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ELECTROPHORETIC SEPARATION OF NEUTRAL AND ACID β-GLUCOSIDASE ISOZYMES IN HUMAN TISSUES

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Summary

An electrophoretic system using cellulose acetate has been developed for the resolution of β-glucosidase isozymes (β-D-glucoside glucohydrolase, EC 3.2.1.21 and D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45) in human tissue homogenates. Electrophoresis of homogenates from normal and Type 1 Gaucher disease tissues revealed two fluorescent bands of β-glucosidase activity which corresponded to the acid and neutral isozymes separated by concanavalin A-Sepharose chromatography. The acid isozyme had only β-glucosidase activity, whereas the neutral isozyme also exhibited α-L-arabinosidase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55), β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) activities, using the appropriate 4-methylumbelliferyl glycoside. In homogenates of cultured skin fibroblasts, only the acid isozyme was observed which co-electrophoresed with the acidic activity in other tissue homogenates. The acidic activity in tissue and fibroblast homogenates from Type 1 Gaucher disease appeared to co-electrophorese with the acid isozyme in normal tissues, but had markedly reduced activity.

Introduction

Two major isozymes with β-glucosidase activity (β-D-glucoside glucohydrolase, EC 3.2.1.21 and D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45) have been identified in normal human tissues using the artificial

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substrate, 4-methylumbelliferyl-β-D-glucopyranoside. Acidic and neutral enzymatic activities have been differentiated by their relative pH optima [1–5], subcellular localization [2,6], substrate specificities [2,6,7], and sensitivity to anionic detergents and acidic phospholipids [6]. The acid isozyme, a membrane-bound activity [2,6] which has an affinity for concanavalin A [8,9], has been shown to be deficient in the various subtypes of Gaucher disease, lysosomal storage diseases characterized by the accumulation of glucosyl ceramide [10].

To date, no electrophoretic system has been developed to visualize the activity of both the acid and neutral isozymes from human tissue homogenates. We describe here an electrophoretic method which resolved these isozymes on cellulose acetate gels. This system permitted visualization of the residual β-glucosidase activity in tissue homogenates from Type 1 Gaucher disease. We also report the separation of the acid and neutral isozymes by 'glyco-affinity' chromatography on immobilized concanavalin A.

Materials and Methods

Materials. 4-Methylumbelliferyl substrates were purchased from Koch-Light Laboratories, Colnbrook, U.K.; sodium taurocholate from Gallard-Schlesinger Corp., Carle Place, NY; concanvalin A-Sepharose from Pharmacia Fine Chemicals, Piscataway, NJ. Cellogel and the horizontal electrophoresis tank from Kalex Scientific Co., Manhasset, NY; ethylene glycol and other solvents were from Fisher Scientific Co., Pittsburgh, PA; 1-*O*-methyl-α-D-glucopyranoside and phosphatidic acid (Grade 1; 99% purity) were from Sigma Chemical Co., St. Louis, MO.

Enzyme assay. β-Glucosidase activity and pH vs. activity profiles were determined using 4-methylumbelliferyl-β-D-glucopyranoside as substrate [4,11].

Sample preparation. Tissues from normal individuals and homozygotes with Type 1 Gaucher disease were collected at surgery or autopsy (within 2 h of death) and were processed immediately or frozen at −70° C prior to use. Optimal results were obtained with fresh tissues, since freezing reduced the acid β-glucosidase activity. Skin fibroblasts were obtained, cultured, and harvested as previously described [4]. For chromatography on concanavalin A-Sepharose [8,9,12], tissues were minced and homogenized in distilled water (3 : 1, w/v) containing 0.05% Triton X-100 and centrifuged at 15 000 × *g* for 30 min at 4° C. The supernatants were diluted in 3 vols. 0.05 M sodium citrate, pH 6.0 with 1 M NaCl/5 mM $MgCl_2$/$MnCl_2$/$CaCl_2$/0.02% NaN_3 (standard chromatographic buffer). For electrophoresis, tissues were homogenized in 40 mM sodium phosphate containing 20% ethylene glycol and 0.05% Triton X-100; the buffer was adjusted to pH 7.0 with 0.5 M citric acid (standard electrophoretic buffer). After homogenization, 1 vol of ethanol/chloroform (2 : 1, v/v) was added dropwise to 2 vols. of the homogenate, gently mixed for 5 min at 25° C, and then centrifuged at 4000 × *g* for 10 min at 4° C [13]; the supernatants were electrophoresed immediately. Fibroblasts were freeze-thawed five times in the standard electrophoresis buffer, ethanol/chloroform treated and the whole cell homogenate was immediately used for electrophoresis. Separation of the acid and neutral isozymes was optimized by systematically varying electrophoretic

conditions including ionic strength (20 mM—50 mM phosphate), pH (6.0—8.0), the concentrations of Triton X-100 (0—0.15%) and ethylene glycol (0—50%). In addition, the presence of phosphatidic acid (0.1 mg/ml) or sodium taurocholate (0.02%) had no effect on isozyme migration.

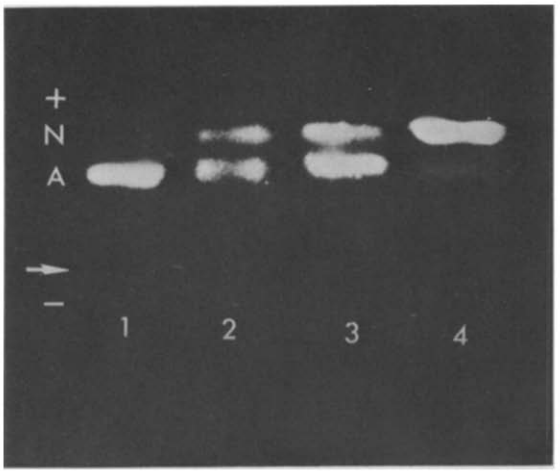
Chromatographic separation of acid and neutral isozymes. Supernatants from about 5 g tissue were diluted in the standard chromatographic buffer, mixed with 2 ml concanavalin A-Sepharose at 25° C for 2 h and then poured into a disposable polypropylene column. The column was washed with this buffer (approx. equal to 20 ml) until no β-glucosidase activity was detected in the eluant. Bound β-glucosidase activity was then eluted with this buffer containing 1 M 1-*O*-methyl-α-D-glucopyranoside (approx. equal to 20 ml); over 90% of the bound activity was recovered in the first 5-ml fraction. The buffer wash and 1-*O*-methyl-α-D-glucopyranoside fractions were pooled separately and dialyzed overnight against 2 l standard electrophoretic buffer. Pooled fractions were concentrated by ultrafiltration, using an Amicon PM10 filter.

Electrophoretic separation of acid and neutral isozymes. A cellulose acetate slab gel (17 × 16 × 0.35 cm, Cellogel) was immersed in the standard electrophoretic buffer for a minimum of 15 min, blotted with Whatman No. 1 filter paper and immediately placed on the electrophoresis apparatus. Samples with at least 100 pmol per min of β-glucosidase activity were applied to the gel. A constant voltage of 240 V and an initial current of 25 mA were applied for 2.5—4.0 h at 4° C. The gels were stained for β-glucosidase activity with a filter paper overlay containing 3 mM 4-methylumbelliferyl-β-D-glucopyranoside in 0.06 M phosphate citrate buffer, pH 4.0 or 6.0, containing 0.012% Triton X-100 and 0.2% sodium taurocholate; for β-galactosidase with 0.8 mM 4-methylumbelliferyl-β-D-galactopyranoside in 0.2 M sodium acetate, pH 4.5; for β-xylosidase with 0.4 mM 4-methylumbelliferyl-β-D-xylopyranoside in 0.2 M sodium citrate, pH 5.0; or for α-L-arabinosidase with 4-methylumbelliferyl-α-L-arabinopyranoside in 0.2 M sodium citrate, pH 5.0. The gel was placed in a sealed, moist chamber and incubated at 37° C for 1—2 h. After incubation, the gel was placed in an NH_4OH chamber for 3 min at 25° C. Activity bands were visualized with long wave ultraviolet light and photographs were taken with Polaroid Type 51 film using a Wratten No. 4 filter.

Results

Fig. 1 shows the cellulose acetate electrophoresis of crude homogenates of normal liver, kidney, brain and cultured skin fibroblasts. Two fluorescent bands of β-glucosidase activity were visualized in liver, kidney, spleen and brain homogenates with 4-methylumbelliferyl-β-D-glucopyranoside. In these tissues, the major band was the more anodal (fast) band. In cultured skin fibroblasts only one band was observed which corresponding to the less anodal (slow) activity.

Two peaks of β-glucosidase activity from splenic and hepatic homogenates were separated by chromatography on concanavalin A-Sepharose and further characterized. As shown in Fig. 2, the activity eluted in the buffer wash electrophoresed as a single band which co-migrated with the fast band of β-glucosidase activity in the crude homogenates. The activity which bound to the lectin column was eluted with 1.0 M 1-*O*-methyl-α-D-glucopyranoside and electro-



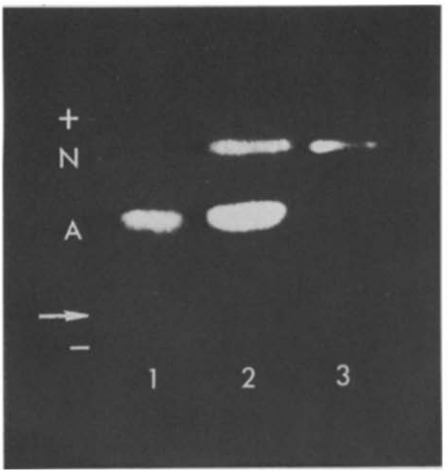


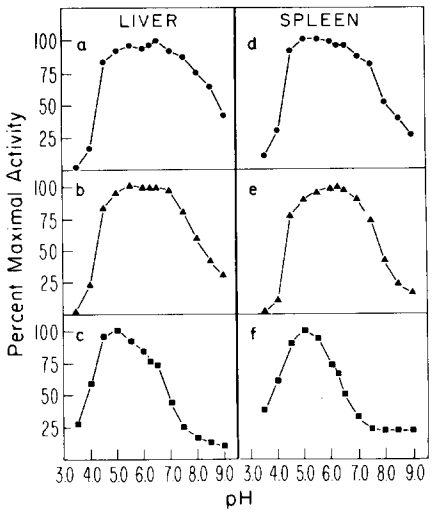
Fig. 1. Fluorescent bands of β-glucosidase activity in normal human tissue homogenates after electrrophoresis on cellulose acetate and staining with 4-methylumbelliferyl-β-D-glucopyranoside. Lane 1, cultured skin fibroblasts; lane 2, kidney; lane 3, liver; lane 4, brain. Arrow, point of application; A, acid β-glucosidase; N, neutral β-glucosidase.

Fig. 2. Electrophoresis of normal hepatic β-glucosidase isozymes before and after separation by chromatography on concanavalin A-Sepharose. Lane 1, 1-*O*-methyl-α-D-glucopyranoside eluate; lane 2, total liver homogenate; lane 3, buffer wash. (See text for details).

phoresed as a single band which co-migrated with the slow activity band.

Fig. 3 shows the pH vs. β-glucosidase activity profiles for the crude homogenate, the buffer wash and the 1-*O*-methyl-α-D-glucopyranoside eluant from concanavalin A-Sepharose chromatography of normal liver and spleen. In each tissue, the β-glucosidase activity which bound to the lectin column had an acid pH optimum (5.0) and co-electrophoresed with the slow band. The hepatic and splenic activities which eluted in the buffer wash had a broader, more neutral pH optima (6.3) and co-electrophoresed with the fast band. Based on these results, the fast band was identified as the neutral β-glucosidase and the slow band as the acid isozyme.

The electrophoretic profiles of the splenic and fibroblast homogenates from homozygotes with Type 1 Gaucher disease and normal individuals are shown in Fig. 4. When equal amounts of protein were electrophoresed, the acid isozyme in the Gaucher tissues appeared to migrate with, but had markedly diminished activity compared to the acidic activity in normal tissues. Prolonged electrophoresis (longer than 4 h) resulted in inactivation of the acid isozyme from Type 1 Gaucher tissues. In contrast, the neutral activity in hepatic and splenic homogenates from Gaucher and normal tissues co-migrated and appeared to have similar activities when the same amounts of homogenate protein were applied. The neutral β-glucosidase activity co-electrophoresed with α-L-arabinosidase, β-D-xylosidase, and the more anodal β-D-galactosidase activities when visualized with the appropriate substrate (data not shown). Electrophoresis of normal fibroblast homogenates revealed only the acid β-glucosidase which did not co-migrate with a band of β-D-galactosidase activity; within the sensitivity of the method, no bands of activity were visualized when incubated with substrates for β-D-xylosidase or α-L-arabinosidase.



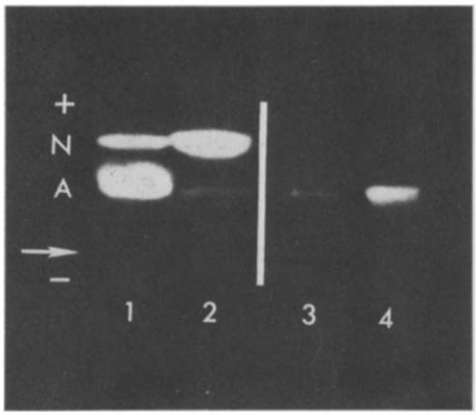


Fig. 3. pH Profiles for β-glucosidase activity from normal human hepatic (a—c) and splenic (d—f) homogenates before and after chromatography on concanavalin A-Sepharose. Total tissue homogenates (a and d); buffer wash (b and e); 1-*O*-methyl-α-D-glucopyranoside eluate (c and f). (See text for details).

Fig. 4. Fluorescent bands of β-glucosidase activity after cellulose acetate electrophoresis of homogenates from normal and Type 1 Gaucher disease tissues. a, lanes 1 and 2, normal and Type 1 Gaucher spleen, respectively; b, lanes 3 and 4, Type 1 Gaucher and normal skin fibroblasts, respectively. Equal amounts of total β-glucosidase activity from normal and Gaucher tissue homogenates were applied. (See text for details).

Discussion

Electrophoresis on cellulose acetate permitted the resolution of both the acid and neutral β-glucosidase isozymes in normal tissue homogenates. The identity of the acid and neutral isozymes was based on the following experimental findings: (1) chromatography on concanavalin A-Sepharose resulted in the separation of the two β-glucosidase activities and permitted characterization of their respective pH profiles. The acidic activity bound to this lectin indicating its glycoprotein nature, similar to other acidic lysosomal hydrolases [12]. The more neutral activity did not bind suggesting a different carbohydrate structure or the lack of post-translational glycosylation. Following electrophoresis, the acidic and neutral activities resolved by concanavalin A-Sepharose each co-migrated with only one of the activity bands present in hepatic or splenic homogenates. (2) Only the acidic band was observed in normal fibroblasts, consistent with the previous demonstration that only the acidic activity is expressed in these cultured cells [4,11]. In addition, (3) the markedly reduced activity of the slow band in tissue homogenates from Type 1 Gaucher homozygotes supports the identification of this electrophoretic band as the acid β-glucosidase isozyme responsible for residual glucocerebrosidase activity. Finally, (4) the neutral β-glucosidase band exhibited activity with other glycoside substrates as previously reported [14], whereas the acidic activity band only hydrolyzed 4-methylumbelliferyl-β-D-glucopyranoside.

The acidic band in Type 1 Gaucher disease was visualized and appeared to

co-migrate with the acidic activity in normal tissues. The most likely explanation for the loss of activity observed on electrophoresis is a point mutation which altered the stability as well as the activity of the residual acid β-glucosidase in Type 1 Gaucher disease. Support for this hypothesis will require biochemical and/or immunologic characterization of homogenous acid β-glucosidase from normal and Type 1 Gaucher tissues.

This electrophoresis method differs markedly from a recently described system for β-glucosidase [15] in which: (1) only one activity band was visualized in normal hepatic and splenic homogenates; (2) the acid and neutral isozymes were not identified, and (3) no activity was observed in Gaucher fibroblasts. In part, this might be explained by our use of ethylene glycol in the electrophoretic buffer and the ethanol/chloroform treatment which enhanced the entrance and migration into the gel of the acidic activity from normal and especially from Type 1 Gaucher tissue homogenates. This finding may be related to the known interaction of the membrane-bound acidic activity with phospholipids [2,6], and suggests that the defective acid isozyme in Type 1 Gaucher disease may also have an altered binding to crucial lipids, substrate, effector molecules or other hydrophobic moieties in the membrane. A more recent report [16], described a method for the polyacrylamide gel electrophoresis of only the acid isozyme. Unfortunately, this system requires large amounts of activity and taurocholate which selectively inhibits the neutral isozyme [6].

In summary, we report the chromatographic and electrophoretic resolution of two β-glucosidase isozymes. These isozymes correspond to the acid membrane-bound activity which is deficient in Type 1 Gaucher disease and the more neutral, soluble activity which may be involved in the post-translational processing of glycoproteins. The electrophoretic separation of these isozymes should provide the means to investigate the molecular nature and interrelationships of the β-glucosidase deficiencies in Gaucher disease subtypes. In addition, this electrophoretic system may be useful to determine the chromosomal localization of the structural genes for the β-glucosidase isozymes using human-rodent hybrid lines which express the human acid and/or neutral activities.

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