SOLUBILIZATION OF GLUCOCEREBROSIDASE FROM HUMAN PLACENTA AND DEMONSTRATION OF A PHOSPHOLIPID REQUIREMENT FOR ITS CATALYTIC ACTIVITY

G.L. Dale, D.G. Villacorte and E. Beutler[1]

Division of Medicine
City of Hope Medical Center
Duarte, California 91010

Received June 23,1976

SUMMARY

Glucocerebrosidase, a membrane bound enzyme, can be solubilized by acetone precipitation and the resultant soluble enzyme activity demonstrated in the presence of acidic phospholipid, e.g. phosphatidylserine. This is the first report of the detergent-free solubilization of glucocerebrosidase.

Gaucher's disease results from a deficiency of glucocerebrosidase, an enzyme which cleaves the β-glucosidic linkage of glucocerebroside (1). The purification of glucocerebrosidase would allow studies of its structure, its relationship to the putative heat-stable cofactor (2), and its properties in normal subjects and persons with Gaucher's disease. Furthermore, purified glucocerebrosidase could be used for experimental replacement therapy in Gaucher's disease patients (3). The purification of glucocerebrosidase has been greatly impeded by the fact that it is a tightly membrane-bound enzyme. Pentchev et al (4) have purified a detergent-solubilized form of glucocerebrosidase; however, a detergent-free, soluble form

[1]This work was supported, in part, by Grant No. AM 14755 from the National Institutes of Health.

of the enzyme would be more suitable for the studies enumerated above as well as for enzyme replacement trials.

Glucocerebrosidase can be assayed conveniently with the fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (5,6,7) and will be referred to here as β-glucosidase. In the course of repeated attempts to purify β-glucosidase, we discovered that as the enzyme was partially purified it lost activity and that the activity could be restored by the addition of crude placental extracts. The factor necessary for activity was found to be a phospholipid. We now report that the preparation of acetone powders from placental extracts solubilizes β-glucosidase activity, an activity which can only be demonstrated in the presence of traces of phospholipids.

MATERIALS AND METHODS

Materials - Taurocholic acid (catalog number 103004, lot number 3176) used for tissue extraction was obtained from ICN Pharmaceuticals, Cleveland, Ohio; taurocholic acid used in the enzyme assays was obtained from Calbiochem, La Jolla, California. Koch-Light Laboratories, Colnbrook, England, was the supplier of 4-methylumbelliferyl-β-D-glucopyranoside. Con-A Sepharose was obtained from Pharmacia, Uppsala, Sweden. Hexokinase, glucose-6-phosphate dehydrogenase, ATP, NADP and phospholipids were obtained from Sigma Chemical Co., St. Louis, Mo. Cutscum detergent was from Fisher Scientific Co., Fair Lawn, New Jersey. Acetone was reagent grade.

Enzyme Assays - β-glucosidase was assayed at 37° by adding 20 µl of enzyme preparation to 200 µl of a reaction mixture comprised, unless otherwise indicated, of 1 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 measured as outlined previously (8). One milliunit of enzymatic activity represents the hydrolysis of one nanomole of substrate per minute.

Glucocerebroside was isolated from the spleen of a Gaucher's patient by the method of Rosenberg and Chargaff (9). Glucocerebrosidase was measured by adding 20 µl of enzyme preparation (Step III, Table II) to 200 µl of 0.3 mM glucocerebroside, 0.5% taurocholic acid, 0.1% Cutscum, 0.2 mM phosphatidylserine, 50 mM Na citrate, pH 6.0 and incubating for one hour at 37°. The reaction was terminated by boiling for one minute. The amount of glucose released from glucocerebroside was measured fluorometrically, phosphorylating the glucose by incubation with hexokinase and ATP and then oxidizing the glucose-6-phosphate formed with glucose-6-phosphate dehydrogenase and NADP. One milliunit, mU, of enzyme activity represents the

release of 1 nanomole of glucose from glucocerebroside per minute.

Solubilization and Purification - Human placenta obtained from a local hospital was stripped of membranes and homogenized in water (25% w/v) for ten minutes at 0°C at top speed in a Sorvall Omnimixer. The homogenate was centrifuged at 10,000 x g for 20 minutes, and the supernatant was discarded. The pellet was homogenized (40% w/v) in 1% taurocholic acid, 50 mM sodium chloride, 50 mM citrate, pH 6.0 with 0.02% sodium azide at top speed in a Sorvall Omnimixer for 60 seconds at 0°C. After centrifugation at 10,000 x g for 20 minutes, most of the β-glucosidase activity was found in the supernatant.

Fifty milliliters of Con-A Sepharose were poured into a 2.5 cm diameter column and equilibrated with 0.5% taurocholic acid, 5 mM $CaCl_2$, 5 mM $MnCl_2$, 50 mM NaCl, 50 mM Na citrate, pH 6.0 with 0.02% Na azide. Three hundred milliliters of taurocholate extract, made 5 mM in $CaCl_2$ and $MnCl_2$, was applied to the column at room temperature at a flow rate of 1 ml/min, and the column was then washed with 500 ml of starting buffer. β-glucosidase was eluted with 500 ml of 0.1 M α-methyl mannoside in starting buffer. The eluate was concentrated to approximately 250 ml by ultrafiltration.

Ten volumes of acetone per volume of protein solution was magnetically stirred and was cooled to -15°C in an ice-NaCl bath. β-Glucosidase solution was dripped into the rapidly stirring acetone at a rate of 1 ml/min. After the precipitation, the solid residue was collected rapidly on a Buchner funnel with Whatman No. 4 filter paper, sucked dry and finally dried in vacuo. The dry powder was either stored dessicated at -20°C or redissolved in 250 ml of 50 mM NaCl, 50 mM Na citrate, pH 6.0.

RESULTS

When an acetone powder of placenta was first prepared in an effort to solubilize β-glucosidase, it appeared that all enzyme activity was lost. However, addition of the original crude placental homogenate, or of a chloroform-methanol (1:1) extract of that homogenate was found to reactivate the redissolved acetone powder. Therefore, the activating effect of phospholipids was tested; Table I shows the stimulatory capacity of five phospholipids. Phosphatidylserine at 0.1 mM gave maximal stimulation of the β-glucosidase.

The acetone precipitation step was then included in the partial purification scheme detailed above and summarized in Table II; seventy-two fold purification was achieved with a yield of 143% (see discussion). After re-dissolving the acetone powder,

TABLE I

PHOSPHOLIPID STIMULATION OF β-GLUCOSIDASE

ADDITION	SPECIFIC ACTIVITY mU/mg PROTEIN	RELATIVE β-GLUCOSIDASE ACTIVITY
Phosphatidylserine	18	100
Phosphatidylinositol	14	78
Phosphatidic Acid	5.2	29
Phosphatidylethanolamine	0.79	4.4
Phosphatidylcholine	0.47	2.6
None	0.45	2.5

Assays were with 1 mM 4-methylumbelliferyl-β-D-glucopyranoside in 25 mM Na citrate, pH 6.0 with additions listed. The β-glucosidase used had been purified through the acetone precipitation step as outlined in the text and Table II. The phospholipids were all used at a concentration of 0.1 mM.

TABLE II

PARTIAL PURIFICATION OF β-GLUCOSIDASE FROM PLACENTA

STEP	VOLUME (ml)	MILLI UNITS	SPECIFIC ACTIVITY mU/mg PROTEIN	YIELD
I. Taurocholate Homogenate	300	945	0.25	100%
II. Con-A-Sepharose	500	1790	24	190
III. Dissolved Acetone Powder	250	1350	18	143

95% of the β-glucosidase remained in the supernatant following centrifugation at 100,000 x g for 45 minutes; in contrast, only 23% of the β-glucosidase of the crude placental homogenate remained in the supernatant after an identical centrifugation. Furthermore, after acetone precipitation the β-glucosidase can be dialyzed extensively against 50 mM Na citrate, pH 6.0, or chromatographed in this same buffer on Sephadex G-200 without precipitation of the enzyme; this indicates that the solubility achieved is not the result of residual taurocholic acid, but represents rather a true solubilization.

The glucocerebrosidase activity of the solubilized β-glucosidase preparation after acetone precipitation was 6 mU/mg of protein compared to 18 mU/mg of protein of β-glucosidase activity.

DISCUSSION

Development of the present method was dependent on the discovery that the enzyme requires phospholipid for optimal catalytic activity. Con-A Sepharose chromatography was included in the procedure since the 95-fold purification achieved in this step increases the subsequent yield of the acetone precipitation step from 30% to 75%. The lower yield from the cruder preparation was due to an inability to completely dissolve that acetone powder.

The high overall yield (143%, Table II) is a consequence of a shift in the pH optimum for β-glucosidase during purification. In crude homogenates and intact cell systems, the enzyme has maximum activity in the pH 4 to 5 range (7), while partially purified enzyme has a pH 6 optimum (4). The Con-A Sepharose purification step completes the shift of the pH optimum to 6, the pH used for all assays reported in this study. When β-glucosidase is assayed at pH 5, the recovery is approximately 75% for the Con-A Sepharose step and 56% for the overall purification.

In the studies on phospholipid stimulation of β-glucosidase (Table I), only the negatively charged phospholipids, phosphatidylserine, phosphatidylinositol and phosphatidic acid, were found to significantly stimulate enzyme activity. The neutral phospholipids employed, phosphatidylethanolamine and phosphatidylcholine, were inactive. Previously, Ho and Light (10) showed that detergent solubilized glucocerebrosidase required phospholipid to interact optimally with a low molecular weight glycoprotein, stimulating factor. However, our study has indicated that the enzyme itself requires phospholipid for activity.

The solubilization of glucocerebrosidase makes possible its purification for physical and immunological studies as well as for replacement therapy in Gaucher's disease.

REFERENCES

1. Brady, R.O., Kanfer, J.N., and Shapiro, D. (1965) Biochem. Biophys. Res. Commun., 18, 221-225.

2. Ho, M.W., and O'Brien, J.S. (1971) Proc. Natl. Acad. Sci. USA, 68, 2810-2813.

3. Desnick, R.J., Thorpe, S.R., and Fiddler, M.B. (1976) Physiol. Rev., 56, 57-99.

4. Pentchev, P.G., Brady, R.O., Hibbert, S.R., Gal, A.E., and Shapiro, D. (1973), J. Biol. Chem., 248, 5256-5261.

5. Hultberg, B., and Ockerman, P.A. (1970) Clin. Chim. Acta, 28, 169-174.

6. Patrick, A.D. (1965) Biochem. J., 97, 17c-18c.

7. Beutler, E., and Kuhl, W. (1970) Lancet 1, 612-613.

8. Beutler, E., and Kuhl, W. (1970) J. Lab. Clin. Med., 76, 747-755.

9. Rosenberg, A., and Chargaff, E. (1958) J. Biol. Chem., 233, 1323-1326.

10. Ho, M.W., and Light, N.D. (1973) Biochem. J. 136, 821-823.