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MICROASSAY FOR GM_1 GANGLIOSIDE β-GALACTOSIDASE ACTIVITY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and sensitive assay for GM_1 ganglioside (GM_1) β-galactosidase activity was devised by direct measurement of released D-galactose using high-performance liquid chromatography (HPLC). GM_1 β-galactosidase activity in crude samples such as brain homogenates could be measured by this method. After incubation of brain homogenate for 1 h with GM_1 at 37°C and pH 4.4 in the presence of sodium taurodeoxycholate, the reaction was terminated by heating at 100°C for 2 min and the supernatant from the centrifuged sample was analysed directly by HPLC. D-Galactose isolated by HPLC was converted into a fluorescent compound by a post-column reaction with arginine at 150°C and the fluorescence intensity at 430 nm was measured with excitation at 320 nm. By this method 10 pmol of D-galactose could be measured and the fluorescence intensity was linear up to 1 mmol of D-galactose. Using this method, the optimal conditions for the activity of this enzyme were re-examined. As an application, the enzyme activity in the brain of a patient with GM_1 gangliosidosis was examined. This method can be applied to any natural substrates, glycolipids or glycoproteins, the terminal galactose of which is hydrolysed by this enzyme.

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INTRODUCTION

Acid β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is known to have a broad substrate specificity; it cleaves the terminal D-β-galactose from glycolipids and glycoproteins [1,2]. Deficiency of acid β-galactosidase elicits a hereditary disease, GM_1 gangliosidosis, in humans. Recently we found that in cultured skin fibroblasts and lymphocytes prepared from patients with the adult form of GM_1 gangliosidosis the enzyme not only reduces the activity but also changes the substrate specificity; the mutant enzyme reduces the activity toward GM_1 ganglioside (GM_1), but maintains the activity toward glycoproteins [3]. On the other hand, in another primary β-galactosidase deficiency, Morquio B syndrome, the mutant enzyme reduced the activity to glycoproteins [3]. For the study of the mutant enzyme, a sensitive and simple method for determining the enzyme activity toward natural substrates, glycolipids and glycoproteins, is required, and usually β-galactosidase activity is measured by use of synthetic substrates such as O-nitrophenyl β-D-galactoside or 4-methylumbelliferyl β-D-galactoside (4MU β-gal). For assay using natural substrates, previous methods required the preparation of the radioactive glycolipids or glycoproteins [4]. In order to measure the activity using non-radioactive substrates, we previously devised an assay procedure for GM_1 β-galactosidase activity toward unlabelled natural substrates [5,6]. This method is based on the measurement of NADH generated from NAD by β-galactose dehydrogenase and D-galactose released from GM_1 or asialofetuin. However, this method was not applicable to human brain homogenates because of interfering compounds. Also, the detergents used for the assay inhibited the analysis of NADH by high-performance liquid chromatography (HPLC).

This paper reports a simple procedure for measuring the enzyme activity of β-galactosidase toward glycolipids and glycoproteins in general. The activity in small amounts of crude sample such as human brain homogenates can be assayed by this method.

EXPERIMENTAL

Chemicals

Sodium taurocholate and taurodeoxycholate were purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.), Triton X-100 and *n*-octyl and *n*-heptyl β-D-glucopyranoside from Sigma (St. Louis, MO, U.S.A.), Tween 20, sodium cholate and deoxycholate from Nakarai Chemicals (Kyoto, Japan) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) from Dojindo Labs. (Kumamoto, Japan). GM_1 was purified from human brain [7]. Boric acid was of the grade for amino acid analysis and other chemicals were of analytical-reagent grade.

Enzyme samples

Human brains were obtained at autopsy within 8 h of death from control patients without neurological diseases, and stored at −80°C until use. Grey and

white matter were isolated from cortex of the frontal lobe and washed with chilled saline and homogenized with 10 volumes of 10 m*M* potassium phosphate buffer (pH 7.4) containing 100 m*M* sodium chloride and 0.02% sodium azide. The sample was washed with 10 volumes of the phosphate buffer by centrifugation at 1000 *g* for 60 min with a Centricut Type 20 centrifuge tube (Biofield, Tokyo, Japan), which excluded molecules of relative molecular mass below 20 000.

The protein concentration was measured according to Bradford [8] using bovine γ-globulin as a standard.

Enzyme assay

GM_1 β-galactosidase activity was measured as follows. The enzyme sample (50 μg to 1 mg of protein) was incubated with 1 m*M* GM_1 in 200 μl of 50 m*M* citric acid–100 m*M* sodium phosphate buffer (pH 4.4) containing 100 m*M* sodium chloride and 0.5% sodium taurodeoxycholate. The mixture was incubated at 37°C for 1 h and the reaction was terminated by heating at 100°C for 2 min; then the mixture was cooled in an ice-bath and 200 μl of 0.5 *M* boric acid–sodium hydroxide buffer (pH 8.7), the mobile phase for HPLC, were added; then the mixture was centrifuged at 800 *g* for 10 min. The supernatant was filtered through a Millipore HV filter and an aliquot was analysed by HPLC.

The enzyme activity of β-galactosidase toward a synthetic substrate, 4-MU β-gal, was carried out as described previously [9].

Apparatus

An LC-3A HPLC apparatus (Shimadzu, Kyoto, Japan) was connected to a Shimadzu RF-500 spectrofluorophotometer. A Shimadzu ISA-07/S2504 prepacked column (250 mm × 4.0 mm I.D.) was used. The column eluate was reacted with arginine solution in a Shimadzu CRB-3A chemical reaction box, which contained a 10-m reaction coil.

Chromatographic parameters

For the HPLC analysis of D-galactose the column was eluted with 0.5 *M* boric acid–sodium hydroxide buffer (pH 8.7) at a flow-rate of 0.6 ml/min at 65°C. According to Mikami and Ishida [10], sugars in the eluate were converted into fluorescent derivatives. The eluate was mixed with 2% L-arginine and 3% boric acid solution, the flow-rate of which was 0.5 ml/min, and the mixture was heated at 150°C. After cooling in a 5-m tube, the fluorescence intensity at 430 nm was measured with excitation at 320 nm. D-Galactose in the sample was determined by comparison of the peak area with that of a standard, using a Shimadzu C-CIA Chromatopac.

RESULTS AND DISCUSSION

Chromatography

As shown in Fig. 1, the fluorescent peaks of the derived D-galactose of the standard and those in the reaction mixture with grey and white matter homogenate were clearly separated from other peaks. The sensitivity with respect to D-

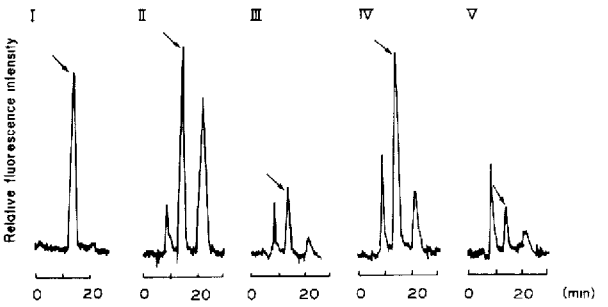

Fig. 1. HPLC patterns of standard D-galactose and the reaction mixture with grey and white matter homogenates with GM_1. I, D-Galactose (1 nmol); II and IV, reaction mixture of 1 m*M* GM_1 with homogenates of grey (250 μg protein) and white matter (340 μg protein); III and V, grey and white matter homogenates incubated without addition of GM_1.

galactose after conversion into a fluorescent derivative by post-column labelling was 10 pmol, the limit of sensitivity being defined as a signal-to-noise ratio of 5. Linearity of the fluorescence intensity up to 1 mmol of D-β-galactose was confirmed. The HPLC pattern of the enzyme samples incubated with GM_1 (Fig. 1, II and IV) showed a peak corresponding to D-galactose in addition to two unidentified peaks. As shown in Fig. 1 (III and V), grey and white matter homogenates incubated without addition of GM_1, as a blank, contained or released D-galactose from endogenous substrates.

TABLE I

EFFECTS OF DETERGENTS ON GM_1 GANGLIOSIDE β-GALACTOSIDASE IN HUMAN BRAIN

Grey matter homogenate from human brain cortex (frontal lobe) was used as a source of β-galactosidase. The final concentration of the detergent was 1%. The values given are means $\pm$S.D. of triplicate measurements in two experiments.

Detergent	Enzyme activity (nmol/h mg protein)
Sodium taurocholate	11.0 ± 1.0
Sodium taurodeoxycholate	20.7 ± 0.8
CHAPS	11.2 ± 2.0
CHAPSO	11.6 ± 1.5
Sodium cholate	1.6 ± 0.3
Sodium deoxycholate	2.5 ± 0.2
Triton X-100	Not detectable
Tween 20	Not detectable
n-Octyl β-D-glucoside	Not detectable
n-Heptyl β-D-glucoside	Not detectable
Control	Not detectable

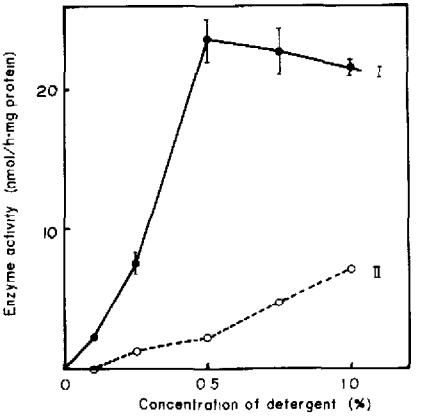

Fig. 2. Effect of the concentrations of sodium taurodeoxycholate and CHAPS on the activity of GM_1 β-galactosidase. Grey matter homogenate (200 μg protein) was incubated with 1 mM GM_1 in the presence of various concentrations of the detergents. The amount of D-galactose produced was assayed as described in the text. I, Sodium taurodeoxycholate; II, CHAPS.

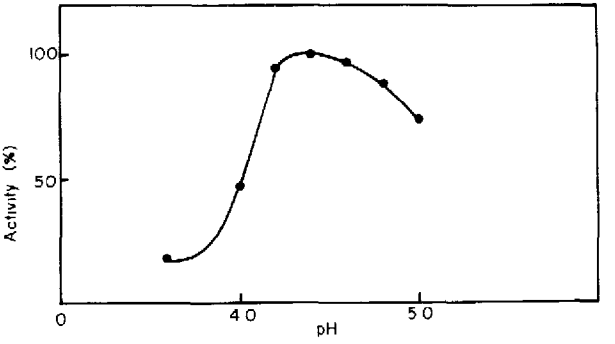

Fig. 3. pH-activity profile of GM_1 β-galactosidase. Grey matter homogenate (150 μg protein) was incubated with 1 mM GM_1 in the presence of 0.5% sodium taurodeoxycholate. The buffer used was 50 mM citric acid-100 mM sodium phosphate buffer (pH from 3.6 to 5.0).

Assay conditions

The optimal reaction conditions for GM_1 β-galactosidase activity were re-examined. As summarized in Table I, the hydrolysis of GM_1 by brain homogenates required a detergent, and sodium taurodeoxycholate was the best of those tested for activation of the enzyme activity. The optimal concentration of sodium taurodeoxycholate was approximately 0.5%, as shown in Fig. 2, and the optimal pH of the hydrolysis of GM_1 ganglioside was 4.4, as shown in Fig. 3. The reaction was linear with reaction time up to 1 h, as shown in Fig. 4, and the linearity was confirmed with regard to the protein amounts of grey and white matter homogenates, as shown in Fig. 5. The kinetic analysis of the hydrolysis of GM_1 by the enzyme sample could be carried out by this method, as shown in Fig. 6. The Michaelis constant (K_M) value and the maximal velocity of the enzyme (V_{max}) in grey and white matter homogenates were 2.08 ± 0.58 mM and 31.7 ± 2.1 nmol/h mg protein and 1.99 ± 0.46 mM and 17.1 ± 1.1 nmol/h mg protein, respectively.

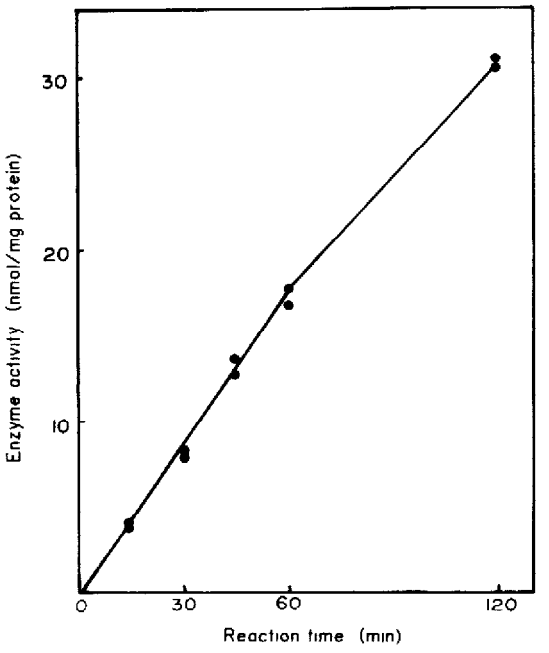

Fig. 4. Effect of reaction time on the amount of D-galactose released by β-galactosidase. Grey matter homogenate (200 μg protein) was incubated with 1 mM GM_1 in the presence of 0.5% sodium taurodeoxycholate at pH 4.4 and 37°C. After various incubation times the reaction was terminated by heating at 100°C and the amounts of D-galactose produced were assayed as described in the text.

The K_M value and the V_{max} of this enzyme in grey matter homogenates toward 4-MU β-gal were 369.7 ± 53.8 μM and 2.19 ± 0.19 nmol/min mg protein, respectively, indicating that the enzyme had a higher activity toward the artificial substrate than toward the naturally occurring substrates under the reaction conditions used.

Application

The method was applied to homogenates of the brain from a patient with the adult form of GM_1 gangliosidosis. The GM_1 β-galactosidase activity in the frontal and cerebellar cortex was reduced to 1.5 and 1.1 nmol/h mg protein from 18.0 and 15.2 nmol/h mg protein, respectively, in control brain.

Our previous assay procedure for the quantification of GM_1 β-galactosidase could be applied to samples such as human skin fibroblasts and lymphocytes [3], but it could not be used for the assay of the enzyme activity in human brain homogenates; for the assay of β-galactosidase in human brain, a high fluorescent blank inhibits the HPLC determination of NADH generated from NAD with galactose dehydrogenase and β-galactose. The separation of sugars by HPLC and post-column labelling to give fluorescent compounds can be applied to the quantitation of almost any hexoses and oligosaccharides, indicating that this method may be generally applicable to other hydrolases that cleave terminal sugars from glycolipids or glycoproteins. The method is simple and sensitive enough to be applied to the kinetic analysis of the enzyme reaction toward natural substrates,

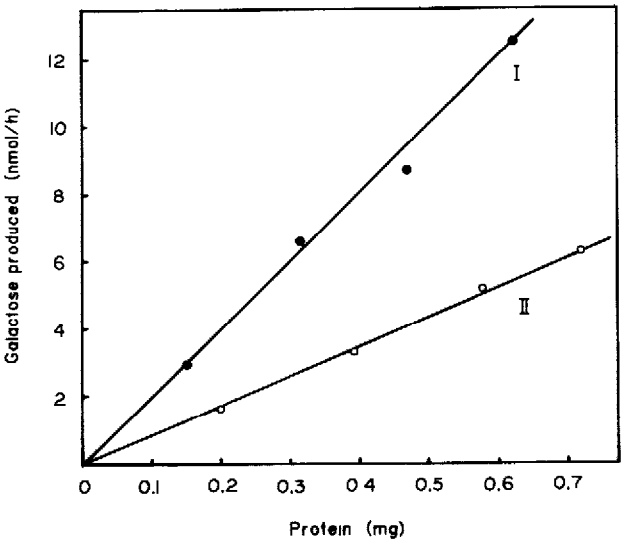

Fig. 5. Effect of amount of protein in grey and white matter on the velocity of GM_1 hydrolysis. Various amounts of grey and white matter homogenate were incubated with 1 m*M* GM_1 for 1 h and the amount of galactose released was measured as described in the text. I, Grey matter; II, white matter.

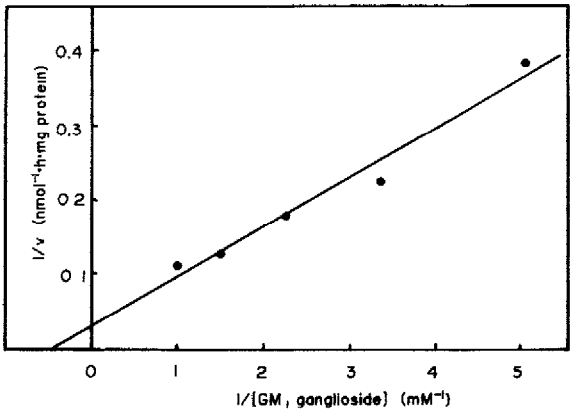

Fig. 6. Lineweaver–Burk plot of the hydrolysis of GM_1 β-galactosidase. Grey matter homogenate (150 μg protein) was incubated with various concentrations of GM_1 at 37°C and pH 4.4 for 1 h in the presence of 0.5% sodium taurodeoxycholate. The reciprocal of the velocity of D-galactose released from GM_1 was plotted against that of the concentration of GM_1 according to Lineweaver and Burk.

glycolipids and glycoproteins, the terminal β-D-galactose of which can be cleaved by this enzyme, and may be useful for the study of the enzyme properties of normal and mutant enzymes.

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