological action of the serum enzyme in *Sanfilippo* B fibroblasts were studied.

Materials and Methods

Materials

Uridine 5'-diphospho-N-acetylglucosamine and adenosine 5'-monophosphate were purchased from Boehringer-Mannheim, *p*-nitrophenyl-N-acetyl- α -D-glucosaminide from Koch-Light.

Phenyl-N-acetyl- α -D-glucosaminide was kindly provided by Dr. G. Mersmann.

Ampholines pH 4–6 and pH 5–8 were obtained from LKB Produkter AB (Stockholm), $\text{Na}_2^{35}\text{SO}_4$ (carrier-free) from Amersham-Buchler. All other reagents were of analytical grade.

Determination of serum α -N-acetylglucosaminidase activity

Sera were assayed either within 9 hours after blood withdrawal, or after storage at -20°C for up to 8 months. Three different substrates were employed in the determination of enzyme activity:

In test I the reaction mixture consisted of 0.01 mol/l *p*-nitrophenyl-N-acetyl- α -D-glucosaminide, 0.0375 mol/l sodium citrate pH 3.8, 0.2 g/l NaN_3 and 0.2 ml serum, final volume 0.5 ml. After incubation for 3–36 hours at 37°C deproteinization was performed with 0.2 ml 0.825 mol/l HClO_4 . 0.5 ml of the supernate was treated with 1.0 ml 0.4 mol/l glycine/NaOH buffer, pH 10.4. Readings were made at 430 nm.

In tests II and III *p*-nitrophenyl-N-acetyl- α -D-glucosaminide was replaced by 0.006 mol/l phenyl-N-acetyl- α -D-glucosaminide or 0.01 mol/l UDP-N-acetylglucosamine, respectively. After deproteinization with 0.2 ml 0.152 mol/l uranyl acetate the N-acetylglucosamine liberated was determined by the *Morgan-Elson* reaction (4). 0.2 ml of the supernate was treated with $10\ \mu\text{l}$ 2 mol/l KOH and $40\ \mu\text{l}$ 0.8 mol/l sodium tetraborate, pH 9.2, and the mixture was kept for exactly 4 minutes in a boiling water bath. Following the addition of 1.2 ml of tenfold diluted *Ehrlich's* reagent, the colour was developed by incubation for 20 minutes at 37°C . Readings were made at 546 nm.

Other methods

Electrofocussing was done in a 440 ml column (LKB Produkter AB) according to the instructions in the producer's manual.

The determinations of *Sanfilippo B* corrective factor activity and α -N-acetylglucosaminidase activity in cultivated skin fibroblasts were performed as described previously (2).

Results

Methodology of serum α -N-acetylglucosaminidase determination

Using *p*-nitrophenyl-N-acetyl- α -D-glucosaminide, phenyl-N-acetyl- α -D-glucosaminide and UDP-N-acetylglucosamine as substrates for the α -N-acetylglucosaminidase, N-acetylglucosamine is liberated at a constant rate for a least 24 hours under the conditions described.

As shown in Table 1 serum α -N-acetylglucosaminidase exhibits the highest activity towards UDP-N-acetylglucosamine, while *p*-nitrophenyl-N-acetyl- α -D-glucosaminide is cleaved at the lowest rate. No age-dependence of the enzyme activity was found. The small number of 0–1 year old individuals investigated ($n = 6$), however, did not allow a correct statistical comparison of this group with other age-groups.

A log normal distribution was found for the serum α -N-acetylglucosaminidase activity (Fig. 1).

Since the determination of *p*-nitrophenolate is easier and quicker to perform than that of N-acetylglucosamine,

Fig. 1. Distribution of serum α -N-acetylglucosaminidase activity of normal individuals (a) and of homozygous and heterozygous carriers of the *Sanfilippo B* gene (b).

Tab. 1. Serum α -N-acetylglucosaminidase activity in normal individuals and homozygous and heterozygous carriers of the *Sanfilippo B* gene

	Test I (<i>p</i> -nitrophenyl-N- acetyl- α -D-glucosaminide)	Test II (phenyl- α -D- glucosaminide) [U/l]	Test III (UDP-N-acetyl- glucosamine)
Normals			
mean value*	0.44	0.70	0.88
median value**	0.42	0.66	0.84
1 s range	0.29–0.59	0.48–0.91	0.63–1.12
	$n = 40$	$n = 142$	$n = 172$
<i>Sanfilippo B</i> disease			
Homozygous			
mean value*	0.01	0.05	0.15
range	0.00–0.01	0.04–0.07	0.11–0.19
	$n = 3$	$n = 4$	$n = 4$
Heterozygous			
mean value*	0.13	0.23	0.36
standard deviation	0.01	0.05	0.08
	$n = 16$	$n = 22$	$n = 22$

* calculated as for a normal distribution

** calculated as for a log normal distribution

p-nitrophenyl-N-acetyl- α -D-glucosaminide is preferred for routine purposes. Determination of N-acetylglucosamine by the *Morgan-Elson* reaction should be used if low activities are expected, e. g. in serum from patients with *Sanfilippo* B disease, since the absorption coefficient of the *Morgan-Elson* chromogen is 5 times greater than that of *p*-nitrophenolate. The measurement of free phenol from the cleavage of phenyl-N-acetyl- α -D-glucosaminide is made difficult by high blank values.

Deproteinization has to be carried out with a neutral salt if the N-acetylglucosamine is to be measured by the *Morgan-Elson* reaction. Unprecipitated uranyl acetate in the supernate, however, depresses colour development in the *Morgan-Elson* reaction by 20% in a sample with a serum content of 70 g/l, and a variation in the serum protein concentration of $\pm 10\%$ modifies the absorbance by $\pm 5\%$. In Table 2 the values are not corrected for the effect of uranyl acetate or for variations in the protein content.

Influence of storage on the enzyme activity

Serum α -N-acetylglucosaminidase shows no detectable decrease of activity after storage at -20°C for up to 8 months and repeated freezing and thawing. Storage at room temperature or at $+4^\circ\text{C}$ for up to one week may lead to changes of activity of $\pm 20\%$. The reason for this is not yet understood.

Properties

The *Michaelis* constants of the serum N-acetylglucosaminidase calculated from *Lineweaver-Burk* plots (Fig. 2) were 0.20; 0.39; and 1.10 mmol/l towards *p*-nitrophenyl-N-acetyl- α -D-glucosaminide, UDP-N-acetylglucosamine, and phenyl-N-acetyl- α -D-glucosaminide respectively.

The pH dependence of the enzyme activity varied with the substrates used (Fig. 3). Using phenyl-N-acetyl- α -D-glucosaminide a broad pH optimum with a maximum at pH 4.3 was found, whereas with *p*-nitrophenyl-N-acetyl- α -D-glucosaminide a second pH optimum at pH 7.6 has invariably been observed in addition to an optimum at

Fig. 2. *Lineweaver-Burk* plot of serum α -N-acetylglucosaminidase using *p*-nitrophenyl-N-acetyl- α -D-glucosaminide ($\bullet$ — $\bullet$), phenyl-N-acetyl- α -D-glucosaminide (Δ — Δ), and UDP-N-acetylglucosamine ($\circ$ — $\circ$) as substrates.

Fig. 3. pH dependence of hydrolysis of *p*-nitrophenyl-N-acetyl- α -D-glucosaminide ($\bullet$ — $\bullet$), phenyl-N-acetyl- α -D-glucosaminide (Δ — Δ), and UDP-N-acetylglucosamine ($\circ$ — $\circ$) by serum.

Tab. 2. Comparison of corrective activity of α -N-acetylglucosaminidase from serum and urine in *Sanfilippo* fibroblasts.

Corrective activity is calculated from the equation: maximal correction/correction - 1 = 1/units. Correction is the reduction of $^{35}\text{SO}_4$ mucopolysaccharide-accumulation brought about by the sample.

α -N-acetylglucosaminidase added	Correction of $^{35}\text{SO}_4$ -mucopolysaccharide accumulation (counts/min $\cdot$ mg cell protein)	<i>Sanfilippo</i> B corrective activity [U]	Corrective activity/added α -N-acetylglucosaminidase activity
Maximal correction (urinary enzyme, 10 mU)	11 000		
Serum enzyme, 0.42 mU	1 730	0.19	0.45
Urinary enzyme, 0.42 mU	7 660	2.33	5.55
Serum enzyme, 0.42 mU + urinary enzyme, 0.42 mU	7 990	2.70	3.21

pH 4.4. UDP-N-acetylglucosamine is cleaved maximally at pH 9.7 and pH 4.7. N-acetylglucosamine is liberated from UDP-N-acetylglucosamine not only by the action of the α -N-acetylglucosaminidase but also by the combined action of a nucleotide pyrophosphatase and orthophosphoric acid monoester phosphatase, the latter of which can be inhibited by addition of ATP, ADP or AMP. Figure 4 shows the hydrolysis of UDP-N-acetylglucosamine in the presence and absence of 10 mmol/l AMP. The addition of AMP reduces the rate of hydrolysis by 73% at pH 9.7 and by 18% at pH 4.5.

The enzyme activity is completely destroyed after 10 minutes heating at 70 °C.

The influence of several bivalent cations at a concentration of 10 mmol/l were tested on α -N-acetylglucosaminidase activity.

Hg^{++} completely inhibits the enzyme activity towards all three substrates, whereas the degree of inactivation by Mn^{++} and Cu^{++} ranges between 40 and 80% depending on the substrate used. Mg^{++} , Ba^{++} , Ca^{++} , Cd^{++} , Pb^{++} have no influence on the activity. SH-protective reagents (cysteine, glutathione, dithiothreitol, 2 mmol/l each), SH-reagents (N-ethylmaleinimide, 1 mmol/l), EDTA 2 mmol/l, and Triton X 100 ($0.04-3.0 \times 10^{-2}$ v/v) do not alter serum α -N-acetylglucosaminidase activity.

The isoelectric point of serum α -N-acetylglucosaminidase, as determined by isoelectric focussing, is at pH 4.34 $\pm$ 0.02.

Corrective activity of serum α -N-acetylglucosaminidase

Addition of α -N-acetylglucosaminidase to the culture medium of *Sanfilippo* B fibroblasts reduces the accumu-

Fig. 4. pH dependence of hydrolysis of UDP-N-acetylglucosamine in the absence (o—o) and presence (▲—▲) of 0.01 mol/l AMP.

Tab. 3. Pinocytosis of serum and urinary N-acetyl- α -D-glucosaminidase by *Sanfilippo* B fibroblasts. The intracellular enzyme activity was determined 16 hours after enzyme addition.

N-acetyl- α -D-glucosaminidase added [mU]	N-acetyl- α -D-glucosaminidase pinocytosed [mU/mg cell protein]	
serum enzyme	1.3	0.0043
urinary enzyme	1.3	0.0516
serum enzyme + urinary enzyme	1.3	0.0544

lation of sulfated mucopolysaccharides as previously described (2). In Table 2a comparison of the corrective activity of serum α -N-acetylglucosaminidase and urinary α -N-acetylglucosaminidase is given. On administration of equal amounts of enzyme activity the corrective activity brought about by the serum enzyme is only one twelfth of that of the urinary enzyme. Mixing experiments gave the expected intermediate values. The reduced corrective activity of the serum enzyme is not due to a diminished activity towards the stored heparan sulfate. As shown in Table 3 the intracellular enzyme level obtained after administration of the serum enzyme is also one twelfth of that obtained after application of an equal amount of urinary enzyme.

Discussion

In this paper the determination of serum α -N-acetylglucosaminidase activities with three different substrates is described. All three substrates are suitable for the diagnosis of homozygous and heterozygous carriers of the *Sanfilippo* B gene. For clinical purposes, however, *p*-nitrophenyl-N-acetyl- α -D-glucosaminide is the most convenient substrate, since the liberated *p*-nitrophenolate is readily measured. The applicability of UDP-N-acetylglucosamine is limited by the fact that even at pH 4.7 the rate of cleavage by enzymes other than α -N-acetylglucosaminidase is more than 20%.

The data obtained for pH optimum and kinetic behavior of serum α -N-acetylglucosaminidase agree well with data found for α -N-acetylglucosaminidase from human urine (4), bovine spleen (5, 6), pig liver (7) and rat testis (8), and the heat stability is also in accordance with reported data (8, 1). On the other hand characteristic differences were found in the isoelectric points of urinary and serum α -N-acetylglucosaminidase, being pH 5.52 (4) and pH 4.34, respectively. It is therefore concluded that the urinary enzyme is not derived from the serum by simple ultrafiltration without further modification.

Fratantoni et al. (10) have shown that in the mucopolysaccharidoses the deranged catabolism of mucopolysaccharides can be normalized in cultivated fibroblasts by the addition of the deficient enzyme to the culture

medium. These observations initiated a number of therapeutic trials to improve the clinical picture by infusions of whole blood (11), plasma (11–16), serum (17) or leukocytes (18). Contradictory results were reported. For the only therapeutic attempt reported for *Sanfilippo* B disease (16) no improvement was observed. Studies on the pinocytotic uptake and intracellular action of α -N-acetylglucosaminidase in cultured *Sanfilippo* B fibroblasts have been reported so far only for the enzyme from human urine (19) and placenta (20). Under the assumption that the serum enzyme has the same properties as

the urinary enzyme with regard to pinocytosis and intracellular action it was calculated that per g cell protein, 80 ml of serum would be needed for half-maximal reduction of the accumulated mucopolysaccharides.

After the administration of the serum enzyme the catalytic activity available intracellularly for metabolic correction is, however, more than one order of magnitude lower than after the administration of the urinary enzyme. It has to be concluded therefore that enzyme replacement therapy in *Sanfilippo* B disease by serum infusions will not result in measurable specific effects.

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