α -D-Mannosidase in the Liver and Brain from Patients with I-Cell Disease

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MINAMI, R. and NAKAO, T. *α -D-Mannosidase in the Liver and Brain from Patients with I-Cell Disease.* Tohoku J. exp. Med., 1982, **136** (1), 43-51 — The properties of α -D-mannosidase in the liver and brain from patients with I-cell disease were investigated, using 4-methylumbelliferyl- α -D-mannopyranoside as a substrate. A combination of chromatographies on DEAE-cellulose, concanavalin A-Sepharose and Sephacryl S-300 showed that intermediate α -D-mannosidase was present in the liver and brain from I-cell disease as well as control liver and brain, although an abnormal distribution of intermediate and acidic α -D-mannosidases occurred in the tissues from I-cell disease. These results suggested that a defect of intermediate α -D-mannosidase is unlikely to be the primary defect responsible for I-cell disease. ——— intermediate α -D-mannosidase; I-cell disease

Mucopolipidosis II (I-cell disease) and III are characterized by a deficiency of many lysosomal hydrolases in cultured fibroblasts and in leukocytes with a concomitant increase in the activities of the same enzymes in culture medium and in plasma. The molecular defect responsible for these diseases is still unknown.

Three types of α -D-mannosidase have been described in mammalian tissues (Phillips et al. 1974; Hultberg et al. 1976; Hirani and Winchester 1979). Acidic α -D-mannosidase with a pH optimum of 4.0-4.5 is a typical lysosomal hydrolase and is divided into forms A and B. Neutral α -D-mannosidase with a pH optimum of 6.0-6.5 is located in the cytosol. Intermediate α -D-mannosidase with a pH optimum of 5.5-5.75, which was first isolated from Golgi membranes of rat liver as Golgi α -D-mannosidase (Dewald and Touster 1973), is present in human plasma and serum (Phillips et al. 1974; Hultberg et al. 1976; Hirani and Winchester 1979). These three forms of α -D-mannosidase are immunologically distinct in rat liver (Opheim and Touster 1978). Moreover, four forms of intermediate α -D-mannosidase components, I₁-I₄, which differ in their molecular weight and affinity for concanavalin (Con) A-Sepharose, have been described in human plasma (Hirani and Winchester 1979).

Patients with mannosidosis have a deficiency of acidic α -D-mannosidase, but neutral and intermediate α -D-mannosidases are unaffected (Masson et al. 1974; Winchester et al. 1976). On the other hand, an absence or an alteration in serum

or urine intermediate α -D-mannosidase has been reported in mucopolipoidosis II and III (Kress and Miller, 1978, 1979).

In this paper, the properties of α -D-mannosidase in the livers and brains from patients with I-cell disease were investigated by a combination of chromatographies on DEAE-cellulose, Con A-Sepharose and Sephacryl S-300.

MATERIALS AND METHODS

All procedures were carried out at 0–4°C unless otherwise stated. Liver and brain specimens were obtained from control individuals and two patients with I-cell disease.

Tissues used as control were obtained postmortem from patients of similar ages with no apparent neurological disease. All the specimens were immediately frozen after autopsy and stored at –70°C for 1–3 years until analysis. Frozen pieces of liver and brain were homogenized in 3–10 volumes of ice-deionized water. The homogenate was sonicated for 1 min and centrifuged at 100,000 $\times$ g.

Assay of α -D-mannosidase activity. α -D-Mannosidase activity in tissue supernatants or in fractions obtained by separation procedures was assayed by using fluorometric techniques with 4-methylumbelliferyl (4MU)- α -D-mannopyranoside (Koch-Light). To examine pH optimum, reaction mixtures consisted of 0.1 ml of 0.1 M citrate phosphate buffer, 0.1 ml of enzyme source and 0.4 ml of 1 mM 4MU- α -D-mannopyranoside. The reaction was stopped by the addition of 3 ml of 0.25 M glycine buffer, pH 10.7. Fluorescence was read immediately with an excitation wavelength 365 nm and an emission wavelength 450 nm. Thermostability of pH-dependent α -D-mannosidase activity was investigated by incubation at 50°C for 60 min.

Ion-exchange chromatography on DEAE-cellulose. DEAE-cellulose (Whatman DE-52) is equilibrated in 10 mM sodium phosphate buffer, pH 6.0, and packed in a column (1 $\times$ 10 cm). After tissue supernatants were applied on the column, elution was started with the equilibration buffer and was continued with a linear NaCl gradient (0–0.3 M) in 100 ml of the same buffer. Fractions of 2.0 ml were collected at a flow rate of 60 ml/hr, and assayed for α -D-mannosidase activities at pH 4.0 and 6.0.

Affinity chromatography on Con A-Sepharose. Tissue supernatants and fractions obtained by chromatography on DEAE-cellulose were applied to a column (1 $\times$ 5 cm) of Con A-Sepharose, which had been equilibrated with 15 ml of 0.1 M acetate buffer containing 1 M NaCl, 0.01 M MnCl₂, 0.01 M MgCl₂ and 0.01 M CaCl₂, pH 6.0. The column was eluted with the equilibration buffer at room temperature. Fractions of 3.0 ml were collected at a flow rate of 14 ml/hr. The material that had bound to Con A-Sepharose was eluted with 30 ml of 0.5 M methyl- α -D-mannoside in the equilibration buffer. α -D-Mannosidase activities from the fractions were assayed at pH 4.0 and 6.0.

Sephacryl S-300 filtration. Gel filtration was performed with a column (2.5 $\times$ 85 cm) of Sephacryl S-300 superfine (Pharmacia) equilibrated with 10 mM sodium phosphate buffer containing 10 mM NaCl, pH 7.0. Fractions of 3.0 ml were collected at a flow rate of 15 ml/hr, and assayed for α -D-mannosidase activities at pH 4.0 and 6.0.

Concentration of fractions. A Minicon Macrosolute Concentrator (Amicon) was used to concentrate some separated fractions.

RESULTS

pH Dependence of α -D-mannosidase

The pH profile curves of α -D-mannosidase in the control liver exhibited two types of activity (Fig. 1a). One had a pH optimum of 4.0 and was unaffected by heating at 50°C for 60 min. The other had a pH optimum of 6.0 and was denatured by heating. The latter showed greater activity than the former. In

contrast, α -D-mannosidase in the livers from I-cell disease revealed a single active site with a pH optimum of 4.0 and showing the pH profile almost unchanged by heating. pH profile curve of the activity in the control brain revealed a single active site with a pH optimum 4.0 and the profile was almost unchanged by heating (Fig 1b). In contrast, the activity in the brains from I-cell disease exhibited pH profile curves similar to that in the control. α -D-Mannosidase activities at pH 4.0 in all control and I-cell tissues had similar K_m values, and total α -D-mannosidase activities at pH 4.0 of the livers and brains from I-cell disease revealed about 2 times increase, compared with control values.

Ion-exchange chromatography on DEAE-cellulose

When an extract of control liver was chromatographed on DEAE-cellulose, two peaks of α -D-mannosidase at pH 4.0 (peaks A and B), and two peaks at pH 6.0 (peaks I and C) were resolved (Fig. 2). Four major peaks of the activity were also obtained in the livers from I-cell disease. However, the activity in peak B was significantly increased when compared with that in the control liver. The profiles of chromatography on DEAE-cellulose in the normal and I-cell brains were similar to that in the control liver (Fig. 3).

Chromatography on Con A-Sepharose

α -D-Mannosidase in the control liver was recovered in both unbound and bound fractions (Fig. 4). To check whether the column had been overloaded, these

Fig. 1. pH-dependence of α -D-mannosidase activity in control subject and I-cell disease. a: α -D-Mannosidase activity in liver. b: α -D-Mannosidase activity in brain. ●, unheated; ○, heated.

Fig. 2. Chromatography of liver α -D-mannosidase activity on DEAE-cellulose. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (○) and pH 6.0 (●). ----, NaCl gradient.

two fractions were rechromatographed on the column, and the activity originally in the unbound fractions and the activity in the bound fractions were obtained in the respective fractions. On the other hand, most of α -D-mannosidase activity in the livers from I-cell disease appeared to bind to the column. The activity in the unbound fractions from the control liver showed virtually no activity at pH 4.0

Fig. 3. Chromatography of brain α -D-mannosidase activity on DEAE-cellulose. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (●) and pH 6.0 (○). ---, NaCl gradient.

Fig. 4. Chromatography on concanavalin A-Sepharose of liver α -D-mannosidase activity. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (●) and pH 6.0 (○). ▲, α -D-mannosidase activity at pH 6.0 after heating at 50°C for 60 min.

and the activity at pH 6.0 was decreased about 60% after heat treatment. In contrast, α -D-mannosidase in the brains from I-cell disease was recovered in both the fractions, but α -D-mannosidase activity in the bound fractions was significantly lower than that in the unbound fractions as compared with the situations in the control liver (Fig. 5). About 65% of the activity at pH 6.0 in the unbound fractions was heat-stable in both the control and I-cell brains. These observations were consistent with the results of the pH activity profile curves and, moreover, showed that the α -D-mannosidase which did not bind to Con A-Sepharose consisted of heat-stable and heat-labile α -D-mannosidases. The heat-labile α -D-mannosidase was the same as neutral α -D-mannosidase, but the heat-stable α -D-mannosidase was clearly different from neutral α -D-mannosidase (Phillips et al. 1974; Hultberg et al. 1976). Then the unabsorbed fractions separated by chromatography on DEAE-cellulose were analysed by chromatography on Con A-Sepharose, and it was found that α -D-mannosidase active at pH 6.0, which did not bind to Con A-Sepharose, was obtainable from both the control and I-cell tissues (Fig. 6) and that, moreover, the activity was activated by Co^{2+} . α -D-Mannosidase unabsorbed to DEAE-cellulose and unbound to Con A-Sepharose was clearly different from acidic and neutral α -D-mannosidase (Phillips et al. 1974; Hultberg et al. 1976), and appeared to be the same to intermediate α -D-mannosidase component I_2 reported by Hirani and Winchester (1979), although they did not described the thermostability.

Fig. 5. Chromatography on concanavalin A-Sepharose of brain α -D-mannosidase activity. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (●) and pH 6.0 (○). ▲, α -D-Mannosidase activity at pH 6.0 after heating at 50°C for 60 min.

Fig. 6. Chromatography on concanavalin A-Sepharose of the peaks of the liver and brain α -D-mannosidases unabsorbed to DEAE-cellulose. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (●) and pH 6.0 (○).

Fig. 7. Gel filtration on Sephacryl S-300 of the liver and brain α -D-mannosidases. The plotted relative activities were the fluorescence values. α -D-Mannosidase activity was measured at pH 4.0 (●) and pH 6.0 (○).

Gel filtration patterns on Sephacryl S-300

In the control liver α -D-mannosidase activity at pH 4.0 was recovered as a single peak. On the other hand, the activity at pH 6.0 was resolved into two

peaks (Fig. 7) and the latter was thought to be α -D-mannosidase component I_2 in view of that α -D-mannosidase I_1 has a lower molecular weight than acidic α -D-mannosidase (Hirani and Winchester 1979). The α -D-mannosidase I_2 was also present in the control and I-cell brains, but its amount was only trace in the liver from I-cell disease. These results were consistent with the pH profile curves and the results of chromatographies on DEAE-cellulose and Con A-Sepharose.

DISCUSSION

Hirani and Winchester (1979) reported that intermediate α -D-mannosidase is divided into four forms of I_1 , I_2 , I_3 and I_4 , and that α -D-mannosidase I_1 has properties very similar to neutral α -D-mannosidase and other three forms do not appear to be present in liver except in trace amounts. In contrast, the occurrence of α -D-mannosidase I_2 , which co-elutes with α -D-mannosidase A from DEAE-cellulose column and does not bind to Con A-Sepharose column, was observed in our experiments. Hirani and Winchester (1979) did not mention about thermostability of α -D-mannosidase I_2 , but α -D-mannosidase I_2 was thought to be heat-stable from our data. Moreover, α -D-mannosidase I_2 was clearly different from acidic α -D-mannosidase, non-bound to Con A-Sepharose, because the activity of α -D-mannosidase I_2 was undetectable at pH 4.0. α -D-Mannosidase I_2 was present in both the control and I-cell tissues. The remaining of intermediate forms of α -D-mannosidase I_1 , I_3 and I_4 were not detectable in any of the tissues studied here, because I_1 was very similar to neutral α -D-mannosidase, and I_3 and I_4 were obscured by the high concentration of acidic α -D-mannosidase that binds to Con A-Sepharose. It was, therefore, suggested that a defect of intermediate α -D-mannosidase is not the primary defect in I-cell disease in agreement with other reports (Hirani and Winchester 1980; Halley et al. 1980).

On the other hand, our study showed that α -D-mannosidase B was increased in the livers from I-cell disease and intermediate α -D-mannosidase component I_2 was conversely increased in the brains. Although there are no data to explain these phenomena, these phenomena might result from an abnormality of post-translational modification of normal enzymes, such as a deficiency of phosphorylation or a defect of glycosylation which is suggested to be the molecular defect responsible for I-cell disease (Hasilik and Neufeld 1980).

Acknowledgments

We are grateful to Ms. M. Toyoda for valuable technical help.

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