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Enzyme replacement therapy in Gaucher's disease: Large-scale purification of glucocerebrosidase suitable for human administration

(hydrophobic chromatography/cholate extraction/β-glucosidase/concanavalin A)

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ABSTRACT **Enzyme replacement therapy for the alleviation of Gaucher's disease has been impeded because of the difficulty in preparing large amounts of glucocerebrosidase, the enzyme that is deficient in patients with this disorder. A large-scale procedure for the purification of human placental glucocerebrosidase has been developed. The method uses cholate extraction, ammonium sulfate fractionation, acid precipitation, butanol extraction, and hydrophobic chromatography; the final enzyme preparation has a specific activity of more than 10^6 units/mg of protein with an overall recovery of 30%. In addition, the contamination of enzyme preparations, intended for human infusion, prepared by isolation procedures involving concanavalin A columns has been studied and is reported here.**

The metabolic basis of Gaucher's disease is a deficiency of the enzyme, glucocerebroside-β-glucosidase, that catalyzes the hydrolysis of glucosylceramide (glucocerebroside) to glucose and ceramide (1). Recent studies (2) have revealed that administration of active glucocerebrosidase from human placenta to patients with this metabolic disorder causes a decrease in the quantity of accumulated glucocerebroside in the liver and bloodstream. However, the difficulty in preparing large quantities of highly purified enzyme from appropriate human sources (e.g., placenta) has hampered the development of a long-term therapeutic study. A procedure for the purification of glucocerebrosidase previously developed in this laboratory yields enzyme of acceptable purity (3); however, the method did not prove amenable to large-scale operation.

Accordingly, we undertook a comprehensive investigation of the physical-chemical characteristics of the protein for possible application of these findings to the purification of the enzyme. Inasmuch as glucocerebrosidase is a membrane-bound lysosomal glycoprotein, two properties deemed worthy of examination were its high degree of hydrophobicity and its interaction with concanavalin A (Con A). Utilizing these attributes, we achieved a detergent-free preparation of glucocerebrosidase with 20,000-fold purification and 20% overall yield (F. S. Furbish, unpublished data). The procedure consisted of cholate extraction, ammonium sulfate fractionation, exhaustive dialysis, butanol extraction, Con A fractionation, gel filtration, and hydrophobic chromatography; up to 15 kg of placental tissue could be conveniently processed with ordinary laboratory equipment. The final product had a specific activity of greater than 10^6 units/mg of protein.

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Although this method was successful for the purpose of producing highly purified enzyme, our concern regarding contamination by Con A made us acutely distrustful of utilizing this product for infusion in humans. Because Con A can interact with a wide range of receptor sites on various cells (4–12), the possibility of untoward reactions resulting from its infusion should be given serious consideration. Although few *in vivo* effects of Con A have been demonstrated in humans, the scope of its potential toxicity is extensive. Hence, it seemed appropriate to determine the extent of Con A contamination, if any, before infusion. We report here that such contamination does readily occur. This potential hazard is also true of the relatively gentle Con A elution conditions used by Dale and Beutler (13) in their glucocerebrosidase preparation. Consequently, we have discontinued the use of Con A in preparing enzyme for human use. We present here a practical large-scale procedure that yields enzyme acceptable for human infusion purposes.

MATERIALS AND METHODS

All pilot plant development studies were done at the New England Enzyme Center.

Materials. Decyl-agarose was purchased from Miles Laboratories. Octyl-Sepharose and Con A-Sepharose were obtained from Pharmacia. Cholic acid for extraction was produced by Eastman. Taurocholic acid was from Calbiochem. All other chemicals were of reagent grade. Fresh human placentas were obtained from local hospitals and stored in 0.3% (wt/vol) streptomycin sulfate at 4° until used. Buffers utilized during the final two steps were prepared from pyrogen-free water or saline.

Purification Procedure. All steps were performed at 4°. Fresh placentas were freed of cords and membranes, washed with distilled water, and passed through a meat grinder equipped with a ⅝-inch cutting plate. The minced tissue was homogenized for 1.5 min in a Gifford Wood colloid mill with 5 volumes of 25 mM sodium phosphate buffer, pH 6.0, and the homogenate was filtered through fiberglass window screening to remove connective tissue debris. The filtrate was centrifuged in a Sharples Md 16 (14,000 × *g* at a flow rate of 25 liters/hr) and the supernatant was discarded. The sedimented material was rehomogenized with two volumes of 25 mM sodium citrate/1% (wt/vol) sodium cholate, pH 7.0, and centrifuged at 14,000 × *g* for 45 min. The supernatant was brought to 35% saturation by the addition of solid ammonium sulfate (195 g/liter) and centrifuged as above; the precipitate was discarded.

Abbreviations: Con A, concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate.

The ammonium sulfate supernatant was brought to pH 5.35 (±0.05) by dropwise addition of 0.5 M citric acid and the suspension was centrifuged as above. The acid precipitate was resuspended in 25 mM sodium citrate, pH 4.7, with the volume adjusted to one-half that of the ammonium sulfate supernatant. This suspension was brought to 20% (vol/vol) butanol concentration by the slow addition of 250 ml of *n*-butanol per liter of suspension. The emulsion was stirred for 30 min and then centrifuged at 5000 × *g* for 30 min. The aqueous lower layer was collected and dialyzed against 20 volumes of 0.1 M sodium citrate/2% butanol, pH 5.0.

A decyl–agarose column (length-to-diameter ratio, 5:1) was packed and equilibrated with 0.1 M sodium citrate buffer, pH 5.0/1 mM 2-mercaptoethanol/5 mM EDTA (this buffer was used in the following steps and will be referred to hereafter as citrate buffer). The dialyzed butanol extract was clarified by centrifugation at 5000 × *g* for 30 min, and the supernatant was applied to the column at a ratio of 50,000 units/ml of column volume. The column was then washed with one-half column volume of citrate buffer followed by elution with an 8 column-volume linear gradient of 30–80% ethylene glycol in citrate buffer. Glucocerebrosidase was eluted on the trailing edge of a protein peak at an ethylene glycol concentration of approximately 60%. Fractions with high specific activity were pooled and diluted 1:3 with citrate buffer. An octyl–Sepharose column (length-to-diameter ratio, 5:1) was packed, washed for 12 hr with 0.1 M NaOH, and equilibrated with citrate buffer prepared in pyrogen-free 0.9% (wt/vol) saline. The enzyme sample was applied at a ratio of 300,000 units/ml of column volume. The column was washed with one-half column volume of pyrogen-free citrate buffer. Approximately 40% of the inactive protein did not adsorb to the column, and the enzyme was eluted at about 60% ethylene glycol concentration.

The fractions from the octyl–Sepharose column with high specific activity were pooled, diluted by one-third with 60% ethylene glycol in 0.9% saline, and made 0.5 mg/ml in human serum albumin. This enzyme solution was added rapidly to three volumes of cold 95% ethanol, stirred well, and held in the cold for 1 hr before centrifugation at 4000 × *g* for 5 min. The supernatant was discarded and the precipitate was suspended in 200 volumes (wt/vol) of an ethanol/glycerol solution (three volumes of 95% ethanol and one volume of 60% pyrogen-free glycerol); the suspension was centrifuged as above. The wash was repeated twice more and the final precipitate was taken up in human serum albumin solution (40 mg/ml in 0.9% saline) to a concentration of approximately 10^6 units/ml.

Enzyme Assays. Glucocerebrosidase activity was measured at 37° by adding 0.1–10 μl of the enzyme preparation to an assay mixture of 25 μl of potassium phosphate buffer containing Cutscum (10 mg/ml), pH 5.9; 150 μl of H_2O; and 5 μl of D-[*1*-^{14}C]glucocerebroside (14) (7.5 mg/ml in sodium taurocholate at 50 mg/ml). The D-[*1*-^{14}C]glucose liberated by the enzyme was recovered and its radioactivity was determined as described (15). One unit of activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 nmol of substrate per hr. A plot of cpm versus incubation time or aliquot of enzyme was obtained to prevent exceeding the proportionality of the assay.

Protein was estimated by the Lowry procedure as modified by Bensadoun and Weinstein (16).

Gel Electrophoresis. Disc electrophoresis on polyacrylamide gels was performed in the presence of sodium dodecyl sulfate ($NaDodSO_4$) according to Laemmli (17). Periodic acid-Schiff staining of glycoproteins in these gels was carried out by the procedure of Segrest and Jackson (18).

Con A Chromatography. A 200 ml Con A–Sepharose column was washed with water and up to 12×10^6 units of glucocerebrosidase was applied. The column was washed with 500 ml of 0.1 M citrate/1.0 M NaCl, pH 5.0, followed with 200 ml of 50 mM α-methylmannoside in the same buffer. Elution of the enzyme was accomplished by increasing the sugar concentration to 1.0 M and raising the column temperature to 37°. This procedure was utilized after butanol extraction and before hydrophobic chromatography. To ascertain the extent of Con A contamination, a blank column was run exactly as above, but without addition of enzyme sample. In this case, the elution volume normally occupied by enzyme was tested for Con A protein.

A blank Con A column was run according to the method of Dale and Beutler (13) and the column eluate was subjected to these authors' ethanol precipitation. Both the column eluate and the ethanol precipitate were examined for Con A contamination.

RESULTS AND DISCUSSION

Inspection of blank Con A column eluates at elution volumes normally containing glucocerebrosidase in our preparations revealed contamination by Con A protein. Subsequent purification by gel filtration and hydrophobic chromatography failed

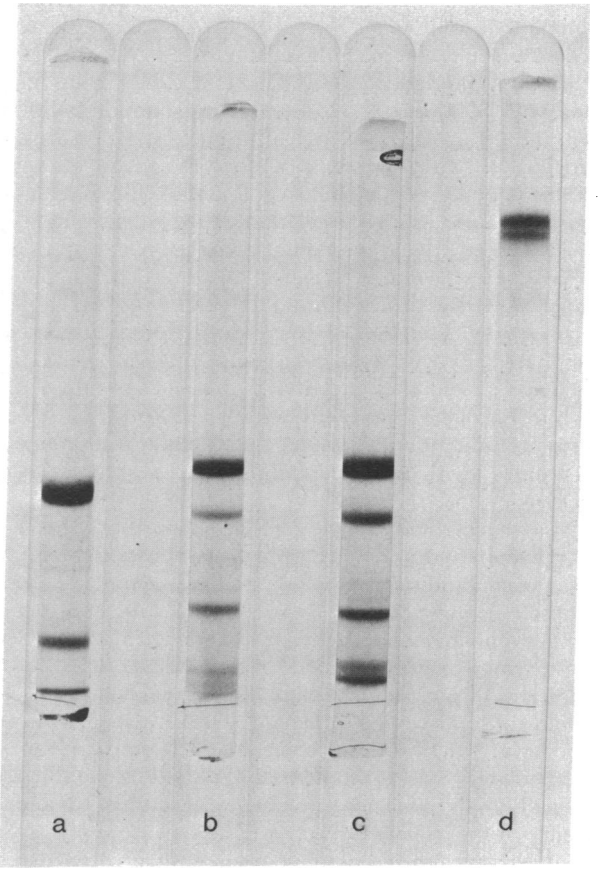

FIG. 1. $NaDodSO_4$/gel electrophoresis of Con A protein and of purified glucocerebrosidase. Gels contained: (a) 40 μg of Con A protein as eluted from a blank 100-ml Con A–Sepharose column; (b) 40 μg of Con A protein after ethanol fractionation; (c) 40 μg of Con A protein after elution from decyl–agarose with buffer containing ethylene glycol; and (d) 20 μg of purified glucocerebrosidase as eluted from octyl–Sepharose (Table 1).

Table 1. Purification of glucocerebrosidase from human placentas*

Fraction	Volume, ml	Activity, units/ml	Protein, mg/ml	Specific activity, units/mg protein	Total units $\times 10^{-6}$	Recovery, %
Cholate extraction	269	5,630	15	375	1.5	100
35% ammonium sulfate supernatant	257	5,010	10	500	1.3	85
Butanol extraction	217	3,260	0.35	9,330	0.71	47
Decyl–agarose chromatography	7.7	71,900	0.49	147,000	0.55	36
Octyl–Sepharose chromatography	7.5	57,700	0.052	1,110,000	0.43	28
Ethanol concentration	0.47	1,000,000	—	—	0.47	31

* Based on 1 kg of starting tissue. In a typical preparation, 15–30 kg of fresh placentas is processed.

to remove the entire amount of contamination. $NaDodSO_4$/disc gel electrophoresis of the final product demonstrated a minor band of Con A protein with a migration rate slightly less than that of the enzyme. Because we could not circumvent the contamination problem arising from the use of Con A–Sepharose, the less stringent elution conditions used by Dale and Beutler (13) were of interest. These authors achieved a more gentle elution of enzyme from Con A columns by including a detergent in the buffer system and by not removing the inherent lipid from the preparation prior to chromatography. In the hope that a judicious rearrangement of our purification procedures could eliminate the contamination, we studied the method in some detail. A blank 100-ml Con A column was eluted with 500 ml of 0.1 M α-methylmannoside in 50 mM NaCl and 50 mM citrate, pH 6.0, containing 0.5% sodium taurocholate. After dialysis to remove the sugar, the eluate contained about 6 mg of con A protein that gave three major bands in $NaDodSO_4$ gels (gel a in Fig. 1). This eluate was subjected to an ethanol precipitation as the next step in Dale and Beutler's procedure. The eluate was dripped into absolute ethanol at $-15°$ with stirring, and the product was collected by centrifugation. One-half of the Con A protein applied was precipitated along with the detergent. On the $NaDodSO_4$/gel, the ethanol-precipitated protein showed increasing dissociation (gel b in Fig. 1). This protein was then adsorbed to a decyl–agarose column; it could not be eluted with buffer alone but was removed with buffer containing ethylene glycol (gel c in Fig. 1).

This last experiment suggested that any purification method that relied on hydrophobic chromatography would fail to remove the contaminating Con A from the enzyme preparation. Because our enzyme isolation was strongly dependent upon such hydrophobic separations, we abandoned all use of Con A in preparing enzyme intended for human use.

The large-scale purification of human placental glucocerebrosidase is summarized in Table 1. The final specific activity exceeded 10^6 units/mg of protein and the overall recovery was approximately 30%. It should be noted that the overall purification of 3000-fold is deceptively low because of the high efficiency of the cholate extraction. The specific activity of the initial extract reported here is considerably greater than that of previous taurocholate extracts (3, 13, 19). Furthermore, the ease of cholate removal by dialysis permits the acquisition of a detergent-free preparation, which is not possible in a taurocholate system. The high selectivity of the two hydrophobic columns is attainable only with a preparation free of detergent and only after the intrinsic lipids have been removed by extraction with butanol.

The purity of the final preparation is shown by its behavior on $NaDodSO_4$/gels (gel d in Fig. 1). Two closely spaced bands corresponding to an apparent molecular weight of 67,000 were obtained. Both gave a positive reaction with periodic acid-Schiff stain for glycoproteins. Further purification of the enzyme, yielding a specific activity of 2.5×10^6 units/mg, did not greatly alter the relative amounts of these two bands. These results suggest microheterogeneity in the carbohydrate moiety of the enzyme rather than gross protein contamination as being responsible for the appearance of the double band profile (see Note Added in Proof).

Pyrogen testing of the final product was done in accordance with U.S.P., volume XIX, page 613, by F. & F. BioResearch, Inc., Ashumet, MA. No pyrogenicity was detected.

It is anticipated that this isolation technique will permit preparation of the large amounts of glucocerebrosidase required for enzyme replacement therapy in Gaucher's disease without extraneous foreign protein.

Note Added in Proof: After this manuscript was submitted, we noticed that the double band profile obtained in $NaDodSO_4$ gels is an artifact arising from the denaturation in $NaDodSO_4$ at 100°. When less stringent conditions of denaturation are used, electrophoresis of the final preparation yields a single band.

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