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β-Glucosidase Activator Protein from Bovine Spleen ("Coglucosidase")[1]

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β-Glucosidase-stimulating proteins ("co-β-glucosidase") have been isolated from bovine spleen by acidification of homogenized spleen, heat denaturation, and chromatography with DEAE-Sephacel, Sephadex G-75, hydroxyapatite, and decyl agarose columns. Gel electrophoresis of the product revealed a trace of inert protein and two fast-moving bands, a major diffuse band and a minor, faster-moving band. The latter two bands could be eluted from the gel and shown to stimulate a glucosidase preparation from bovine spleen. They both stained with Stains All and fast green, but poorly with Coomassie blue. The bands could also be visualized by ultraviolet scanning. Periodate-Schiff stain was positive for the major band. The M_r of the coglucosidase was about 20,400 as measured with the gel permeation column, but 4900 as measured with a Sephacryl S-200 column containing guanidine hydrochloride and roughly 6200 as measured by gel electrophoresis with Na dodecyl sulfate. A p*I* of 4.3–4.4 was indicated by isoelectric focusing. Neutral sugar was found to be present, but no sialic acid. It was destroyed by Pronase, but not by lyophilization, *N*-ethylmaleimide, or alkaline phosphatase. Stimulation of the basal activity (1 nmol/h assayed with methylumbelliferyl glucoside) was 50% when 0.15 μg/ml of coglucosidase was included in the incubation. The activating protein raised the *V* values and lowered the K_m values when both glucosyl ceramide and the artificial substrate were used. In contrast, phosphatidyl serine raised both the *V and* the K_m for cerebroside hydrolysis. The activator protein was found to occur in the soluble part of spleen as well as in the mitochondrial and lysosomal fractions.

Several hydrolases are known to be stimulated by specific activator proteins, which could be called "cohydrolases" in analogy with the term, "colipase," a protein which acts in the digestive tract as an adjunct to the action of pancreatic lipase. Whereas most hydrolase activators have been isolated from normal tissues, co-β-glucosidase has been studied primarily in spleens of patients with Gaucher's disease (a genetic deficiency of glucosyl ceramide glucosidase) since normal human spleen appeared to contain a coglucosidase having only a relatively low specific activity (1, 2). Action of glucosidase on both glucocerebroside (glucosyl ceramide) and an unnatural glucoside is stimulated by the Gaucher protein (3), and the effect is enhanced by the addition of an acidic phospholipid, such as phosphatidyl serine (4). Because of the difficulty of obtaining human spleen, we decided to isolate and characterize the coglucosidase of bovine spleen. This material proved to be somewhat more active than the preparation from Gaucher spleen.

MATERIALS AND METHODS

Materials used. MUG,[2] phosphatidyl serine from bovine brain, Triton X-100, and guanidine-HCl were from Sigma Chemical, and Stains All, from Polysciences (Warrington, Pa). Glucosyl ceramide was isolated from a Gaucher spleen (5) and [6-^{3}H]glucosyl ceramide was prepared from it chemically (6). Coglucosidase from human Gaucher spleen was kindly furnished by Dr. Robert H. Glew and Lydia B. Dan-

[1] The authors would like to dedicate this paper to Professor David Shemin in honor of his 70th birthday.

[2] Abbreviation used: MUG, methylumbelliferyl-β-D-glucopyranoside.

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iels, University of Pittsburgh, and partially purified cohexosaminidase from human liver was the kind gift of Drs. Su-Chen Li and Yu-Teh Li, Tulane University. Glucocerebrosidase from human placenta was supplied by Henry Blair, New England Enzyme Center. Dialyses were performed with Spectra/Por cellulose tubing (Spectrum Medical Industries Inc., Type 1, 6000–8000 M_r cutoff).

Isolation of coglucosidase. All steps were carried out at 0° or 4°C except where indicated. Two frozen bovine spleens from a slaughterhouse were thawed in cold water. The spleen pulp was scraped from the large blood vessels and cartilage at room temperature with the use of a notched steel trowel, the type sold for applying tile cement to floors. No section of spleen was exposed to room air for more than 10 min. The water used for thawing was added back to the pulp to make a 15% homogenate (Polytron homogenizer, Brinkmann Instruments). Acetic acid (2 M) was added to adjust the pH to 6 and, after 15 min, the homogenate was centrifuged 15 min at 25,000*g*. The supernatant, in 500-ml portions in a 1000-ml flask, was heated 20 min in a boiling water bath while stirring, then cooled and centrifuged 10 min at 10,000*g*. The resultant supernatant liquid was mixed with Analytical Reagent Celite and filtered through a sintered glass funnel.

After adjustment to pH 7 with 1 M NaOH, the active protein was concentrated by binding to DEAE-Sephacel (Pharmacia), previously equilibrated with Buffer A (5 mM Na phosphate, pH 7, 40 mM NaCl, 0.02% Na azide). For every liter of filtrate, 24 ml of a 50% suspension of the gel were added, stirred overnight and filtered through a sintered glass funnel. The gel was scraped into a beaker and stirred 30 min with 5 mM Na phosphate, pH 7, 0.5 M NaCl, 0.02% Na azide, using 2 ml/ml of DEAE-Sephacel. The suspension was filtered and the gel washed again, but with one-half the volume of buffer. The combined filtrates were dialyzed against two portions of Buffer A. A portion of the dialyzed filtrate corresponding to 1000 g of spleen pulp was used for further purification and the remaining solution was saved at 4°C.

The coglucosidase solution was pumped at 50 ml/h through a DEAE-Sephacel column, 2.6 × 30 cm, previously equilibrated with Buffer A. Elution was carried out with 1000 ml of 5 mM Na phosphate (pH 7) containing a linear gradient between 0.04 and 0.5 M NaCl (Fig. 1). Fractions rich in coglucosidase were combined and concentrated to about 20 ml by ultrafiltration with a pressurized, stirred solution device (Amicon Corp., Lexington, Mass.) through an Amicon UM2 membrane (1000 M_r cutoff). The column packing was washed with 500 ml of 1 M NaCl between runs.

The concentrate was then pumped at 50 ml/h through a Sephadex G-75f column, 2.6 × 92 cm, packed in Buffer A (Fig. 2). The coglucosidase effluent was concentrated by ultrafiltration as above, then dialyzed against Buffer B (5 mM K phosphate, pH 6.8, 0.02% NaN_3).

The solution was next pumped at 80 ml/h through a 1.5 × 10-cm column containing hydroxyapatite (Bio-Gel HTP, Bio-Rad Labs., Richmond, Calif.), packed in Buffer B and operated at room temperature. Elution was made with 40 ml of Buffer B and then with a 250-ml linear gradient ending up with Buffer B containing *30* mM phosphate. Ultraviolet-absorbing material eluted as two peaks, an early one and a subsequent one that trailed until the end of the run. The active material eluted in the early part of the second peak.

After each run the packing was washed with 0.4 M phosphate-azide until the A_{280} in the effluent reached the baseline.

The eluted coglucosidase was concentrated with a small column of DEAE-Sephacel (0.7 × 2 cm) packed in Buffer A. It was eluted with 4 ml of 5 mM Na phosphate, pH 7, 0.5 M NaCl, 0.02% NaN_3, then dialyzed against Buffer A.

The coglucosidase solution was next applied at room temperature at 20 ml/h to a column of decyl agarose (7) (Sigma Chemical Co.), 1 × 10 cm, in Buffer A. Elution was carried out with 15 ml of Buffer A, then with 100 ml of a linear gradient from Buffer A to Buffer A made up in ethylene glycol–water, 90:10 (v/v). This was followed by elution with 1 M NaCl. Here too ultraviolet-absorbing material came out in the form of an early peak and a later, slowly rising peak. The fractions eluting near the end of the gradient contained the active material.

The eluted coglucosidase was concentrated (and freed of ethylene glycol) as above, with a 0.7 × 1-cm column of DEAE-Sephacel, followed by dialysis against Buffer A. The preparation was stored at 4°C.

Glucosidase preparation. About 30 g of pulp from frozen spleen were homogenized as described above but the suspension was centrifuged at 200,000*g* for 30 min. The pellet was extracted by the method of Ho and Light (4) and the enzyme was precipitated at 4°C with ammonium sulfate (0.25 g/ml of liquid), left 15 min, and centrifuged 10 min at 10,000*g*. The pellet was mixed with 25 ml of water and particles removed by a similar centrifugation. The extract was then chromatographed in Buffer A with the Sephadex G-75f column described above and the milky, enzyme-containing fractions were combined and stored in portions at −70°C.

Assays. The standard assay for glucosidase activity was carried out in a volume of 0.2 ml with 2 mM MUG in 50 mM Na acetate, pH 4.5, containing 0.05% Triton X-100 (3). The solution was incubated 30 min at 37°C. One unit of activity is 1 nmol/h of methylumbelliferone formation.

The standard assay for activity of the stimulating protein was carried out by adding various amounts of the test sample to the glucosidase preparation. The

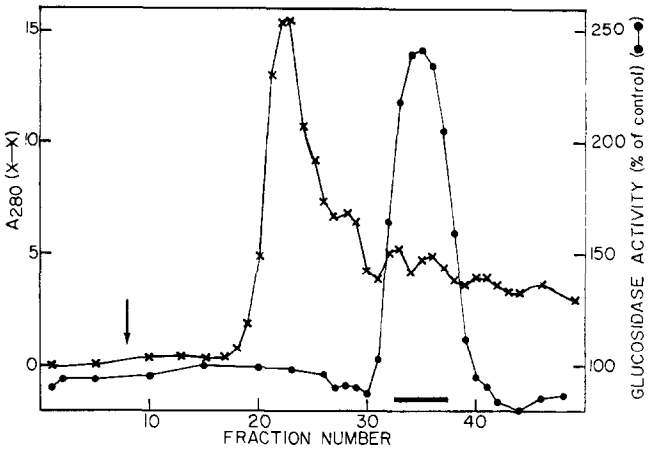

FIG. 1. Chromatography of bovine spleen coglucosidase with DEAE-Sephacel. The sample was added to the column and eluted with a solution of increasing NaCl concentration, starting at the point marked by the arrow. The fractions collected (25 ml each) were assayed for absorbance at 280 nm and for ability to stimulate glucosidase. The horizontal bar indicates the fractions pooled for size exclusion chromatography.

amount of glucosidase chosen (16-22 μg) was sufficient to produce 1 nmol of methylumbelliferone per hour in the assay for glucosidase described above. One unit of coglucosidase is defined as the amount of material which produces 150% of the basal enzyme activity.

Glucocerebrosidase activity was measured under the same conditions as the aryl glucosidase except for the use of 150 μM [6-^{3}H]glucosyl ceramide instead of MUG. The labeled glucose liberated was separated from the substrate by partitioning with isopropanol-castor oil-chloroform-aqueous glucose containing 1 mg of $CaCl_2$/ml of water (8). (Calcium chloride was useful in preventing emulsions in experiments in which phosphatidyl serine was included in the incubation tubes.) One unit of activity is 1 nmol/h of glucose liberated.

Protein determinations were made on precipitated proteins by the Peterson modification (9) of the method of Lowry *et al.* (10), with BSA as standard.

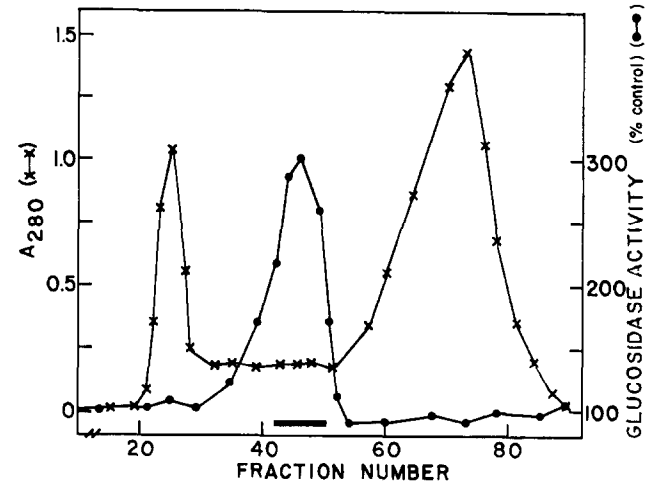

FIG. 2. Chromatography of coglucosidase with Sephadex G-75f. The fractions (6.7 ml) were assayed for A_{280} and glucosidase stimulation. The horizontal bar identifies the fractions pooled for hydroxyapatite chromatography. The first protein peak appears at the void volume.

The proteins, in 1 ml, containing 0.25 mg Na deoxycholate (11), were precipitated with 1 ml of 12% trichloroacetic acid/1% phosphotungstic acid prior to carrying out the color reaction. The phosphotungstic acid was necessary to achieve complete precipitation of coglucosidase (only 30% was precipitated when this acid was omitted). Similar results were obtained when the modified method was tested with bovine plasma glycoprotein (fraction VI, Pentex).

RESULTS

Developing the coglucosidase assay. Stimulation of membrane-bound glucosidase by water-extractable coglucosidase was demonstrated with human spleen (1). We found a similar, but much smaller, effect with bovine spleen. Unfortunately the stimulation decreased on storing the enzyme fraction and we accordingly turned to soluble glucosidase preparations. Attempts to prepare soluble glucosidase according to Ho and Light (4), inactive except in the presence of coglucosidase, did not work with the bovine enzyme. However, partial purification of the enzyme did give a stable preparation which responded to very low concentrations of activator.

A complication in the assay problem was the finding that the observed enzyme activity was not linearly dependent on the concentration of enzyme (Fig. 3, bottom curve). The shape may indicate the presence of endogenous stimulator(s) in the enzyme preparation. The addition of coglucosidase at all enzyme concentrations produced stimulation and, at high concentrations of coglucosidase, linearized the relationship between enzyme activity and concentration of enzyme.

When the data from this experiment were calculated to show the percentage of basal activity produced by coglucosidase (Fig. 4), the response to the activator was found to be proportional to the concentration of coglucosidase up to about 170% of the basal activity. The response in this region was independent of the enzyme concentration over a sixfold range. Curve B, which is derived from the standard coglucosidase assay condition (1 nmol/h basal activity), was found to plateau at about 300% of the basal activity with high concentrations of coglucosidase. Different glucosidase preparations were found to vary slightly in their sensitivity to coglucosidase.

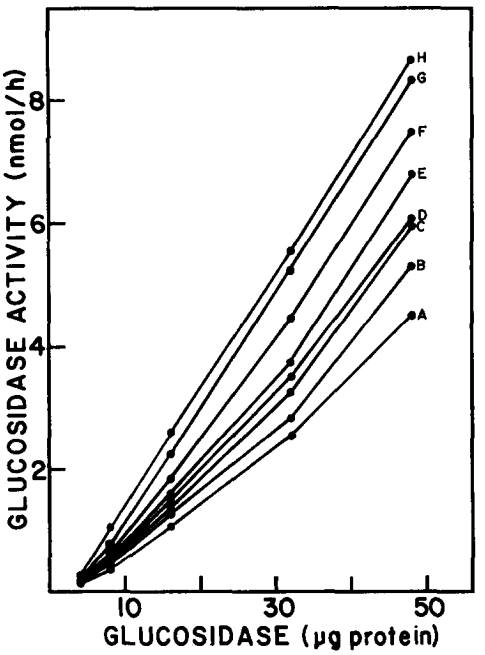

FIG. 3. Effect of glucosidase concentration on MUG hydrolysis with various amounts of coglucosidase (A, 0; B, 8.5; C, 17; D, 25; E, 34; F, 64; G, 127; H, 254 ng of protein in 0.2 ml of incubation solution).

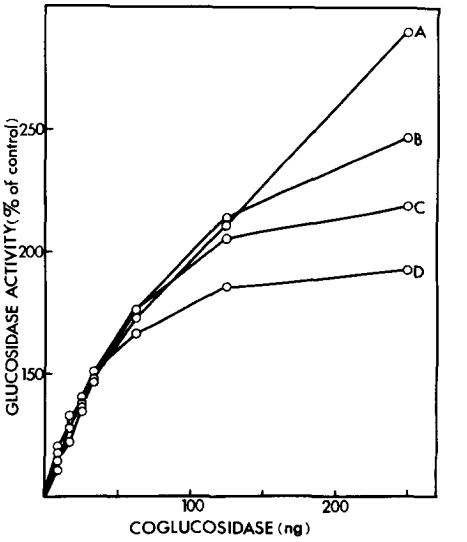

FIG. 4. Effect of coglucosidase concentration on glucosidase activity at various glucosidase levels (A, 8; B, 16; C, 32; D, 48 μg of protein in 0.2 ml).

An interesting feature of Fig. 4 is that the percentage stimulation at high coglucosidase levels was greatest with the least amount of enzyme. Based on the above proposed interpretation of Fig. 3—that some endogenous stimulatory material is present in the enzyme preparation—we suggest that the endogenous stimulator is less effective in the dilute enzyme solution, so that the added stimulator produces a larger effect.

Purification of coglucosidase. Table I shows the progress of the purification steps, with omission of the first four steps. The coglucosidase could not be assayed at the first four stages of purification because of the presence of glucosidase and inhibitory material. A similar problem with crude activator was observed by Li *et al.* in the isolation of cohexosaminidase (12).

Preliminary trials of the initial extraction step showed that pH was an important factor: pH 7 was a little less effective than 6 but pH 5 yielded 1/10th the activity (assayed after DEAE-Sephacel chromatography).

The decyl agarose column, with each reuse of the packing, released the coglucosidase in the form of a broader peak having a lower retention volume. The packing had to be discarded after five uses. Such a limited use span for lipophilic columns has been noted before (13).

The second uv-absorbing peak with the decyl agarose column was also seen in blank runs and was found to be due, in part at least, to a contaminant in the glycol. The uv-absorbing material was removed from the coglucosidase by the final step. (In retrospect, it would seem wiser to use unplasticized tubing in operating this column since the organic solvent might extract plasticizer and deposit it in the lipoidal packing.)

TABLE I

PURIFICATION OF COGLUCOSIDASE[a]

Column step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
DEAE-Sephacel	285	333,000	1,170
Sephadex G-75f	34	308,000	9,070
Hydroxyapatite	9.6	230,000	23,700
Decyl agarose	4.2	137,000	32,500

[a] From 970 g of bovine spleen pulp.

The possibility that coglucosidase is an artifact produced during the heating step was investigated by comparing heated and unheated spleen extracts that were then purified by gel permeation. Both preparations yielded peaks of activity that were very similar in shape and elution volume. The yield of active material from the heated sample was 81% of that from the unheated sample.

Electrophoretic characterization of coglucosidase. Polyacrylamide electrophoresis was performed in alkaline 22.5% gels according to Davis (14) with persulfate - tetramethylethylenediamine - catalyzed stacking and separating gels. Stained gels showed a minor band at R_f 0.3 and a pair of close but distinctly separated bands at R_f 0.6 and 0.7 (Fig. 5). The latter bands were stained only weakly by Coomassie blue G (15), a characteristic of some glycoproteins, but relatively strongly with Stains All (16) and fast green FCF (17). Stains All, which gives characteristic colors for various kinds of substances, produced the blue color typical of acidic polymers. Staining for glycolic compounds with periodate-Schiff reagent (18) yielded a positive reaction with the major band. Some gels, immersed in 7.5% HOAc and scanned at 280 nm (Fig. 6), revealed the same three bands with relative areas approximately matching the visually evaluated intensity of the Stains All bands. The measured areas were 5, 83, and 12 (in arbitrary units). Repeated scanning of a single gel gave decreasing peak heights, indicating that the proteins were poorly fixed by the acetic acid.

Some gels, with or without prior immersion in 7.5% HOAc, were sectioned into 1-mm slices and left in 5 mM Na-phosphate, pH 7, 5 mM NaCl, 0.1% Triton X-100 for 1 h at 37°C. Assay of the extracts for coglucosidase activity showed a close match between the uv absorbance and glucosidase stimulation in the two faster bands (Fig. 6). The slow band appeared to

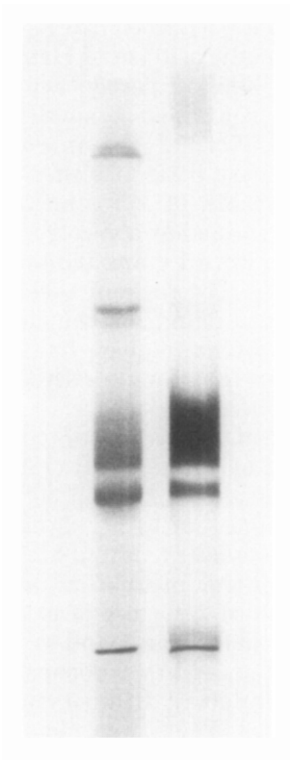

FIG. 5. Polyacrylamide gel electrophoresis of coglucosidase (0.1 mg) in alkaline buffer. Gel A was stained with Coomassie blue G and gel B, with Stains All. The bromphenol blue band at the bottom of the photo is marked with a wire.

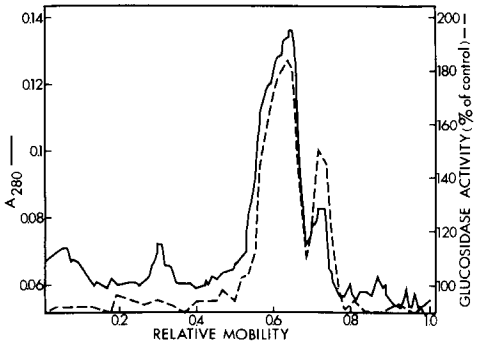

FIG. 6. Localization of coglucosidase after alkaline electrophoresis of 100 μg protein. One gel was scanned at 280 nm, the other was sectioned, extracted, and assayed for glucosidase stimulation. The mobility of the protein with respect to bromphenol blue is indicated.

be inactive. Combining the extracts from each active band and assaying for protein content and activity showed that the faster component comprised 15% of the total activity. The two components had the same specific activities.

Electrophoresis of coglucosidase at pH 2.3 (19) and assay for coglucosidase activity in gel slices showed an active fraction at R_f 0.7 in the direction of the cathode. Attempts to stain the protein in the gel with Coomassie blue and Stains All proved unsuccessful; the latter stain produced a faint, short-lived band in the active region.

Electrophoresis of coglucosidase was also carried out in Na dodecyl sulfate according to Laemmli (20) with the addition of 8 M urea to the sample. Staining with fast green and scanning at 625 nm revealed a broad, fast-moving band near the tracking dye (apparently a partially resolved pair of peaks) followed by a very small band. Comparison with standards (aldolase, α-chymotrypsinogen, ribonuclease, and aprotinin; M_r's = 40,000, 25,700, 13,700, and 6,500 respectively) suggested that the major peak had a M_r of 6200. Prestaining with fluorescamine (21) gave the same results as fast green.

Isoelectric focusing of coglucosidase in 2% Ampholine, pH 3.5-5 (LKB Instruments, Inc.) (22) yielded two very close, opaque bands, visible without staining, which disappeared on standing or on immersion in 12.5% trichloroacetic acid. The bands could be visualized with fast green but not with Stains All or Coomassie blue. Tests with glucosidase showed that coglucosidase was present in a relatively wide band with most of the activity in the region of pH 4.3 to 4.4. This is similar to the reported pI of 4.1 for the cogalactosidase of human liver (23), the pI of 4.3 for human liver cosulfatase (24), and the pI of 4.8 for cohexosaminidase of human kidney (25).

Other properties of coglucosidase. The elution volume for coglucosidase with the preparative Sephadex column was compared with the values obtained with bovine α-chymotrypsinogen (M_r 25,700) and whale myoglobin (M_r 17,800). The active material eluted between them as a rela-

tively broad peak with M_r 20,400. This is close to the M_r of 22,000 for human cogalactosidase (23) and 21,500 for cosulfatase (24), and a little lower than the 25,000 reported for cohexosaminidase (25) (all measured by size exclusion).

The M_r of coglucosidase was estimated also by the method of Belew *et al.* (26), which involves *S*-carboxymethylation with labeled iodoacetate and size exclusion chromatography in 6 M guanidine-HCl with Sephacryl S-200. This method has the advantage over ordinary gel permeation in its insensitivity to protein shape. The derivatization step did not affect the stimulatory activity but the yield of radioactive material was low compared with other proteins that were treated similarly. The column was standardized with aprotinin (58 amino acid residues), ribonuclease (124), and chymotrypsinogen (245). Elution of derivatized coglucosidase revealed a major radioactive peak corresponding to a protein having 104 residues and a minor peak corresponding to 45 residues. Assay of the column fractions for stimulatory activity, after dialysis, showed that the latter peak contained 71% of the initial activity. The first peak was presumably derived from the trace protein impurity present in our coglucosidase. Treatment of the data by the method of Fish *et al.* (27) gave a M_r of 4900 for the active peak, in reasonable agreement with the value from the dodecyl sulfate electrophoresis experiment.

Chromatography of underivatized coglucosidase in the same column, equilibrated with Buffer A, resulted in the same elution volume as in the guanidine-HCl system.

The uv spectrum of coglucosidase was typical of proteins, showing a maximum at 274 nm with a molar absorbance of 3000 (based on a M_r of 4900, disregarding the slow-moving impurity). The 280/260 absorbance ratio was 1.14, signifying a negligible or zero content of nucleic acid (28).

Analysis with anthrone/sulfuric acid (29) yielded a neutral sugar content of 1.5 residues per mole of coglucosidase, based on galactose as standard. No significant content of sialic acid could be found after hydrolysis for 1 h in 50 mM H_2SO_4 at 80°C (30). The coglucosidase of Gaucher spleen (2) had a similar percentage of neutral sugar but contained a small amount of sialic acid. Normal human coglucosidase contained very little of either (2).

The contents of tyrosine and tryptophan, determined by a spectrophotometric assay (31), were 1.0 and 0.3 residues/mol, respectively. The ratio of these values resembles that found for the coglucosidase of normal human spleen but is very different from that found with the Gaucher preparation (2).

Incubation of 17 μg of coglucosidase with 1 mg of Pronase-CB (120,000 PUK/mg, Calbiochem) for 4 h at 50°C in 10 mM Tris-Cl/5 mM $CaCl_2$, pH 8, destroyed all of the stimulatory activity of coglucosidase. A control incubation without Pronase produced 30% loss of activity.

Coglucosidase was found to be stable to lyophilization and to exposure to 1 mM *N*-ethylmaleimide or dithioerythritol for 30 min at 22°C. There was also little or no effect on incubation with alkaline phosphatase of calf intestinal mucosa (bound to beaded agarose, Sigma Chemical) and alkaline electrophoresis of the treated coglucosidase showed no change in the migration of the two stimulatory peaks or the staining pattern with Stains All.

To test the possibility that coglucosidase owes its activity to the presence of a lipid, 0.3 mg was extracted with chloroform-methanol, 2:1 (32). The extract was filtered through Celite on a sintered glass funnel and a partition created by addition of water in order to remove buffer. Examination of the lipids by thin-layer chromatography with ninhydrin and charring sprays revealed no difference from the buffer blank. The Celite was washed with water and filtered. The filtrate contained 53% of the glucosidase-stimulating activity and 51% of the protein in the original sample. It therefore appears that coglucosidase is at least partially stable to treatment with chloroform-methanol and that lipid in the preparation does not account for its activity.

While the activator can be stored indefinitely at 4°C in Buffer A (pH 7.0), it

rapidly loses effectiveness at room temperature in acetate, pH 4.5. In one experiment, 4 to 34 ng of coglucosidase in 80 μl of acetate buffer was stored 60 min, then assayed for activity in the usual way after adding Triton. Only 46% of the control stimulatory activity was found. If, however, the Triton was included prior to storage, 90% of the control activity was found. In a parallel experiment, an aliquot of each stored sample was transferred to a separate tube for assay (instead of being assayed in the same tube). In this case, the loss of activity in the absence of Triton was complete, and the protective effect of the detergent was less (75% of control). These results indicate that Triton prevents loss of the protein by adsorption on the glass at pH 4.5.

Further evidence for interaction between Triton and coglucosidase came from chromatography with Sephadex G-75 at pH 4.5 and 37°C with and without the inclusion of 0.05% Triton in all the solutions. In the absence of detergent, the factor eluted close to the position seen at pH 7, but the yield of recovered material was low. In the presence of detergent, the coglucosidase eluted in the void volume in good yield. This indicates that coglucosidase forms a high molecular weight complex with Triton, possibly by binding to detergent micelles, and is thus protected against adsorption to glass and, perhaps, to Sephadex.

Interactions between coglucosidase and glucosidase. The activity of glucosidase, with or without added coglucosidase, was found to be constant with time over a 2-h period, the first measurement being made at 5 min. This shows that the activator does not act by stabilizing glucosidase against spontaneous denaturation and that its action is very rapidly inaugurated.

The stimulatory action of coglucosidase on glucoside hydrolysis could be seen over a range of two pH units (Fig. 7). The percentage stimulation was relatively insensitive to the pH.

Sodium chloride reduced the basal enzyme activity and also interfered with the action of coglucosidase (Fig. 9). The same

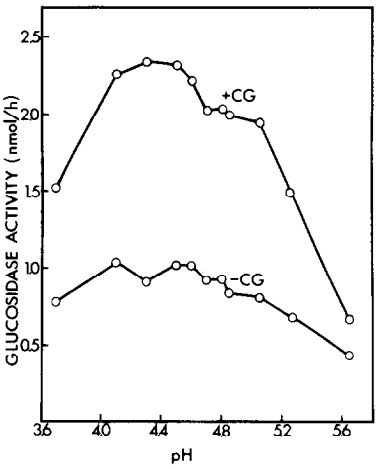

FIG. 7. pH dependence of glucosidase activity. The enzyme was incubated 30 min at 37°C in 50 mM Na acetate, 2 mM MUG, and 0.05% Triton X-100 alone or in the presence of 210 ng of coglucosidase.

phenomenon was observed with KCl, NaBr, and NaOAc. This makes it important to control ionic strength in assays for the enzyme and for its activator. The inhibitory effect of salt on fibroblast "β-glucosidase" has been reported previously (33).

The inclusion of Triton X-100 in glucosidase incubations produced some stimulatory effect (Fig. 8). The stimulation produced by the addition of coglucosidase to the incubation mixtures was maximal at

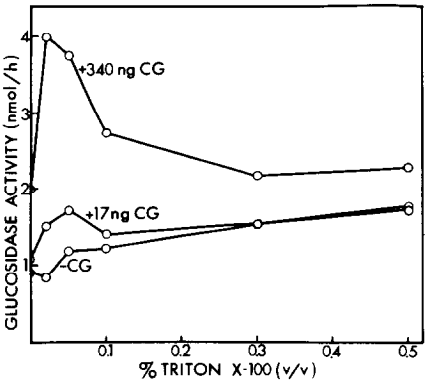

FIG. 8 Effect of Triton X-100 concentration on glucosidase activity, in the presence of different amounts of coglucosidase. The standard coglucosidase assay procedure was used, except for the variation in detergent concentration.

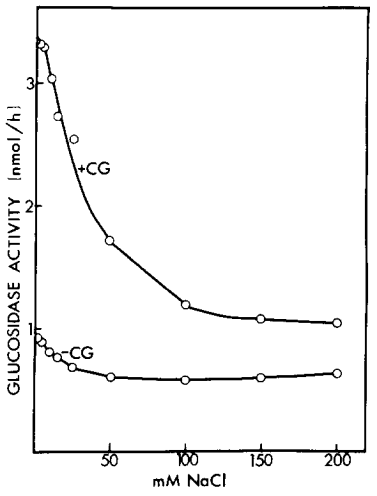

FIG. 9. Effect of NaCl concentration on glucosidase activity, with and without the presence of 210 ng of coglucosidase. The standard coglucosidase assay procedure was used.

TABLE II

KINETIC ANALYSIS OF GLUCOSIDASE ACTIVITY WITH MUG AND GLUCOSYL CERAMIDE[a]

Additions	"Aryl glucosidase" K_m (mM)	"Aryl glucosidase" V (nmol/mg/h)	Cerebrosidase K_m (μM)	Cerebrosidase V (nmol/mg/h)
None	2.21	104	19.6	19
Coglucosidase (0.21 μg)	1.43	245	6.1	46
Phosphatidyl serine (20 μg)	1.54	456	39.1	147

[a] Enzyme activities were measured as described under Methods except for the use of 0.25 to 4 mM MUG or 7.5 to 150 μM [6-^{3}H]glucosyl ceramide. The K_m and V were determined from Lineweaver-Burk plots.

0.02-0.05% Triton, further additions of Triton acting to interfere with the stimulatory effect of coglucosidase. With a low concentration of coglucosidase, high concentrations of Triton completely abolished the stimulatory effect.

Specificity of coglucosidase action. At least two glucosidases which hydrolyze MUG have been found to occur in mammalian tissues. One of these acts on glucocerebroside and is selectively inactivated by conduritol B epoxide (anhydroinositol) (34, 35). The glucosidase used in the present study was completely inactivated by incubation with 6.2 mM conduritol B epoxide for 30 min at 37°C. The activity (toward MUG) could not be restored by the addition of 20 μg of phosphatidyl serine or of 210 ng of coglucosidase. It appears therefore that the enzyme preparation used here is glucocerebrosidase.

The enzyme preparation did indeed hydrolyze glucosyl ceramide (Table II) when tested with [^{3}H]glucosyl ceramide under the standard assay conditions. The V for the natural substrate was somewhat lower than for MUG, but the K_m was about 1/100th that for MUG. When coglucosidase was included in the incubations, the hydrolysis rate of both substrates was increased to a similar degree and the K_m values for both were decreased. This may mean that coglucosidase acts on glucosidase to enhance the enzyme's binding to the substrates. The kinetic values shown here are rather similar to those reported for human glucosidase with MUG and cerebroside in the presence of Gaucher coglucosidase (36).

The addition of phosphatidyl serine, at about 100 times the coglucosidase level, also changed the kinetic properties of the enzyme but, unlike coglucosidase, *increased* the K_m for cerebroside.

Because of the limited solubility of MUG, the concentration used in the standard assays (and in the assays in other laboratories) is in the region of the K_m's. Thus, the degree of stimulation produced in any assay involving MUG is a reflection of the effect of the stimulator on *both* the K_m and V, and will then depend on the substrate concentration.

The effect of a large amount of coglucosidase (0.85 μg, which yielded 380% of basal activity when tested with MUG) on a variety of "acid" hydrolases was examined, using our somewhat crude glucosidase preparation as the source of the enzymes, and the corresponding methylumbelliferyl derivatives as the substrates.

Our standard assay with Triton buffer was used, with substrate concentrations at 2 mM except where solubility limits required a lower concentration (0.3 mM β-galactoside and 0.5 mM α-glucoside and phosphate). Enough enzyme preparation was used to produce a basal activity of 0.4 to 1.8 nmol/h. Coglucosidase had no effect on sulfatase, phosphatase, α-glucosidase, β-galactosidase, and β-glucosaminidase but *inhibited* β-glucuronidase 40%. Additional tests with glucuronidase showed that slightly higher inhibition could be produced with higher coglucosidase concentrations and lower inhibitions with lower concentrations (e.g., 11% with 0.2 μg). These tests are admittedly incomplete since the previously described cohydrolases have been reported to be active only with natural substrates, not the methylumbelliferyl derivatives. However, the interesting effect on glucuronidase is consistent with the observation (37) that the inactivation of glucocerebrosidase in mice injected with conduritol B epoxide leads to a decrease in glucuronidase activity. It may be that coglucosidase is elevated in these mice in response to the loss of glucosidase activity.

A preparation of coglucosidase from Gaucher spleen (2) was found to stimulate our bovine glucocerebrosidase, but with a specific activity that was 29% of the bovine protein. The curves obtained by plotting the enzyme activity against cofactor units were identical for the human and bovine cofactors. A preparation of highly purified glucocerebrosidase from human placenta was stimulated by the splenic factor, but higher coglucosidase concentrations were required to produce stimulations equal to those obtained with the bovine enzyme. The bovine activator protein also stimulated the hydrolysis of MUG by glucosidase prepared from mouse liver by the procedure we use for bovine spleen.[3] These observations suggest that coglucosidase and glucosidases from different organs and species are able to interact.

Another activator from human tissue, which stimulates hexosaminidase (11), proved to be inactive in our coglucosidase assay when tested at the 0.8-μg level. Also nonstimulatory was a group of glycoproteins, tested at concentrations up to 50 μg/ml: ovalbumin, fetuin, thyroglobulin, serum albumin, and glycoprotein fraction VI from plasma.

Subcellular distribution of coglucosidase. Fresh bovine spleen was homogenized in sucrose-mannitol and fractionated by differential centrifugation (38) and the cytosol and membranous preparations (in water) were stored at −20°C. Prior to assay for protein and enzymes, the preparations were freeze-thawed five times. Triton (1 mg/ml) was added to samples used for the assay of glucosidases and the suspensions were sonicated with a probe sonicator.

Because of the presence of inhibitory materials in all of the fractions, it was necessary to purify the coglucosidase before assaying it. The samples were sonicated briefly with Triton X-100 (1 mg/ml), freeze-thawed three times, and centrifuged at 25,000*g* for 15 min. The resultant pellets were reextracted similarly and the pooled supernatants were heated at 100°C for 10 min, filtered through Celite, and adjusted to pH 7. The coglucosidase was concentrated with DEAE-Sephacel and purified with a column of Sephadex G-75f as before, but with a second pass through the permeation column to further remove interfering material. At this stage of purification, linearity in the coglucosidase assay could be seen up to at least 150% of the basal activity toward methylumbelliferyl glucoside, depending on the particular subcellular fraction.

Coglucosidase activity was found in all fractions (Table III), the highest relative specific activities being in the M and L fractions (1.75 and 1.80, respectively). A considerable amount of activity was seen in the soluble fraction, in which the relative specific activity was not much lower (1.52). The distribution patterns for aryl glucosidase and cerebrosidase were quite similar to each other and to the pattern for coglucosidase, except for the relatively low amounts of enzyme in the cytosol.

In this experiment, the nuclear debris

[3] Unpublished study, D. Pang, S. L. Berent, and N. S. Radin.

TABLE III

SUBCELLULAR DISTRIBUTION OF COGLUCOSIDASE AND β-GLUCOSIDASES IN BOVINE SPLEEN

Material measured	Total activity[d]	Observed activity (% of total)					Total recovered activity (%)
		ND[e]	M	L	P	S	
Coglucosidase[a]	440	34	26	15	4	29	109
Aryl glucosidase[a]	7430	40	34	18	4	7	102
Glucocerebrosidase[a]	392	41	29	17	4	7	98
Lactate dehydrogenase[b]	38	22	12	5	1	51	90
Succinate:cytochrome *c* dehydrogenase[c]	10	29	48	13	0	3	93
Protein (mg)	102	54	15	8	5	19	101

[a] Units are described under Methods. Cerebrosidase and aryl glucosidase were assayed in the presence of 20 μg of phosphatidyl serine.

[b] Unit is μmol/min.

[c] Unit is one absorbance unit change per minute.

[d] Calculated by adding the units in the nuclear debris (ND) and supernatant derived from 1 g of spleen.

[e] Fraction M is the mitochondria-rich fraction, L is the lysosome-rich fraction, P is the microsomal fraction, S is the soluble (cytosol) fraction.

fraction contained a relatively large amount of all the materials that were measured because of the toughness of spleens (39) and because the pellets were not washed. Despite this, the relative specific activities of the soluble and mitochondrial marker enzymes, lactate dehydrogenase (40), and succinate:cytochrome *c* reductase (41), showed the expected values. The distribution patterns for the β-glucosidases showed the characteristics of lysosomal enzymes which, in spleen, are more diffuse than in liver (42).

DISCUSSION

Our findings on the properties of coglucosidase suggest that it is a highly acidic small protein in the form of a relatively open, elongated structure. The high charge is inferred from the high ionic strength needed to elute it from DEAE columns. The shape is inferred from the elution experiments with Sephacryl, which showed that the elution volume was unaffected by the presence of guanidine-HCl. This points to a "linear random coil" (43) in neutral pH even in the absence of guanidine-HCl. Our value of M_r 20,000 obtained by gel permeation in the absence of chaotropic agents is high because standards of globular shape were used to standardize the column. The heat stability of coglucosidase is consistent with the picture of a small protein lacking a complex structure.

The occurrence of two active bands and the diffuseness of the major band seen when coglucosidase was electrophoresed cannot be attributed solely to heterogeneity in the carbohydrate side chains, since diffuseness was evident also after isoelectric focusing. The absence of sialic acid eliminates this sugar as a source of heterogeneity. Diffuse electrophoretic bands have been noted for other cohydrolases. Perhaps the explanation lies in the existence of varying degrees of amidation of the acidic amino acid residues.

The physiological significance of coglucosidase is yet to be established. Its concentration in bovine spleen, judging from our yield of isolated protein, is over 4 μg/g. This concentration is far above the saturating concentration needed for our assay system, so the protein is likely to be functionally stimulatory in the intact animal. Moreover, coglucosidase and the enzyme on which it acts have both been found to occur in the same membranous particles, isolated as "tritosomes" from rat liver (44). In Gaucher's disease, a coglucosidase appears to accumulate in spleen

lysosomes together with the substrate and glucosidase (38). In normal bovine spleen (Table III), there is a marked parallelism in the distributions of the two proteins in the membrane fractions. We did find a relatively large amount of coglucosidase in the cytosol, so it is possible that there is a second pool of the cofactor that acts as a reserve for times when faster hydrolysis is needed. However, mechanical disruption of the lysosomes during homogenization may have released coglucosidase more readily than cerebrosidase, which is tightly membrane bound. We tried to minimize this effect by the use of a brief homogenization and by omitting the usual rehomogenization steps.

Further support for a physiological function comes from the finding that other hydrolases are stimulated or otherwise "enhanced" by specific proteins (23-25, 45) although the mechanisms of action seem to differ. The most striking evidence for the importance of cohydrolases is the discovery of a patient with a variant form of Tay-Sachs disease in which the genetic disorder was due to lack of *co*hexosaminidase rather than to a defective hexosaminidase (46). This is also a strong hint that the cohydrolases are quite specific for individual hydrolases.

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Note added in proof: A recent report by D. A. Wenger, M. Sattler, and S. Roth in *Trans. Amer. Soc. Neurochem.* **12,** 210 (1981) describes a protein from Gaucher spleen very similar to the one described here. The protein activates not only glucocerebrosidase, but also galactocerebrosidase and sphingomyelinase. A test of our protein by Dr. Wenger (personal communication) has disclosed that it too stimulates all three enzymes.

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