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Articles

Studies on Glucocerebrosidase from Bovine Brain
II: Molecular and kinetic characterization of the solubilized and immobilized enzymes

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Glucocerebrosidase (EC 3.2.1.45) is a lysosomal and membrane-bound β-glucosidase. Bovine brain glucocerebrosidase was immobilized on decyl-Sepharose. Immobilization did not change temperature and pH dependence of the enzyme activity. The solubilized enzyme was a little more stable to heat inactivation than the immobilized enzyme. Immobilization did not or little change kinetic parameters, *K*m and Vmax, and the inhibition by conduritol-B-epoxide, but the immobilized enzyme was less susceptible to ceramide inhibition. Thus, bovine brain glucocerebrosidase can be immobilized on decyl-Sepharose with little changing molecular and kinetic properties.

Key words: Glucocerebrosidase; Immobilization; Decyl-Sepharose; Ceramide; Conduritol-B-epoxide; Bovine brain

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 glucosyl-ceramide (glucocerebroside) to yield glucose and ceramide[1]. Glucocere-brosidase has been purified from a variety of mammalian tissues[2,3]. Furbish *et al*. purified human placental glucocere-brosidase using hydrophobic chromato-graphy[2]. In the preceding paper[4], we reported inhibition of bovine brain glucocerebrosidase by Dextran sulfate.

We here reported immobilization of the bovine brain enzyme to acyl-Sepharose, and indicated some of the properties of the solubilized and immobilized enzyme.

Materials and methods

Materials. 4-Methylumbeliferyl-β-D-glucopyranoside (MUG), and cyanogen bromide were obtained from Nacalai Tesque Inc. Sepharose 4B was obtained from Amersham Biosciences Corp.

Preparation of bovine brain enzyme. Glucocerebrosidase was partially purified from bovine brain by ammonium sulfate fractionation, acid precipitation at pH 5.35 and butanol extraction as described previously[4].

Preparation of acyl-Sepharose. All steps were carried out at 4˚C. Sepharose 4B (80 ml) was washed with distilled water and suspended in 200 ml of 2 M Na_2CO_3 (pH 12.1). Cyanogen bromide solution (25 g/500 ml in distilled water) that was made just before using was added to Sepharose gel suspension slowly with stirring. During the addition of cyanogen bromide solution, the pH of the gel suspension was maintained at 11.0-12.0 by addition of NaOH solution. The gel suspension was stirred gently for 30 min. Activated Sepharose 4B was washed with 2 l of cold water and 2 l of cold 0.1 M $NaHCO_3$ (pH 9.5), and then suspended in 0.1 M sodium carbonate buffer (pH 9.5).

For preparation of decyl-Sepharose, 25 ml of *N*-decylamine was mixed with 100 ml of *N*,*N*'-dimethylformamide and 100 ml of 0.1 M $NaHCO_3$ and pH was adjusted to 9.5 by addition of citric acid. Activated Sepharose 4B was added to the decylamine solution and stirred for 20 h. Decylated Sepharose 4B was washed with 1 l of distilled water and suspended in 200 ml of 1 M 2-aminoethanol (pH 9.5). The suspension was stirred for 4 h and washed with 500 ml of distilled water, and then 0.2 M acetic acid, distilled water, 50 mM NaOH, distilled water, 50% (v/v) 1,4-dioxane, 0.2 M acetic acid, and finally washed with 1 l of distilled water, respectively.

Other acyl-Sepharose was prepared by the same method as decyl-Sepharose.

Immobilization of glucocerebrosidase to decyl-Sepharose. Partially-purified glucocerebrosidase was dialyzed against 20 mM sodium phosphate buffer (pH 5.5) containing 5 mM EDTA and 1 mM 2-mercaptoethanol. Ethylene glycol (final 30% (v/v)) was added to the dialysate and the sample was mixed with decyl-Sepharose. The gel suspension was washed with distilled water, and then suspended in the same buffer that was used for dialysis of the enzyme. The gel

suspension was stirred for 4 h and centrifuged at 15,000xg for 15 min. The pellet was washed with sodium phosphate buffer (pH 5.5) containing 5 mM EDTA and 1 mM 2-mercaptoethanol, and suspended in the same buffer.

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[4]. Time courses of the enzyme-catalyzed hydrolysis of MUG were measured at 30˚C. The amount of produced 4-methylumbeliferon was determined by fluorescence intensity exited at 340 nm. The progress curves of the enzyme reaction were practically straight lines over 8 to 16 min.

Protein determination. Protein concentration was determined by the method of Bradford[5] using bovine serum albumin as standard.

Kinetic studies. The apparent *K*m of enzyme for MUG was determined by standard assay with varying substrate concentrations. *K*m values were determined by *s*/*v*~*s* plots. Inhibitor constants, *K*i, were determined by standard assay using MUG as substrate in the presence of various concentrations of inhibitor. *K*i values were determined by Dixon plots.

Results

Glucocerebrosidase is a membrane-bound enzyme. Human placental glucocerebrosidase has been purified using hydrophobic chromatography, and it was shown that the placental enzyme was eluted at 60% ethylene glycol during chromatography on octyl-Sepharose[2]. Reddy *et al*.[3] indicated that bovine brain glucocerebrosidase was more hydrophobic than the human placental enzyme.

Adsorption of bovine brain glucocerebrosidase to various acyl-Sepharose was examined by batch

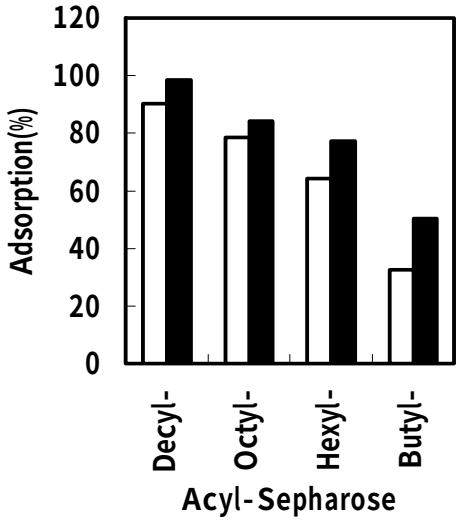

Fig. 1. Adsorption of glucocerebrosidase to acyl-Sepharose.
The enzyme was mixed with the indicated acyl-Sepharose in the absence (open bar) and presence (closed bar) of 30% ethylene glycol, and was allowed to stand for 15 min at 4˚C. After centrifugation, the enzyme activity of the supernatant was assayed.

method in the absence and presence of 30% ethylene glycol (Fig. 1). In the absence of ethylene glycol, 80% or more of the enzyme was adsorbed to decyl- and octyl-Sepharose, and addition of 30% ethylene glycol increased the amount of the adsorbed enzyme. The results suggested that bovine brain enzyme cannot be eluted from octyl-Sepharose column by addition of ethylene glycol.

We here adsorbed the bovine brain enzyme to decyl-Sepharose, and characterized the immobilized enzyme.

Molecular characterization of solubilized and immobilized glucocerebrosidase

The property of immobilized enzyme was compared with the solubilized enzyme, using the enzyme-catalyzed hydrolysis of MUG as the guidance. Immobilized enzyme retained the activity in 1% Triton X-100, 2% sodium cholate, 1% sodium taurocholate, and 1% CHAPS. In 80% ethylene glycol, 10% of enzyme was eluted from the gel. By addition of 100 mM sodium thiocyanate, about 20% of glucocerebrosidase was eluted. These results suggested that decyl-Sepharose-immobilized glucocerebrosidase had enough stability to make comparison with solubilized glucocerebrosidase.

Optimum temperature. The effects of temperature on the activities of the glucocerebrosidase were examined with MUG in 50 mM sodium phosphate buffer, pH 5.5, containing 0.15% Triton X-100, 0.125% sodium taurocholate. The highest activity of both enzyme was

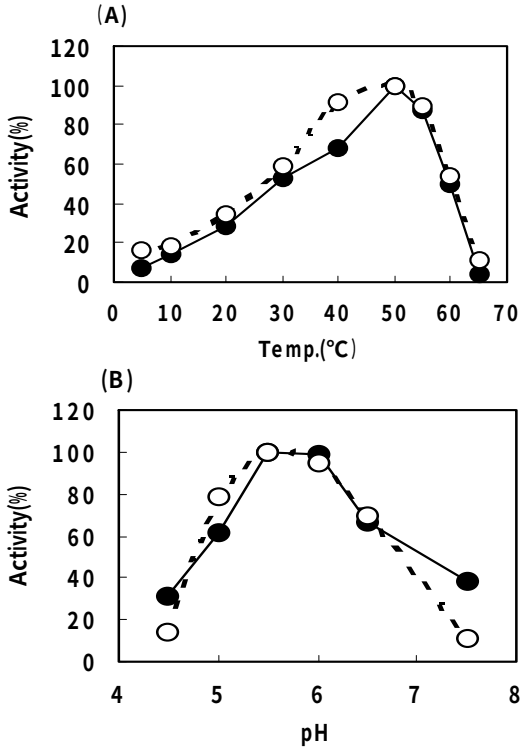

Fig. 2. Temperature (A) and pH (B) dependence of solubilized (●) and immobilized (○) glucocerebrosidase activity.
The enzyme activity was assayed in 50 mM sodium phosphate buffer (pH 5.5) containing 0.15% Triton X-100 and 0.125% sodium taurocholate (A) or in wide-range citrate/phosphate buffer containing 0.15% Triton X-100 at 30˚C. The amount of produced 4-methyl-umbeliferon was determined at 8 to 15 min. The maximal activity was taken as 100%.

obtained at 50˚C (Fig. 2A) under the conditions. At the higher temperatures, the heat inactivation was observed during the enzyme assay as shown below. The temperature dependence of activity was not so much changed between solubilized and immobilized glucocerebrosidase.

Optimum pH. The effects of pH on solubilized and immobilized glucocerebrosidase activity were examined with MUG as the substrate in wide-range buffer, containing 0.15% Triton X-100. The optimum pH of both enzymes were estimated to be pH 5.5 (Fig. 2B). The pH dependence of solubilized and immobilized glucocerebrosidase activity were not different.

Thermal stability. Thermal stability of immobilized glucocerebrosidase was compared with that of solubilized glucocerebrosidase. When enzymes were incubated at 5˚C, solubilized and immobilized glucocerebrosidase kept almost all the activities at least for 150 min. Both enzymes also retained more than 90% activity after incubation at

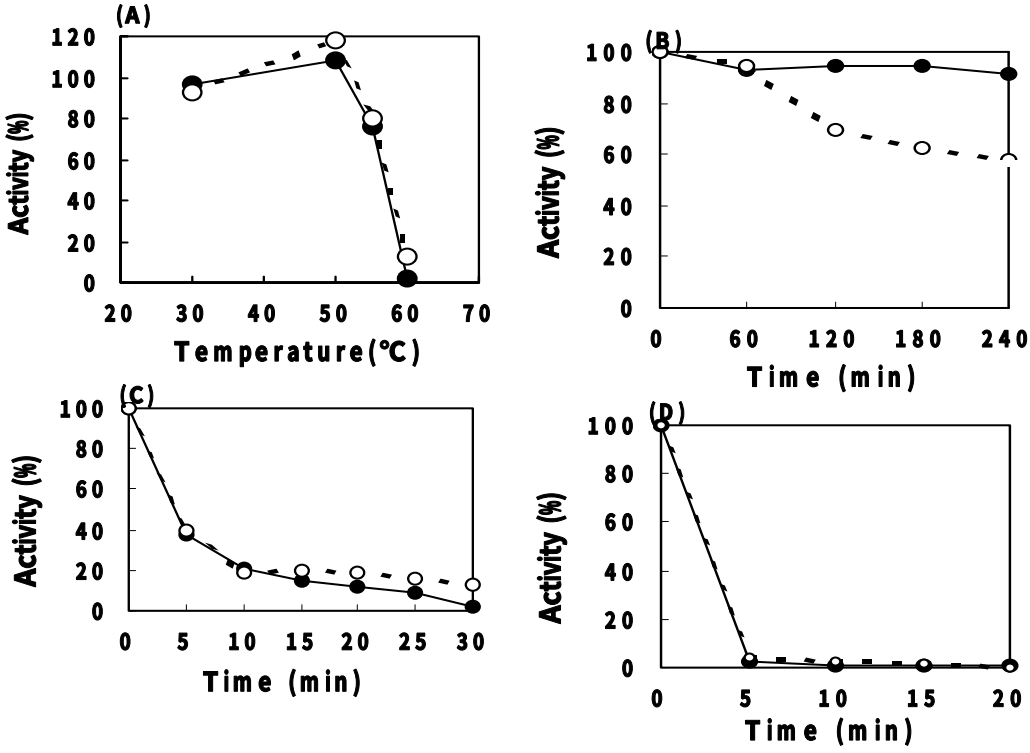

Fig. 3. Thermal stability of solubilized and immobilized glucocerebrosidase.
(A) The enzyme was incubated at various temperature for 30 min in sodium phosphate buffer (pH 5.5) and the enzyme activity was assayed. (B)—(D) The enzyme was incubated at 50˚C (B), 60˚C (C) and 65˚C (D). At various intervals samples were removed and assayed for glucocerebrosidase at 30˚C. The initial activity without incubation was taken as 100%.

30˚C for 30 min (Fig. 3), and more than 70% activity for 120 min. Thermal stability of both enzymes incubated at 50, 60 and 65˚C were examined. The solubilized enzyme kept the activity with incubation at 50˚C at least for 240 min, while the immobilized enzyme remained about 60% activity at 240 min. The results indicated that the solubilized enzyme was a little more stable with incubation at 50˚C than the immobilized enzyme. While both enzymes were relatively stable at 50˚C, the enzymes incubated at 60˚C were lost most activity until 30 min, and at 65˚C the activities were not detected at 5 min.

These results suggested a possibility that the immobilized enzyme was eluted from the gel with incubation at high temperature. Therefore, the extent of the enzyme immobilized at 50˚C was evaluated from the remaining protein: About 71% of the enzymes was yet immobilized on the decyl-Sepharose gel with incubation for 90 min at 50˚C.

Table 1. Kinetic parameters of the solubilized and immobilized glucocerebrosidase-catalyzed hydrolysis of MUG.

	Glucocerebrosidase	
	Solubilized	Immobilized
*K*m (mM)	1.1	1.3
V*max* (μM/min)	0.55	0.54

Kinetic characterization of solubilized and immobilized glucocerebrosidase

Kinetic parameters. The Michaelis constant, *K*m, and maximum velocity, V*max*, were obtained with MUG as a substrate. The plot of the initial velocity, *v*, against $[MUG]_0$ apparently fitted a Michaelis-Menten fashion. The *K*m and V*max* values evaluated with MUG are summarized in Table 1. The immobilized glucocerebrosidase showed almost the same values as solubilized one. The results suggested that immobilization did not affect the activity of glucocerebrosidase-catalyzed hydrolysis of MUG.

Inhibition by ceramide. Effects of

Table 2. Effects of ceramide on the solubilized and immobilized glucocerebrosidase-catalyzed hydrolysis of MUG.

	Solubilized		Immobilized	
	Ceramide (3 μM)			
	+	−	+	−
Km^{app} (mM)	4.7	1.0	4.7	0.86
V^{app} (μmol/min)	6.6	3.8	6.0	4.0
*K*i for ceramide (mM)	0.055		0.22	

Table 3. Effects of conduritol-B-epoxide on the solubilized and immobilized glucocerebrosidase-catalyzed hydrolysis of MUG.

	Solubilized		Immobilized	
	Conduritol-B-epoxide (3 μM)			
	+	−	+	−
Km^{app} (mM)	5.3	0.98	4.8	1.0
V^{app} (μmol/min)	6.6	3.3	6.0	3.0

ceramide on the solubilized and immobilized glucocerebrosidase-catalyzed hydrolysis of MUG were examined. To obtain inhibitor constant, *K*i, Dixon plots of this reaction were figured, and indicated that ceramide acted as a mixed-type inhibitor of both solubilized and immobilized glucocerebrosidase. Inhibitor constant, *Ki*, values were estimated to be about 0.055 mM for the solubilized glucocerebrosidase and 0.22 mM for the immobilized glucocerebrosidase (Table. 2). Thus, the immobilized enzyme was less susceptible to ceramide inhibition

Inhibition by conduritol-B-epoxide. Conduritol-B-epoxide (CBE) is a specific active-site inhibitor of glucocerebrosidase. Effects of CBE on the glucocerebrosidase-catalyzed hydrolysis of MUG were examined (Table. 3). Immobilization of the enzyme little affected the inhibition of CBE.

Discussion

In the preceding paper[4], we partially purified glucocerebrosidase from bovine brain. The properties of bovine brain glucocerebrosidase, reported here for the "solubilized" enzyme, were similar to those reported for bovine brain glucocerebrosidase[3] and human placental[2] and brain[6] glucocerebrosidase, but pH optimum for these enzymes, reported to be around 6.2, was a little higher than that for the bovine brain enzyme characterized here (Fig. 2B). This difference (6.2 vs 5.5) may be due to the difference of substrate used for the enzyme assay; the natural substrate was used for the reported enzymes, while the artificial compound, MUG, was used here.

Bovine brain glucocerebrosidase was adsorbed to decyl-Sepharose, and the immobilization was practically stable. The molecular and kinetic properties of the solubilized and immobilized glucocerebrosidase were similar, such as temperature and pH dependence of

activity, thermal stability, kinetic parameters, and inhibition by conduritol-B-epoxide.

Conduritol-B-epoxide binds covalently to the active site of glucocerebrosidase, resulting in irreversible inhibition[7]. Since no equilibrium exists to permit dissociation of the enzyme-inhibition complex, any *K*i value calculated will be meaningless, but Table 3 indicated that conduritol-B-epoxide similarly inhibited the solubilized and immobilized glucocerebrosidase.

Thus, bovine brain glucocerebrosidase can be immobilized on decyl-Sepharose with little changing molecular and kinetic properties.

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