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HUMAN β-GLUCOSIDASE: INHIBITION BY SULPHATES AND PURIFICATION BY AFFINITY CHROMATOGRAPHY ON DEXTRAN-SULPHATE-SEPHAROSE

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Summary

The acid β-glucosidase (D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3 2.1 45) from human placenta is inhibited by sulphated macromolecules such as Dextran sulphate or chondroitin sulphate. This inhibition is alleviated by compounds such as crude taurocholate or phospholipids, which are known activators of acid β-glucosidase. Partially-purified human β-glucosidase will bind to Dextran sulphate linked to Sepharose 4B and can be eluted with low concentrations of crude sodium taurocholate. This procedure gives a 10—15 fold purification with good yield and has been included in a scheme giving an approx. 4000-fold purification of placental β-glucosidase. Evidence is presented which suggests that phospholipids bind to β-glucosidase by both ionic and hydrophobic interactions The inhibition of enzyme activity caused by sulphated compounds and non-ionic detergents may be attributed to interference with, respectively, the ionic and hydrophobic binding of phospholipid to the enzyme

Introduction

Glucosylceramidase (D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1 45) is a membrane-bound lysosomal hydrolase which catalyzes the hybrolysis of glucocerebroside into ceramide and glucose. Purification of this enzyme is of particular interest because of its potential use for replacement

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In this paper β-glucosidase refers to glucosylceramidase

therapy in cases of Gaucher's disease [1—3]. However, purification has been hampered by the hydrophobic characteristics of the enzyme and conventional methods give poor recovery of activity [4]. Higher yields have recently been achieved by affinity chromatography on Con A-Sepharose [1] and on hydrophobic gels such as octyl-Sepharose and decyl-agarose [2], and phosphatidylserine-agarose [1] These affinity methods are not highly specific but can be used in combination to obtain good purification Ho [5] reported a more specific affinity technique employing an immobilized glycoprotein effector (purified from the spleen of a patient with Gaucher's disease) which bound glucosylceramidase in the presence of phospholipids. Although successful, the applicability of this method to large-scale purifications is limited by the availability of the effector glycoprotein

Development of a widely applicable affinity technique requires a specific, reversible and readily available enzyme inhibitor During studies on the inhibition of human acid β-glucosidase we found that the enzyme was inhibited by sulphated macromolecules In this report we present data concerning the mechanism of this inhibition and describe the use of immobilized Dextran sulphate as an affinity gel for placental β-glucosidase

Materials and Methods

Enzyme assays β-Glucosidase activity was assayed with the artificial fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (4MU-β-Glu, Koch-Light) as described previously [6]. Glucosylceramidase activity was determined under similar assay conditions but using as substrate *N*-stearoyl-dihydroglucocerebroside (Miles-Yeda) in the form of a micellar suspension. Glucose released from this compound was assayed with the 'Glucostat' test kit (Worthington Biochemicals)

Assays to measure the effect of phospholipids and inhibitors on the purified enzyme were performed as follows Phospholipid suspension in 30 μl 0 06% Triton X-100, 5 mM 4 methylumbelliferone-β-glucopyranoside in 60 μl H_2O, 30 μl 0.2 M phosphate/citric acid, pH 6 0, plus inhibitors as required, 10 μl 0.12% Triton X-100, enzyme, diluted at least 10-fold in 20 μl H_2O. Phospholipids were obtained from Sigma Chemical Company Crude sodium taurocholate was from British Drug Houses. All work described in this paper was carried out with a single batch of taurocholate

Protein was estimated by the procedure of Lowry et al. [7] or by the fluorescamine method [8] using bovine serum albumin as a standard.

Preparation of Dextran-sulphate-Sepharose. Dextran sulphate (Pharmacia) was bound to Sepharose 4B via a lysine spacer using a method based on that described by Eichmann and Greenblatt [9]. In a typical preparation 10 g CNBr-activated Sepharose 4B (Pharmacia) was swollen in 1 mM HCl and washed sequentially with 1 l 1 mM HCl and 100 ml 0.05 M $NaHCO_3$ The activated Sepharose was suspended in 40 ml 0 5 M $NaHCO_3$, pH 8 5/20 mg/ml lysine The pH was adjusted to 8 5 and the mixture stirred gently overnight at room temperature The gel was then washed with 500 ml aliquots of 0.2 M $NaHCO_3$/0 1 M sodium acetate, pH 4 5/water/0.5 M $NaHCO_3$, pH 8.5. The washed gel was resuspended in 40 ml 0 5 M $NaHCO_3$, pH 8.5. Activation of Dextran sulphate

was carried out by mixing 20 ml CNBr (Pierce Chemical Co), 100 mg/ml in water, with 20 ml Dextran sulphate, also at 100 mg/ml in water. The mixture was stirred for 15 min at room temperature The pH was then raised to 11 0—11 5 by addition of 1 N NaOH and maintained in this range When the pH had stabilized (5—10 min) the mixture was added to the lysine-Sepharose suspension and stirred gently at room temperature for 8—16 h. Unreacted sites were then blocked by addition of 2 vol. 1 M glycine and the gel was put through the washing cycle described above The gel was washed extensively with 'binding buffer' (0.05 M phosphate/citric acid, pH 6.5/0.02% Triton X-100) prior to use

Purification procedures Placental tissue was homogenized in 2 vol. distilled water. Particulate material was spun down (10 000 × *g*, 30 min) washed once in distilled water and resuspended in a volume of extraction buffer (0.05 M phosphate/citric acid, pH 6.0/0.06% Triton X-100/0.2% crude sodium taurocholate) equal to 3-times the original weight of tissue After 15 min at room temperature particulate material was spun down as above The solubilised β-glucosidase was partially purified by $(NH_4)_2SO_4$ precipitation (33% supernatant, 55% pellet) and dialysis at pH 4.2 [4]. Butanol extraction was carried out by gradual addition of *n*-butanol to the rapidly stirred sample at —2°C. Butanol concentration was brought up to 20% (v/v) over a 30 min period. After stirring for a further 30 min, the extract was centrifuged (10 000 × *g*, 30 min) and the lower aqueous layer removed and dialysed overnight against binding buffer. The sample was then applied to a Dextran-sulphate-Sepharose column.

In a typical purification, such as that described in Table I, 8—10 I.U of β-glucosidase were applied, in a total volume of about 120 ml, to a 100 ml column The flow rate was 80—100 ml/h The column was washed with binding buffer until absorbance at 280 nm fell almost to baseline. The enzyme was then eluted with crude sodium taurocholate, 3 mg/ml in binding buffer.

Active fractions were combined, diluted with an equal volume of distilled water and adjusted to pH 4.5 with 0.5 M citric acid The sample was then extracted with *n*-butanol and dialysed overnight against 0.1 M sodium citrate, pH 5 0/2% (v/v) *n*-butanol/5 mM 2-mercaptoethanol/5 mM EDTA. The sample was then applied to an octyl-Sepharose column, prepared as previously described [2], and eluted with 80% (v/v) ethylene glycol. Active fractions were pooled and dialysed overnight against citrate buffer (as above) containing 25% (v/v) glycerol. The enzyme was concentrated by ultra-filtration (Amicon PM-10) and stored at —20°C before the final purification step of sucrose-gradient centrifugation. Linear 5—20% (w/w) gradients, 15 ml vol., were made up in 0.15 M phosphate/citrate buffer, pH 6.0/0.05% Triton X-100/5 mM EDTA/1 mM 2-mercaptoethanol. Prior to application to the gradients the enzyme was either diluted 8—10-fold and reconcentrated (Amicon-PM-10) or dialysed overnight in order to reduce the glycerol concentration. 1 ml enzyme was applied to each gradient. Tubes were spun in an SW27.1 rotor at 4°C and 26 000 rev./min for about 40 h to a final w^2t of 11 0 · 10^{11} rad^2/s. Active fractions were concentrated (Amicon PM-10), dialysed overnight against the gradient buffer containing 25% glycerol and stored at —20°C

Results

Purification procedure

The results of a typical purification are shown in Table I The procedure incorporates two novel steps. These are chromatography on Dextran-sulphate-Sepharose (step 5) and sucrose-density gradient centrifugation (step 8).

The use of immobilized Dextran sulphate as an affinity column for human acid β-glucosidase is based on our initial observations that the human enzyme is strongly inhibited by this compound and that this inhibition is alleviated by the enzyme activator, crude sodium taurocholate A more detailed description of this is given below As shown in Fig. 1, the Dextran sulphate column bound human β-glucosidase efficiently. Activity was eluted by 3 mg/ml taurocholate as a sharp peak at the leading edge of a peak of ultraviolet-absorbing material Most of this absorption is attributable to the taurocholate and a discrete peak coinciding with enzyme activity was not detected. Subsequent elution with 5 M NaCl gave a sharp peak of ultraviolet-absorbing material but did not release additional enzyme

Purification after Dextran sulphate chromatography was at least 10-fold and recovery was 60—70% More significantly, the procedure separated β-glucosidase from several other lysosomal hydrolases Recovery of β-glucuronidase and hexosaminidase B (two major contaminants) was less than 20%, while binding of other lysosomal hydrolases was negligible It is important to note that efficient binding of acid β-glucosidase to the column could be achieved only after butanol extraction of the sample

Chromatography on octyl-Sepharose was found to give a useful further purification of the material eluted from Dextran sulphate However, the sample remained contaminated with hexosaminidase B and β-glucuronidase activity. The final purification step, density gradient centrifugation, gave only a slight increase in specific activity, but removed 90—95% of the hexosaminidase and

TABLE I

PURIFICATION OF β-GLUCOSIDASE FROM HUMAN PLACENTA

	β-Glucosidase					Gluco-cera-midase Activ-ity (units)	Hexo-samin-idase Activity (units $1\ 10^{-3}$)
	Activity		Protein (mg/ml)	Specific activity (units/mg)	Yield (%)		
	(units *)	(units/ml)					
1 Extraction	13 700	4 8	4 2	1 1	100	—**	409
2 $(NH_4)_2SO_4$ ppt	10 200	99 1	18 0	5 5	75	2040	32
3 Acid dialysis	10 000	89 4	8 7	10 2	73	1848	34
4 Butanol extractions	8300	82 8	7 3	11 3	61	1471	36
5 Dextran sulphate col	5200	58 7	0 53	110 7	38	748	6 9
6 Butanol extraction	3900	42 0	0 40	105 0	29	586	—
7 Octyl-Sepharose col	1900	64 8	0 042	1543 0	14	236	2 1
8 Sucrose gradient	1030	245 0	—	—	9	—	—
9 Dialysis	643	189 0	0 053	3570 0	5	44	0 2

* nmol/min using 4-methylumbelliferyl-β-glucoside

** Activity was too low for accurate measurement

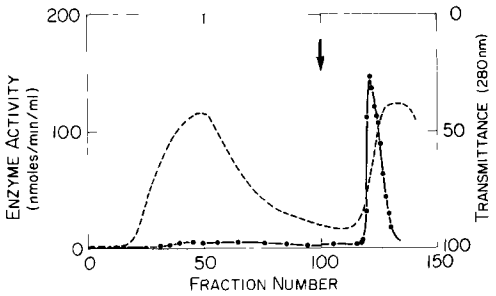


Fig 1 Elution of human acid β-glucosidase from Dextran-sulphate-Sepharose by crude sodium taurocholate (3 mg/ml) (•———•) Column bed-vol was approx 100 ml 116 ml enzyme, 81 nmol/min per ml, were applied at a flow rate of 88 ml/h and a temperature of 5°C Fraction volume was 5 5 ml

all the β-glucuronidase. The ratio of β-glucosidase to hexosaminidase activity in the final preparation was at least 3·1, compared to 0 03 1 in the original extract.

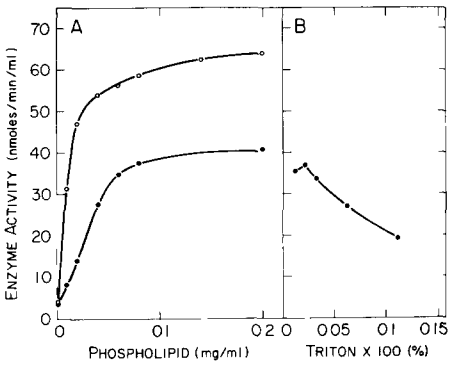
The procedure outlined in Table I gives a 3900-fold purification with a yield of 5%. Considerable loss of activity occurred during the final stages of purification However, the purified enzyme retained activity for several months when stored at −20°C in 25% glycerol. We detected no difference in the elution profiles of 4-methylumbelliferyl-β-glucosidase and glucosylceramidase activities from Dextran-sulphate-Sepharose or octyl-Sepharose. Unfortunately, we were unable to compare sedimentation of these activities in sucrose gradients because of interference by sucrose in our assay system for glucosylceramidase. The purified enzyme showed approx. equal activity towards *n*-stearoyl-, *n*-palmitoyl- or *n*-lignoceroyl-dihydroglucocerebroside and towards deoxycorticosterone-β-glucoside However, at all stages of the purification the enzyme cleaved the artificial substrate 4-methylumbelliferyl-β-glucoside more efficiently than the natural glycolipid substrates, the ratio ranging from 5 1 to 15·1. This may be due to a lack of sensitivity in the glucose detection system used in the natural substrate assay. It should be noted that both glucocerebroside and deoxycorticosterone-β-glucoside were found to be competitive inhibitors of placental 4-methylumbelliferyl-β-glucosidase activity and that these different substrates are therefore presumably cleaved by the same enzyme.

SDS-polyacrylamide gel electrophoresis of the purified enzyme

The purified enzyme contained four distinct proteins after polyacrylamide gel electrophoresis under dissociating conditions These ran as two sets of two closely-adjacent bands with molecular weights of about 69 000 and 32 000, respectively. A fifth band, molecular weight 52 000 was occasionally present The 69 000 doublet probably corresponds to the two closely adjacent bands of about 67 000 described by Furbish et al. [2]. It remains to be established whether the doublet at 32 000 is a contaminant, a breakdown product or an additional subunit.

Interaction of the enzyme with phospholipids

The purified acid β-glucosidase required either phospholipid or crude sodium



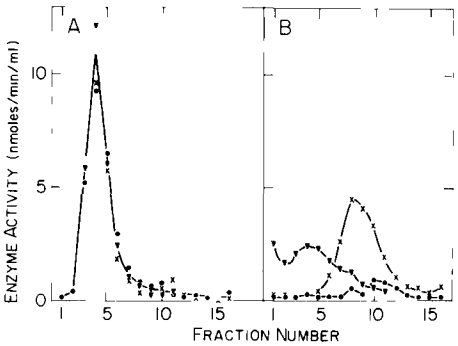


Fig 2 Effect of varying concentrations of phospholipid (A) and Triton X-100 (B) on the activity of purified β-glucosidase Phospholipid activation was assayed at 0 02% Triton X-100 while inhibition by Triton X-100 was assayed at 0 1 mg/ml phosphatidylinositol ○———○, phosphatidic acid, ●———●, phosphatidylinositol

Fig 3 Sedimentation of purified human β-glucosidase through sucrose density gradients Enzyme (0 2 ml at 50 nmol/min per ml) was applied to 4 ml, 5—20% gradients and spun at 35 000 rev /min for 18 h in an SW-56 rotor Final w^2t = 8 2 10^{11} rad^2/s Gradients were made up in 0 05 M phosphate/citric acid, pH 6 0/5 mM EDTA/1 mM 2-mercaptoethanol/with or without 0 05% Triton X-100 Enzyme was preincubated briefly at 4°C in the presence of 4 vol of the gradient buffer containing either no additions (●———●), 2 mg/ml crude taurocholate (▼———▼) or 0 2 mg/ml phosphatidylserine (X———X) (A) Gradient + Triton X-100, 0 05%, (B) Gradient without Triton X-100

taurocholate for activity towards either the natural or the artificial substrate Of the phospholipids tested phosphatidic acid was the most effective, both in terms of maximum activity and activity at low concentrations of phospholipid (Fig 2). Maximum activities achieved with phosphatidylserine, phosphatidylinositol (both at 0.2 mg/ml) and with crude taurocholate (1 mg/ml) were 80, 65 and 70%, respectively, of that obtained with phosphatidic acid Addition to the reaction mixture of both phospholipid and taurocholate together caused no further activation of the enzyme

All assays in which the concentration of phospholipid was varied contained Triton X-100 (0 01 or 0.02%). Control of detergent concentration is important as Triton X-100 is inhibitory if the concentration in the assay exceeds 0.02% (Fig 2).

In an attempt to obtain more direct evidence for interaction of phospholipids and taurocholate with the enzyme we studied the effect of preincubation with these compounds on the sedimentation of the enzyme through sucrose gradients. Two sets of gradients were run, either with or without Triton X-100 (0.05%). As shown in Fig. 3, when Triton X-100 was present in the gradient, preincubation with either phospholipid or taurocholate had no effect on either recovery of activity or sedimentation rate. However, in the absence of Triton X-100 recovery of enzyme activity was extremely low except in the sample preincubated with phospholipid In this sample enzyme activity sedimented more rapidly than enzyme run in the presence of Triton X-100 (Fig. 3). It is clear that taurocholate effects neither the stability of the enzyme nor its sedimentation rate to the same extent as phospholipid.

Thus, preincubation in the presence of phospholipid both stabilizes the enzyme and causes an increase in sedimentation rate. The latter effect is

TABLE II

EFFECT OF SULPHATES ON THE ACTIVITY OF PURIFIED ACID β-GLUCOSIDASE

Substrate	Posphohpid conc * (mg/ml)	None	Inhibitor (4 mg/ml)		
			Dextran sulphate	Chondroitin sulphate	Sodium sulphate
4-methylumbelliferyl-β-glucoside	0 1	38 5 **	23 2	32 0	39 1
	0 05	34 4	8 1	25 0	28 6
	0 02	21 4	2 6	8 0	16 6
Glucocerebroside	0 05	9 1	3 5	7 2	8 3
	0 02	5 3	0 8	3 1	2 9

* Phospholipids used were phosphatidic acid for glucocerebrosidase activity and phosphatidylinositol for β-glucosidase activity

** Activity expressed as nmol/min per ml

reversed by Triton X-100. It is of interest that, while phospholipid and taurocholate have similar effects on enzyme activity, they differ in their effects on stability and sedimentation properties

Inhibition of enzyme activity by sulphates

Dextran sulphate was found to be an effective inhibitor of purified acid β-glucosidase assayed with either natural or artificial substrates. Inhibition was independent of substrate concentration, but was found to be sensitive to the concentration of enzyme activators, either phospholipid or crude taurocholate. Typical results are shown in Table II The sulphated mucopolysaccharide chondroitin sulphate was also inhibitory as was sodium sulphate, although to a lesser degree (Table II). Neither of these compounds was as effective an inhibitor as Dextran sulphate. Inhibition by Dextran sulphate was pH dependent, with a maximum at pH 5.5 (88% inhibition at 5 mg/ml, 0 33 mg/ml taurocholate) falling progressively to 50% at pH 4 5 and 44% at pH 6.5.

Discussion

Affinity chromatography on Dextran-sulphate-Sepharose has proven to be a useful step in the purification of acid β-glucosidase from human placenta. Butanol extraction of the partially purified β-glucosidase preparation is essential for efficient enzyme binding to the Dextran sulphate column. This enhanced binding may be explained by the exposure of sites which were previously shielded by lipids. The column would be expected to bind cationic proteins and, despite the use of the highest pH consistent with enzyme stability, considerable ionic binding did occur. A major peak of ultraviolet-absorbing material was eluted with 5 M NaCl. The enzyme itself could be eluted with salt gradients, but such elution gave only a 2-fold purification and a low yield. This is in contrast to elution with crude sodium taurocholate which gave a 10—15-fold purification. Thus, the ability of the column to give good purification is dependent on the specificity of the eluant

The inhibition of β-glucosidase activity by sulphated macromolecules could

be attributed to the interaction of these compounds with the enzyme or with the activators which are essential for activity of the solubilized enzyme However, the binding of the enzyme to immobilized Dextran sulphate and its specific elution with sodium taurocholate, argues for a direct interaction between the inhibitor and the enzyme itself.

Some pure phospholipids are effective activators of purified human β-glucosidase and give a maximum enzyme activity comparable to that obtainable with taurocholate preparations. Phosphatidic acid was found to be slightly more effective than the substituted phospholipids phosphatidylserine and phosphatidylinositol. Phospholipids were also effective in alleviating inhibition of the enzyme by Dextran sulphate suggesting that the inhibitory effect of sulphated macromolecules on purified β-glucosidase probably occurs through binding to a site normally involved in binding the negatively-charged phosphate moiety of the phospholipid This conclusion is consistent with the observation that the neutral phospholipids phosphatidylethanolamine and phosphatidylcholine are ineffective activators of β-glucosidase [10,11].

It would be expected that hydrophobic forces would also play a role in stabilizing the enzyme-phospholipid interaction. Evidence for this can be deduced from the effects of the non-ionic detergent Triton X-100 on the enzyme. The influence of this detergent on the sedimentation rate of the putative enzyme-phospholipid complex (Fig. 3) and the inhibition of enzyme activity at relatively low detergent concentrations (Fig 2), may both be attributable to disruption of the enzyme-phospholipid interation

It seems clear that the stability and activity of human β-glucosidase are dependent on a complex array of ionic and non-ionic interactions. Recent advances in purification procedures should allow for the preparation of purified enzyme in amounts sufficient for a more detailed analysis of these interactions

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