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***Articles***

# Studies on Glucocerebrosidase from Bovine Brain I: Inhibition by Dextran sulfate

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**Glucocerebrosidase (EC 3.2.1.45) is a lysosomal β-glucosidase that catalyses the hydrolysis of glucocerebroside to glucose and ceramide. A deficiency of glucocerebrosidase is the cause of Gaucher's disease. Glucocerebrosidase was partially purified from bovine brain with cholate extraction followed by ammonium sulfate fractionation, acid precipitation at pH 5.35 and butanol extraction. Molecular and kinetic properties of this enzyme were characterized with 4-methylumbeliferyl-β-D-glucopyranoside as substrate. The enzyme activity was increased by addition of 0.05%—0.2% Triton X-100 and sodium taurocholate. Dextran sulfate noncompetitively inhibited the reaction. The *K*i value was estimated to be 1.5 mM. Inhibition by Dextran sulfate was independent of the substrate concentration, but was pH dependent. Maximal inhibition was observed at pH 4.1.**

Key words: Glucocerebrosidase; Dextran sulfate; Bovine brain

Enzyme activities have been studied in various conditions, such as pH, temperature and ionic strength. These are important environmental factors to regulate enzyme activities. The environment of membrane and intracellular organelles is much different from experimental conditions. Analysis of protein function in these intracellular organelles is important to reveal biological function of proteins. We attracted attention to membrane-bound

proteins participating in biological function. We have examined reaction mechanisms of enzymes in the hydrophobic circumstances. We here selected an enzyme acting on sugar chain.

Glucocerebrosidase (glucosyl-ceramide-β-glucosidase, EC 3.2.1.45) is a membrane-bound lysosomal hydrolase that participates in the degradation of glycosphingolipids by cleaving glucosylceramide (glucocerebroside) to yield glucose and ceramide[1)]. The metabolic defect in patients with Gaucher's disease is a deficiency of this enzyme activity, which results in the accumulation of glucocerebroside in tissues of Gaucher's patients.

Glucocerebrosidase has been purified from a variety of mammalian tissues[2,3)]. The enzyme is able to hydrolyze not only its natural lipid substrate, glucocerebroside, but also artificial water-soluble compounds such as 4-methylumbeliferyl-β-D-gluco-pyranoside (MUG). Delipidation and purification, that make the enzyme water-soluble, markedly impair the enzyme activity towards both lipid and water-soluble substrates; the activity can be restored by the addition of detergents or acidic lipids to assay mixture.

Human[4,5)] and mouse[6)] cDNA clones encoding glucocerebrosidase were isolated. The likely precursor protein of human glucocerebrosidase is 536 amino acids long. The N-terminal 39 amino acids constitute a leader sequence that is cleaved from the mature protein.

In this paper, we describe the characterization of glucocerebrosidase from bovine brain

## Materials and methods

**Materials**. Phenylmethylsulphonyl fluoride (PMSF), cholic acid sodium salt, taurocholic acid sodium salt, MUG, *p*-nitrophenyl-β-D-glucopyranoside and cellobiose were obtained from Nacalai Tesque Inc. Conduritol-B-epoxide and ceramide were purchased from Wako Pure Chemical Ind., Ltd. Glucocere-broside and Dextran sulfate (average *M*r 5000) were obtained from Sigma.

**Preparation of bovine brain enzyme.** Bovine brains were obtained from Keiji slaughter house immediately after the sacrifice of the animals and kept frozen at –80˚C. Brain (about 100 g) was homogenized in 3 volumes of 25 mM sodium phosphate buffer (pH 6.0) containing 1 mM 2-mercaptoethanol and 1 mM PMSF. All procedures were performed at 4˚C. The homogenate was centrifuged at 40,000xg for 30 min. The pellet was rehomogenized in 2

volumes of 25 mM sodium phosphate buffer (pH 7.0) containing 1% sodium cholate, 5 mM EDTA and 1 mM 2-mercaptoethanol, and centrifuged at 100,000xg for 1 h.

Glucocerebrosidase was partially purified and delipidated by ammonium sulfate fractionation, acid precipitation at pH 5.35 and butanol extraction as described by Reddy *et al.*[3] with modifications. The supernatant obtained above was adjusted to 30% saturation with ammonium sulfate. The suspension was gently stirred for 30 min and centrifuged at 10,000xg for 1 h. This supernatant was adjusted to pH 5.35 by addition of 1 M citric acid, and the suspension was centrifuged at 10,000xg for 1 h. The acid precipitate was suspended in 50 mM sodium phosphate buffer (pH 5.0) containing 25 mM citric acid, 5 mM EDTA and 1 mM 2-mercaptoethanol. This suspension was brought to a concentration of 20% (v/v) butanol by slow addition of 1-butanol, stirred for 30 min, and centrifuged at 20,000**xg** for 30 min. The lower aqueous layer was removed and dialyzed overnight against 50 mM phosphate/citric acid buffer (pH 6.5) containing 0.02% Triton X-100.

**Enzyme assays**. The standard assay mixture with artificial substrate contained 5 mM MUG in 0.1 M sodium phosphate buffer (pH 5.5), 0.15% Triton X-100 and 0.125% sodium taurocholate. Time courses of the enzyme-catalyzed hydrolysis of MUG were measured at 30˚C. The reaction was initiated by addition of 100 µl of the enzyme solution to 900 µl of the substrate solution. The aliquots (200 µl) were removed at appropriate intervals and mixed with 50 mM NaOH (1 ml) to inactivate the enzyme. The amount of produced 4-methylumbeliferon was determined by fluorescence intensity exited at 340 nm. The fluorescence intensity was measured using Union RS-401 fluorescence spectrophotometer.

**Protein determination**.

Protein concentration was determined by the method of Bradford[7] using bovine serum albumin as standard.

**Molecular weight determination**. The purity of preparations and molecular weight were determined by SDS-10% polyacrylamide gel electrophoresis. Reference proteins used as molecular weight standards were phosphorylase b, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000 and trypsin inhibitor, 21,500.

**Kinetic studies**. Inhibitor constant, *K*i, of Dextran sulfate was determined by standard assay using MUG as substrate in

the presence of various concentrations of inhibitor. The *K*i value was determined by Dixon plots.

## Results

**Purification of enzyme**. Glucocerebrosidase was partially purified from bovine brain with cholate extraction followed by ammonium sulfate fractionation, acid precipitation at pH 5.35 and butanol extraction. Upon SDS-10% polyacrylamide gel electrophoresis, the final preparation of enzyme gave two main bands corresponding to apparent molecular weights of 65,000 and 55,000 (Fig. 1). The final enzyme preparation had the specific activity, determined by using the standard assay, of 80 nmol/min/mg protein. Addition of 21 μM conduritol-B-epoxide, a specific active site inhibitor of glucocerebrosidase[8], to the assay mixture resulted in 90% inhibition of the β-glucosidase activity. The enzyme hydrolyzed a natural substrate, glucocerebroside, but was inactive for cellobiose and *p*-nitrophenyl-β-D-glucopyranoside.

These results indicated that the activity measured in this study was due to glucosylceramide-β-glucosidase, and not to some other β-glucosidases. The final enzyme preparation was used for the subsequent characterization.

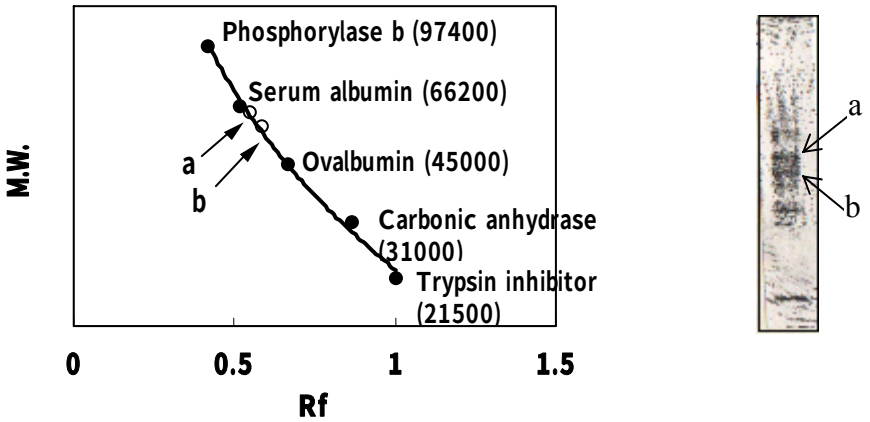


**Fig. 1. SDS-gel electrophoresis of glucocerebrosidase preparation after butanol extraction**. The apparent molecular weights of two main protein bands were 65,000 (a) and 55,000 (b).

**Table 1. Glucocerebrosidase activity assayed in presence (A) and absence (B) of detergents (0.15% Triton X-100 and 0.125% sodium taurocholate).**

| | Specific activity | | |
|---|---|---|---|
| | (A) | | (B) |
| (A)/(B) Purification step | +Detergent | –Detergent | |
| | (nmol/min/mg protein) | | |
| Homogenate | 1.8 | 0.91 | 2.0 |
| Cholate extraction | 5.4 | 0.97 | 5.6 |
| 30% ammonium sulfate supernatant | 13.6 | 1.42 | 9.5 |
| Acidic precipitation | 36.9 | 4.14 | 8.9 |
| Butanol extraction | 80.2 | 8.73 | 9.2 |

**Effect of detergent on enzyme activity**. In studies with membrane-bound enzyme, lipid or detergent is usually added for reconstitution of enzyme activity. Table 1 shows β-glucosidase activity assayed in the presence and absence of detergents (0.15% Triton X-100 and 0.125% sodium taurocholate) for enzyme preparation samples obtained after each purification step. The enzyme activity was increased by addition of Triton X-100 and sodium taurocholate. Figure 2 shows the change of glucocerebrosidase activity of the final preparation by addition of Triton X-100 and sodium taurocholate (1:1). High activity was obtained when 0.05%—0.2% detergent was added, but if the concentration in the assay exceeded 0.5%, the enzyme activity was decreased. The enzyme was assayed below in the presence of 0.125% sodium taurocholate, under which the highest enzyme activity was obtained as shown in Fig. 2. The final

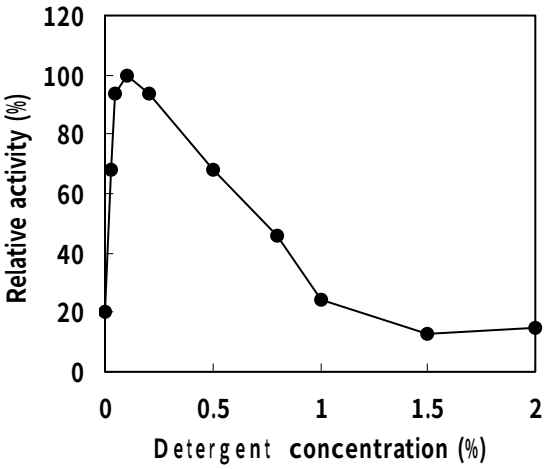


**Fig. 2. Effects of detergent on glucocerebrosidase activity**.
The enzyme activity was measured by standard assay as described under Materials and methods, but with the indicated concentration of detergents (Triton X-100 and sodium taurocholate (1:1)). The maximal activity was taken as 100%.

enzyme preparation had the specific activity of 80 nmol/min/mg protein in the presence of detergents (Table 1).

**Inhibition of enzyme by Dextran sulfate**. Shafit-Zagardo and Turner[9] reported that the enzyme from human placenta was inhibited by sulfated macromolecules such as Dextran sulfate, and that Dextran sulfate was an effective inhibitor of the enzyme assayed with either natural or artificial substrates. Effects of Dextran sulfate on the bovine brain glucocerebrosidase-catalyzed hydrolysis of MUG were examined. Dextran sulfate noncompetitively inhibited the reaction. The *K*i value was estimated to be 1.5 mM. Effects of substrate concentration, [MUG], in the inhibition of enzyme activity by Dextran sulfate were examined. The assay mixture contained 5 mg/ml Dextran sulfate and various concentrations of MUG. As shown in Fig. 3, inhibition by Dextran sulfate was independent of substrate concentration.

Effects of pH on the inhibition by Dextran sulfate were examined in phosphate/citrate buffer. The assay mixture contained 1 mM MUG, and 5 mg/ml Dextran sulfate. As shown in Fig. 4, inhibition by Dextran sulfate was pH dependent. Maximal inhibition was observed at pH 4.1.

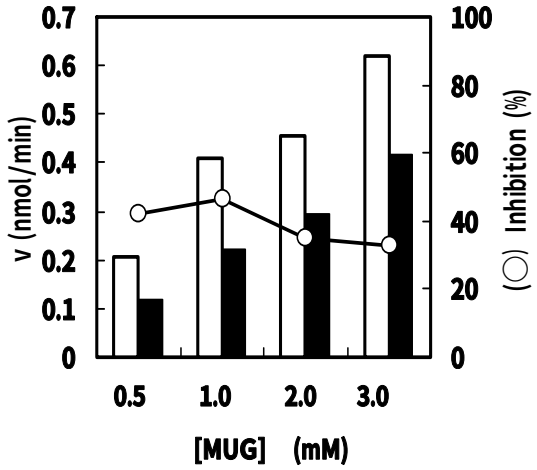


**Fig. 3. Effect of concentrations of substrate, [MUG], on inhibition of glucocerebrosidase-catalyzed hydrolysis of MUG by Dextran sulfate.**
The enzyme was assayed with the indicated concentrations of MUG in the absence (open bars) and presence (closed bars) of 5 mg/ml Dextran sulfate.

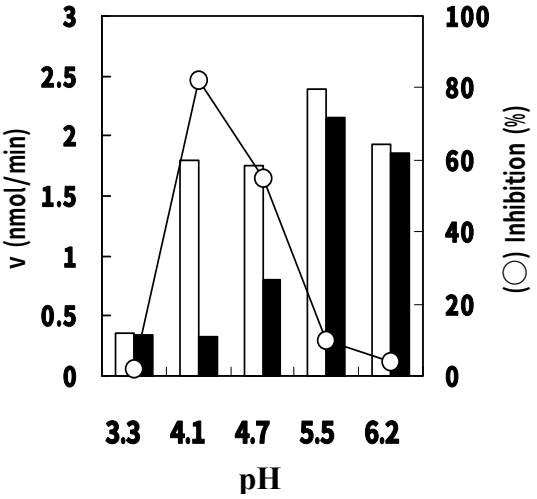


**Fig. 4. Effect of pH on inhibition of glucocerebrosidase-catalyzed hydrolysis of MUG by Dextran sulfate.**
The enzyme was assayed at indicated pH in the absence (open bars) and presence (closed bars) of 5 mg/ml Dextran sulfate.

**Discussion**

Glucocerebrosidase is localized in the lysosome and is a membrane-bound enzyme. Human skin fibroblast glucocerebrosidase requires detergents for its solubilization[10]. Thus, cholate was used for extraction in the initial step of purification of this enzyme. The enzyme activity was increased by addition of Triton X-100 and sodium taurocholate (Table 1 and Fig. 2). The enzyme was assayed here in the presence of 0.15% Triton X-100 and 0.125% sodium taurocholate.

The final preparation of enzyme gave two main bands corresponding to apparent molecular weights of 65,000 and 55,000 upon SDS-polyacrylamide gel electrophoresis (Fig. 1). These two bands may be the bands of glucocerebrosidase, and the observed heterogeneity in apparent molecular weight may be due to the presence of complex carbohydrate chains in the enzyme. From the deduced amino acid sequence, the molecular weight of the mature human glucocerebrosidase is calculated to be 55,384 without glycosylation or carboxyl-terminal processing[4,5]. Placental glucocere-brosidase contains approximately 7% carbohydrate. The enzyme contains high mannose-type oligosaccharides as well as triantennary and biantennary complex- type oligosaccharides[11]. The potential glycosylation sites (Asn-X-Ser/Thr) are identified in the nucleic acid sequence of cDNA. Of these five possible sites for *N*-linked glycosylation, the four sites are glycosylated in the expression experiments of the cDNAs in insect (Sf9) and COS-1 cells[12].

Glucocerebrosidase from human placenta is inhibited by Dextran sulfate[9]. Dextran sulfate also inhibited the bovine brain enzyme noncompetitively. Inhibition was independent of substrate concentration, but was pH dependent, with a maximal inhibition at pH 4.1. The inhibition by sulphated macro-molecule could be attributed to the interaction of this compound with the enzyme or with the activators which are essential for activity of the solubilized enzyme[13]. However, the binding of human β-glucosidase to immobilized Dextran sulphate and its specific elution with sodium taurocholate[9], argues for a direct interaction between the inhibitor and the enzyme itself.

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