SUBSTRATE SPECIFICITY OF CERAMIDE TRIHEXOSIDASE

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1. Introduction

Artificial substrates such as 4-methylumbelliferyl glycosides and *p*-nitrophenyl glycosides have been employed commonly to determine the levels of glycosyl hydrolase activities in plasma, urine and tissues [1–7]. They have provided a simple and rapid method for the diagnosis of some lysosomal storage diseases, whereas in other instances inconsistencies have been noted in the results obtained with these artificial substrates and with the natural substrates. Two examples which may be cited are Krabbe's globoid cell leucodystrophy, characterized by a galactocerebroside β-galactosyl hydrolase deficiency, for which *p*-nitrophenyl β-galactoside cannot be used to detect decreased enzymatic activity in the homozygous patients [8]; and the isolation from Tay-Sachs brain of hexosaminidase which has a much higher K_m for the G_{M2} ganglioside substrate than for the *p*-nitrophenyl β-*N*-acetylgalactosaminide used to monitor the enzyme during purification [9].

We have reported preliminary studies of α-galactosidase activity in Fabry plasma following infusion of normal plasma, and suggested that the enzymatic activities measured by the artificial substrates and by α-galactosyl-(1→4)-βgalactosyl-(1→4)-glucosylceramide (ceramide trihexoside) are not due to the same enzyme. The present report provides unequivocal experimental evidence for the existence of unique α-galactosidases for the artificial substrates and for ceramide trihexoside.

2. Experimental

2.1. *Enzyme isolation*

A mixture of α-galactosidases (human plasma) was isolated from 250 g of Cohn fraction IV-1 (Method 6) as previously described [10]. The enzymes obtained in the acetone precipitate were dialyzed against 10^{-3} M MES buffer containing 5% butanol (pH 5.4), concentrated by dialysis against polyethylene glycol 6000, and chromatographed on an affinity column prepared by coupling melibiose to succinylated Sepharose [11].

2.2. *Enzyme assays*

Boiled enzyme controls were used for all assays. The pH optimum for each substrate was determined using crude Cohn fraction. The conditions used for individual substrates are as follows:

a) 4-Methylumbelliferyl α-galactoside (4-MU): The standard reaction mixture contained 100 μl of enzyme solution, 2.2 μmoles of substrate and 0.1 M citrate-phosphate buffer (pH 4.5) to a constant volume of 0.5 ml. After incubation at 37° for 2 hr the reaction was terminated by addition of 2.5 ml of 0.1 M ethylenediamine buffer (pH 11.2). The fluorescence of the liberated methylumbelliferone was measured using a Turner fluorimeter with 365 nm excitation and 450 nm fluorescence filters.

b) *p*-Nitrophenyl α-galactoside (pNPG): Enzyme solution (100 μl) was added to 0.5 ml of 0.1 M citrate-phosphate buffer (pH 3.0) containing 2.0

μmoles of substrate. After 2 hr at 37° the reaction was terminated by addition of 0.1 ml of 0.01 N sodium hydroxide and 0.5 ml of 0.2 M glycine-carbonate buffer (pH 9.5). The absorbance was read at 420 nm using a Gilford 2400 spectrophotometer.

c) Measurements of enzymatic activity employing ceramide trihexoside (CTH) as substrate were performed as previously described [10].

2.3.

Protein was measured by the method of Lowry et al. [12] as modified by Hess and Lewin [13].

3. Results and discussion

The purification of galactosidase(s) from Cohn fraction IV-1 was monitored with CTH, 4-MU and pNPG. The specific activities in the crude Cohn fraction and the dialyzed acetone precipitate, as determined with the various substrates, are shown in table 1.

During the purification, two discrepancies indicated that the glycolipid and artificial substrates were not measuring the same enzymatic activity. There was partial loss of the α-galactosidase activity detected with the artificial substrates whereas there was no decrease in the enzymatic activity measured with ceramide trihexoside. Furthermore, the degree of purification measured with artificial substrates could not be correlated with that obtained using ceramide trihexoside, even after taking into account this loss of activity.

Table 1
α-Galactosidase activity in Cohn fraction IV-1.

Substrate	Specific activity (nmoles/mg protein/hr)	
	Cohn frac. IV-1	Dialyzed acetone ppt.
CTH	0.07	138
4-MU	0.12	92
pNPG	0.10	76

Fig. 1 shows the elution profile of the dialyzed acetone precipitate from the affinity column. Two fractions of ceramide trihexosidase were obtained [11], neither of which had detectable activity toward the artificial substrates. In addition, a single fraction was obtained that hydrolyzed 4-MU and pNPG, but had no detectable activity toward CTH. Thus affinity chromatography resulted in complete separation of the ceramide trihexosidase-cleaving α-galactosidases from the enzyme hydrolyzing the artificial substrates.

Although artificial substrates may have a fortuitous benefit as diagnostic tools in various heritable lysosomal storage diseases, presumably due to the genetic alteration of more than one hydrolase, they should not be used routinely for the purification of specific

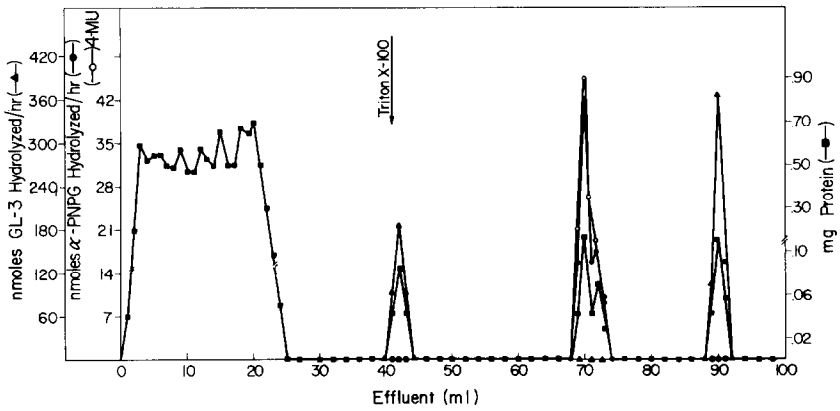

Fig. 1. Affinity chromatography of dialyzed acetone precipitate prepared from Cohn fraction IV-1. The column (0.6 × 6.5 cm) was equilibrated with 10^{-3} M MES buffer (pH 5.4) and the sample was applied in the same solution. Elution was carried out, at a constant rate of 1 ml/min, with buffer until non-adsorbed proteins were eluted, then with 0.1% Triton X-100 (w/v) added to the buffer. Enzymatic hydrolysis of α-pNPG and GL-3 (CTH) was measured as described in the text.

glycosphingolipid hydrolases without unambiguous proof that the enzymes have equivalent activity for the artificial and natural substrates. It is likely that the glycosyl hydrolases for glycolipids will be uniquely active with these lipophilic substrates and that purification based on assays with artificial substrates will be inappropriate.

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