

Enzyme replacement therapy in Gaucher's disease: A rapid, high-yield method for purification of glucocerebrosidase

(β -glucosidase/erythrocyte entrapment of enzyme)

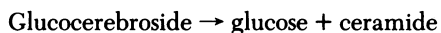
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ABSTRACT Gaucher's disease is caused by a deficiency of the lysosomal enzyme glucocerebrosidase (glucosylceramidase; D-glucosyl-N-acylsphingosine glucohydrolase, EC 3.2.1.45); this disorder has been a leading candidate for enzyme replacement trials. A rapid, high-yield method for purification of glucocerebrosidase has been developed. Detergent extraction of human placenta was followed by salt fractionation, concanavalin A-Sepharose chromatography, organic solvent precipitation, and affinity chromatography on phosphatidylserine-agarose; the total yield is 60% with 6000-fold purification. Purified glucocerebrosidase has been administered intravenously to a volunteer Gaucher's patient on two separate occasions. For the first injection, the enzyme was entrapped in resealed erythrocytes; for the second injection, the enzyme was given without any carrier. The enzyme infusions caused no untoward effects.

Glucocerebrosidase (glucosylceramidase, EC 3.2.1.45) is a lysosomal enzyme that cleaves the β -glucosidic bond of the glycosphingolipid glucocerebroside:



A deficiency of this enzyme results in the accumulation of glucocerebroside, the characteristic feature of Gaucher's disease. Lysosomal storage disorders, such as Gaucher's disease, have been prime targets for enzyme replacement therapy, the administration of exogenous enzyme in an effort to hydrolyze the stored material. Earlier reports of enzyme replacement in Gaucher's disease have been encouraging (1, 2) but this therapy has depended upon enzyme partially purified by a laborious method that provides a very low yield of enzyme (3). A relatively rapid, high-yield method for the purification of glucocerebrosidase is essential to investigation and implementation of enzyme replacement therapy.

The purification of glucocerebrosidase has been hindered by the fact that the enzyme is tightly membrane-bound and requires detergent to be solubilized. This problem has been resolved by the use of organic solvent precipitation to produce a detergent-free, soluble enzyme (4). We now describe the preparation, in high yield, of human glucocerebrosidase. Purification was carried out by monitoring the acid β -glucosidase activity, which appears, for the most part, to be identical to glucocerebrosidase (5). Direct assay of the purified preparation provided verification that it was a highly active glucocerebrosidase. The enzyme has been infused into a patient with Gaucher's disease without any untoward effects.

MATERIALS

Phosphatidylserine and taurocholic acid (catalog number 103004, lot no. 3176) used for tissue extraction were obtained from ICN Pharmaceuticals, Cleveland, Ohio. Taurocholic acid used in enzyme assays was obtained from Calbiochem, La Jolla,

Calif. Research Products International, Elk Grove, Ill., supplied 4-methylumbelliferyl- β -D-glucopyranoside. Concanavalin A (Con-A)-Sepharose and Sepharose 4B were products of Pharmacia, Uppsala, Sweden. Hexokinase, glucose-6-phosphate dehydrogenase, ATP, and NADP were obtained from Sigma Chemical Co., St. Louis, Mo. Cutscum detergent (isooctylphenoxy polyoxyethanol) was from Fisher Scientific Co., Fair Lawn, N.J. All other reagents were reagent grade.

METHODS

Enzyme Assays. β -Glucosidase was assayed at 37° by adding 20 μ l of enzyme preparation of 200 μ l to a reaction mixture comprised of 1.7 mM 4-methylumbelliferyl- β -D-glucopyranoside, 0.1 mM phosphatidylserine, 0.25% taurocholic acid, 25 mM sodium citrate, pH 6.0. The reaction was stopped and fluorescence was measured as outlined previously (6). One milliunit of enzymatic activity represents the hydrolysis of 1 nmol of substrate per min.

Glucocerebroside was isolated from the spleen of a patient with Gaucher's disease by the method of Rosenberg and Chargaff (7). Glucocerebrosidase was measured by adding 20 μ l of enzyme preparation to 200 μ l of 0.3 mM glucocerebroside, 0.5% taurocholic acid, 0.1% Cutscum, 0.2 mM phosphatidylserine, and 50 mM Na citrate, pH 6.0, and incubating for 1 hr at 37°. The reaction was terminated by boiling for 1 min. The amount of glucose released from glucocerebroside was measured fluorometrically: the glucose was phosphorylated by incubation with hexokinase and ATP, and the glucose 6-phosphate formed was oxidized with glucose-6-phosphate dehydrogenase and NADP.

Synthesis of Phosphatidylserine Agarose. A modification of the procedure of March *et al.* (8) was used for coupling phosphatidylserine (PS) to agarose (Sepharose 4B). Fifty milliliters of agarose was washed extensively with water and suspended in a total volume of 100 ml. One hundred milliliters of 2 M Na₂CO₃ and 20 g of CNBr dissolved in 10 ml of acetonitrile were added. The suspension was stirred for 2 min with a glass rod and the gel was then washed on a sintered glass funnel with 200 ml of 0° 0.5 M NaCl, 0.2 M NaHCO₃ at pH 9.0, and 200 ml of 0° water. After all excess water had been removed by vacuum suction the moist cake was added to 50 ml of 1 mM PS, 0.2% sodium dodecyl sulfate, 0.5 M NaCl, 0.2 M NaHCO₃, pH 9.0. After this suspension had been rotated for 2 hr at room temperature, 50 ml of 2 M ethanolamine, pH 9.0, was added to inactivate any sites on the gel that had not reacted with PS. After 45 min the gel was washed extensively with water.

This synthetic procedure normally couples approximately 40 nmol of PS to each ml of agarose, as measured by acid hydrolysis and amino acid analysis (9). Since this degree of substitution was too high for use in the purification method, the PS-agarose was diluted with sufficient unreacted agarose to

Abbreviations: PS, phosphatidylserine; Con A, concanavalin A.

provide a concentration of approximately 20 nmol of PS per ml of agarose.*

Purification. All purification steps were carried out at 4° unless otherwise stated. Frozen human placenta obtained from a local hospital was stripped of membranes and homogenized in water (25% wt/vol) for 10 min at top speed in a Sorvall Omnimixer. The homogenate was centrifuged at 10,000 × *g* for 20 min, and the supernatant was discarded. The pellet was homogenized (40% wt/vol) at top speed in a Sorvall Omnimixer for 60 sec at 0° in 50 mM sodium chloride, 50 mM sodium citrate, at pH 6.0, with 0.02% sodium azide (Buffer A) containing 1% taurocholic acid. After centrifugation at 10,000 × *g* for 20 min, 2200 ml of the taurocholate supernatant, containing most of the β-glucosidase activity, was adjusted to pH 5.0 with dilute acetic acid, and 194 mg of solid ammonium sulfate was added per ml of solution to give 35% saturation. The suspension was centrifuged at 10,800 × *g* for 20 min and the pellet was discarded. The supernatant was adjusted to pH 6.0 with dilute NaOH and then taken to 55% ammonium sulfate per milliliter of original solution. After centrifuging at 10,800 × *g* for 20 min, the supernatant was discarded, and the pellet was dissolved in 900 ml of Buffer A containing 0.5% taurocholate.

Fifty milliliters of Con-A-Sepharose was poured into a 2.5 cm diameter column at room temperature and equilibrated with Buffer A containing 0.5% taurocholate, 5 mM MnCl₂, and 5 mM CaCl₂. Three hundred milliliters of the ammonium sulfate fraction was made 5 mM in both MnCl₂ and CaCl₂ and applied to the Con-A-Sepharose column at room temperature. The column was washed with 500 ml of Buffer A containing 0.5% taurocholate and 0.1 M α-methyl-mannoside. The eluate was concentrated to approximately 250 ml over an Amicon XM-50 membrane.

Ten volumes of absolute ethanol per volume of protein solution was magnetically stirred and was cooled to -15°. The Con-A-Sepharose eluate was dripped into the rapidly stirring ethanol at a rate of 1 ml/min. The precipitate was collected by centrifugation at 2300 × *g* for 20 min at -10° and the supernatant was discarded. The pellet was dissolved in approximately 200 ml of Buffer A and dialyzed two times against the same buffer. The preparation was concentrated to approximately 150 ml over an Amicon PM-30 membrane.

A 2.5 × 7.5 cm column of PS-agarose was equilibrated at 4° with Buffer A. The concentrated enzyme preparation was added at a flow rate of 1 ml/min. After sample application, the column was washed with 250 ml of Buffer A and then with 50 ml of 0.5 M NaCl, 50 mM Na citrate, pH 6.0. The enzyme was eluted with 250 ml of 1.0 M NaCl, 40% (vol/vol) ethylene glycol, 1 mM 2-mercaptoethanol, 50 mM Na citrate, pH 6.0 (Fig. 1).

The active fractions of the PS-agarose column, comprising approximately 60 ml, were combined and were made 5 mM in 2-mercaptoethanol. This solution was then concentrated very rapidly over a PM-10 membrane, first in a large 400 ml Amicon concentrator and then in a small 10 ml Amicon concentrator. When the β-glucosidase had been concentrated to 1 ml, 4 ml of Buffer A was added and the solution was quickly concentrated again to 1 ml. The 1 ml was dialyzed against 250 ml of Buffer A with 1 mM 2-mercaptoethanol.

RESULTS AND DISCUSSION

The purification of human placental β-glucosidase is summa-

* The dilution of a substituted Sepharose gel with unsubstituted Sepharose has an effect that is similar, although not identical, to synthesis of substituted Sepharose with a lower ligand concentration (10).

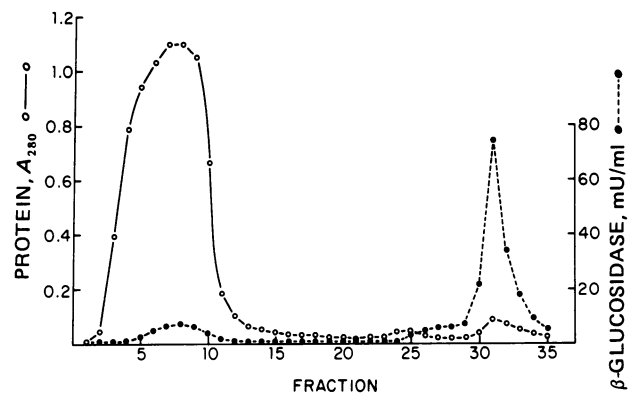


FIG. 1. Chromatography of β-glucosidase on phosphatidylserine-agarose. Protein was monitored by absorbance at 280 nm, and β-glucosidase activity is given in milliunits/ml. Fractions are approximately 15 ml; elution was begun at fraction 27.

rized in Table 1. Although the overall yield is 60%, this represents a modest exaggeration of the true yield; some increase in activity results from the fact that the pH optimum of glucocerebrosidase shifts from 5 to 6 during purification and all assays are performed at pH 6. The purification was monitored using the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside. The purified β-glucosidase preparation was assayed for glucocerebrosidase activity, and 1000 nmol of glucocerebrosidase were hydrolyzed per min/mg of protein.

PS-Agarose chromatography (Fig. 1) was used because of our finding that β-glucosidase interacts with acidic phospholipids (4). It is important that the PS concentration does not exceed 20 nmol of PS per ml of agarose. At higher concentrations of PS, we have been unable to elute the bound glucocerebrosidase in active form. β-Glucosidase activity is very labile during concentration of the PS-agarose eluate; concentration and subsequent dialysis must be performed rapidly to avoid large losses of enzyme activity. This lability, also observed by Pentchev *et al.* (3), appears to be a result of vulnerability to oxidation of glucocerebrosidase in the presence of high-salt concentrations and organic additives (i.e., ethylene glycol, glycerol). It is not yet clear whether the PS-agarose chromatography represents truly bio-specific affinity chromatography or the more general hydrophobic chromatography (11).

The purity of the enzyme preparation is difficult to establish. Polyacrylamide electrophoresis cannot be used as an indicator of purity because, as previously reported by Pentchev *et al.* (3), glucocerebrosidase does not migrate into the gel. However, we found that polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate shows multiple bands, which indicates that our preparation of glucocerebrosidase is not yet homogeneous after 6000-fold purification.

To test for possible toxicity two preparations, sterilized by Millipore filtration, have been injected intravenously into rabbits. In the first test, 200 milliunits of β-glucosidase, which had been purified to a specific activity of 830 milliunits/mg of protein by steps I-IV (Table 1) followed by a phosphocellulose chromatography, was injected into an adult male rabbit with no effect on body temperature or behavior. The rabbit was subsequently sacrificed. Tissues examined included the heart, lung, liver, spleen, kidneys, intestinal tract, brain, spinal cord, bone marrow, bone, lymph nodes, skeletal muscle, thyroid, and eye. Autopsy findings showed no abnormality that could be related to the enzyme infusion.

Multiple injections of purified glucocerebrosidase have also been given on two separate occasions to a patient volunteer with

Table 1. Purification of β -glucosidase

Purification step	Total milliunits*	Volume (ml)	Protein (mg/ml)	Specific activity (milliunits/mg)	Total fold purification	Yield (%)
I. Taurocholate homogenate	14,500	2200	16	0.412	1	100
II. $(\text{NH}_4)_2\text{SO}_4$ fraction	30,200	950	3.0	10.6	26	208
III. Con-A-Sephadex chromatography [†]	24,000	750	0.39	82.1	199	166
IV. Ethanol precipitation [†]	19,300	450	0.66	65.0	158	133
V. PS-agarose chromatography [†]	8,700 (3,480) [‡]	3.0	1.16	2500 (1000) [‡]	6000	60

* One unit of enzyme represents the hydrolysis of 1 μmol of substrate per min. Activity in this table is for the artificial substrate 4-methylumbelliferyl- β -D-glucopyranoside at 1.7 mM, the K_m value for this substrate.

[†] For convenience, these steps were actually carried out with one third of the ammonium sulfate preparation, step II, and the appropriate numbers were converted for this table to show total recoveries.

[‡] Glucocerebrosidase activity was determined as outlined in *Methods*. One milliunit of activity represents 1 nmol of substrate cleaved per min.

far-advanced Gaucher's disease. For the first administration, the enzyme was entrapped in resealed red blood cells as suggested by Ihler *et al.* (12). Ten milliliters of the patient's own packed erythrocytes were mixed with 590 milliunits of enzyme in Buffer A in a sterile dialysis bag. The mixture was dialyzed for 2 hr at 4° against 1200 ml of 5 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ at pH 7.4. The dialysis solution was then changed to 500 ml of 0.154 M NaCl, 5 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ at pH 7.4 and dialysis was continued for 2 hr at room temperature. After washing, 6 ml of resealed cells containing a total of 96 milliunits of β -glucosidase were injected intravenously into the patient. The procedure was repeated on a larger scale 2 days later; an additional 435 milliunits of enzyme was entrapped in erythrocytes and administered to the patient. Three months later a total of 3700 milliunits of purified β -glucosidase (step V, Table 1) was injected directly intravenously into the same patient on 2 successive days. No pyrexia, hypotension, change in heart rate, or effect on coagulation factors was observed following any of the injections. A complete description of the replacement trials is in preparation.

For trials of enzyme replacement therapy of Gaucher's disease to be properly conducted, supply of active nontoxic enzyme must not be a limiting factor. The development of a rapid high-yield purification procedure for glucocerebrosidase has been an essential step in implementing such trials.

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