Ceramide glycanase from the earthworm, *Lumbricus terrestris*

Bing Zhou CARTER, Su-Chen LI and Yu-Teh LI*

Department of Biochemistry, Tulane University School of Medicine, New Orleans, LA 70112, U.S.A.

Ceramide glycanase (CGase) is an enzyme that cleaves the linkage between the sugar chain and the ceramide. To make this enzyme readily available, we have developed a simple method for preparing it from the earthworm, *Lumbricus terrestris*. The method involves Bio-Gel A-0.5m, octyl-Sepharose and *p*-aminophenylthiogalactoside-agarose column chromatography. By gel filtration, the molecular mass of earthworm CGase was found to be 43.7 kDa. With ganglioside G_{M1} as substrate, the optimal pH of this enzyme was found to be between pH 3.5 and 4.0. Earthworm CGase hydrolyses glycolipids only in the presence of a detergent. Among various bile salts tested, sodium cholate was found to be the most effective in stimulating the hydrolysis of G_{M1} by this enzyme. Earthworm CGase released intact glycan chains from various glycosphingolipids in which the glycan chain is linked to the ceramide through a β -glucosyl linkage. It also detached glycan chains from lactosyldialkylglycerol and alkyl- β -lactosides.

INTRODUCTION

Ceramide glycanase (CGase) is an enzyme that cleaves the linkage between the ceramide and the glycan chain in various glycosphingolipids. This enzyme has been found in leeches [1], *Rhodococcus* sp. [2] and earthworms [3], and has been extensively purified from the first two sources [4,5]. As this enzyme has been shown to be very useful for the structural elucidation of glycosphingolipids [6,7], it is important to make it readily available. In this paper, we describe a simple method for the isolation of CGase from the earthworm, *Lumbricus terrestris*.

EXPERIMENTAL

Materials

Earthworms (*L. terrestris*) were purchased from Millbrook Cricket Farm, Jackson, MS, U.S.A. They were gutted and stored at -60°C until use. The following glycosphingolipids were isolated in our laboratory: GbOse₅Cer from dog intestine [8]; GbOse₄Cer and GbOse₃Cer from human erythrocytes [9]; G_{M2} from Tay-Sachs brain [10]; G_{M4} from chicken egg yolk [11]; DiGalCer from the kidney of a patient with Fabry's disease [12]. GgOse₅Cer and GgOse₃Cer were prepared from G_{M1} and G_{M2} respectively by formic acid hydrolysis [13]. The following compounds were generously given: *n*-butyl- β -lactoside, *n*-octyl- β -lactoside, myristoyl- β -lactoside, stearoyl- β -lactoside and phytol- β -lactoside from Dr. R. T. C. Huang, Institut für Molekularbiologie und Biochemie, Freie Universität, Berlin 33, Germany; lactosyldialkylglycerol [14] from Dr. H. Jarrell, National Research Council of Canada, Ottawa, Ontario, Canada; nLcOse₄Cer and LcOse₃Cer, from Dr. S. Basu, University of Notre Dame, Notre Dame, IN, U.S.A. The following materials were purchased from commercial sources: G_{M3}, G_{M1}, G_{T1b}, GlcCer, GalCer and sphingomyelin from Supelco (Bellefonte, PA, U.S.A.); G_{D1a} from Biocarb Chemicals, Lund, Sweden; OTE- β -lactoside, CETE- β -lactoside and Triton X-100, from

Pierce Chemical Co.; octyl-Sepharose from Pharmacia; *p*-nitrophenylglycosides, 4-methylumbelliferyl-NeuAc, methyl- β -lactoside, ethyl- β -lactoside, *n*-propyl- β -lactoside, benzyl- β -lactoside, ceramide type III (mainly non-hydroxy fatty acids), sodium cholate, sodium deoxycholate, octyl- β -glucoside and PAP-S-Gal-agarose, from Sigma; sodium taurodeoxycholate, sodium taurodehydrocholate, sodium tauroolithocholate, sodium taurocholate and sodium taurochenodeoxycholate, from Calbiochem; Rotofor cell isoelectrofocusing unit and Bio-Gel A-0.5m, from Bio-Rad; Silica-gel-60-precoated plates from Merck, Darmstadt, Germany.

Assay of earthworm CGase activity

The incubation mixture contained the following components in 200 μl of 50 mM-sodium acetate buffer, pH 4.0: glycolipid substrate, 30 nmol; sodium cholate, 200 μg ; an appropriate amount of earthworm CGase. After incubation at 37°C for a preset time, the reaction was terminated by adding 5 vol. of chloroform/methanol (2:1, v/v). The mixture was vortex-mixed and centrifuged at 700 *g* for 10 min to form the organic phase (lower) and the aqueous phase (upper). When glycosphingolipids were used as substrates, the organic phase which contained the released ceramide was evaporated to dryness and analysed by t.l.c. using chloroform/methanol (9:1, v/v), as the developing solvent [1]. The ceramide was detected by staining the plate with Coomassie Brilliant Blue [15]. G_{M1} was used as substrate for routine assay and for examining the properties of earthworm CGase. One unit is defined as the amount of enzyme that releases 1 nmol of ceramide from G_{M1}/min at 37°C [4]. The specific activity is expressed as units/mg of protein. Protein concentrations were determined by the method of Lowry *et al.* [16] with BSA as standard. When synthetic β -lactosides were used as substrates, the aqueous phase which contained the released oligosaccharides was dried and analysed by t.l.c. with butan-1-ol/acetic acid/water (2:1:1, by vol.) as the developing solvent [17]. The oligosaccharides were revealed by diphenylamine spray [18]. For quantitative determination of ceramide and oligo-

Abbreviations used: CGase, ceramide glycanase; GgOse₅Cer, gangliotetraosylceramide; G_{M1}, II³ α NeuAc-GgOse₅Cer; G_{D1a}, II³ α NeuAc-IV³ α NeuAc-GgOse₄Cer; G_{T1b}, II³ α (NeuAc)₂IV³ α NeuAc-GgOse₄Cer; GgOse₃Cer, gangliotriaosylceramide; G_{M2}, II³ α NeuAc-GgOse₃Cer; GbOse₅Cer, globopentaosylceramide; GbOse₄Cer, globotetraosylceramide; GbOse₃Cer, globotriaosylceramide; nLcOse₄Cer, neolactotetraosylceramide; LcOse₃Cer, lactotriaosylceramide; LacCer, lactosylceramide; G_{M3}, II³ α NeuAc-LacCer; GlcCer, glucosylceramide; DiGalCer, galactobiosylceramide; GalCer, galactosylceramide; G_{M4}, I³ α NeuAc-GalCer; OTE, 2-(octadecylthio)ethyl; CETE, 2-(2-carbomethoxyethylthio)ethyl; lactosyldialkylglycerol, 3-*O*- β -lactosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol; PAP-S-Gal-agarose, *p*-aminophenyl- β -D-thiogalactopyranoside-agarose.

* To whom correspondence should be addressed.

Fig. 1. (a) Bio-Gel A-0.5m gel filtration of crude earthworm CGase and (b) thin-layer chromatogram showing CGase activity in the column eluate

(a) The crude earthworm CGase (32 ml, 1.37 g of protein) obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation was applied to a Bio-Gel A-0.5m column (5 cm $\times$ 85 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0. The column was eluted with the same buffer at a flow rate of 53 ml/h and 18 ml fractions were collected. The black bar shows the active fractions. (b) To check the CGase activity, 20 μ l of each fraction was incubated with G_{M1} at 37 $^\circ\text{C}$ for 3 h under the assay conditions described in the Experimental section. Cer is the standard ceramide.

saccharides on the t.l.c. plates, the plates were scanned by using a Shimadzu CS-930 t.l.c. scanner as described by Ando *et al.* [19].

Other methods

Proteinase activities were assayed with Azocoll (Sigma, St. Louis, MO, U.S.A.) as substrate [20]. Exoglycosidases were assayed with *p*-nitrophenylglycosides [21] and 4-methylumbelliferyl-NeuAc [22] as substrates. Sphingomyelinase activity was assayed by the same method as was used for assaying the CGase activity, except that the glycosphingolipid was replaced by sphingomyelin. The isoelectric point of earthworm CGase was determined by isoelectrofocusing using a Rotofor cell isoelectrofocusing unit [23]. The purity of earthworm CGase preparations was examined by PAGE using 8–25% gradient gels under non-denaturing conditions with Coomassie Blue stain [15].

Purification of CGase from *L. terrestris*

All operations were conducted at a temperature between 0 $^\circ\text{C}$ and 5 $^\circ\text{C}$ except chromatography on octyl-Sepharose and PAP-S-Gal-agarose columns which was performed at room temperature. Centrifugation was routinely carried out at 20000–30000 *g* for 20–30 min in a Sorvall RC5C refrigerated centrifuge. Ultrafiltrations were performed in Amicon stirring cells with PM-10 membranes.

Gutted earthworms, 660 g derived from 1210 pieces of earthworm, were homogenized with 5 vol. of distilled water in a Waring blender at 20 s intervals for 1 min and centrifuged to obtain a creamy water extract. To this solution, protamine sulphate (2 g/100 ml of water) was added dropwise to give a concentration of 0.7 mg/ml. The precipitate was removed by centrifugation and the clear supernatant was adjusted to 60% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (390 g/l). After standing for 3 h, the precipitate was collected by centrifugation and dissolved in 130 ml of 50 mM-sodium phosphate buffer, pH 7.0, and designated as crude earthworm CGase. This preparation was divided into four portions, and each was fractionated on a Bio-Gel A-0.5m column (5 cm $\times$ 85 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0. The column was eluted with the same buffer at 53 ml/h, and 18 ml fractions were collected. The fractions containing CGase (Fig. 1, fractions 72–84 as indicated by the black bar) were pooled and precipitated by adding solid $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation (472 g/l). After standing overnight, the precipitate was harvested by centrifugation. The precipitates collected from four runs were pooled and dissolved in 55 ml of 50 mM-sodium phosphate buffer, pH 7.0. This solution was then divided into two portions (1028 mg of protein each) and separately applied to an octyl-Sepharose column (2.5 cm $\times$ 18 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0. After the column had been washed with the same buffer to remove the unadsorbed proteins, CGase was eluted with water (Fig. 2). The active fractions (as indicated in Fig. 2 by the horizontal bar) from the two batches were pooled and concentrated by ultrafiltration to 5 ml. The CGase obtained from the octyl-Sepharose column was mixed with an equal volume of 100 mM-sodium phosphate buffer, pH 8.0, and further concentrated by ultrafiltration to 3 ml. This preparation was divided into two portions and separately applied to a PAP-S-Gal-agarose column (1 cm $\times$ 15 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 8.0, and the column was eluted with the same buffer; 0.8 ml fractions were collected. The CGase activity was recovered in the tail portion of the unadsorbed fraction (Fig. 3), and the active fractions (fractions 18–28 and 29–60 indicated by black bars in Fig. 3) were concentrated separately by ultrafiltration to obtain the enzyme preparations designated as PAP-S-Gal-agarose I and II respectively. The adsorbed β -galactosidase was eluted from the column with 50 mM-sodium acetate buffer, pH 4.0. Table 1 summarizes a typical purification and recovery of the CGase based on 660 g of gutted earthworms.

RESULTS AND DISCUSSION

Purification of earthworm CGase

The main emphasis of this work is to devise a simple method for the isolation of CGase from the earthworm. By following the simple purification scheme described in the Experimental section, we were able to isolate a highly purified earthworm CGase free from exoglycosidases. The enzyme was enriched 1124-fold, with 26% recovery in the PAP-S-Gal-agarose II fraction (Table 1).

Before protamine sulphate treatment, the texture of the earthworm extract was very creamy, turbid and reddish in colour. Protamine sulphate precipitated the reddish turbid materials and yielded clear extract which facilitated the subsequent purification of the enzyme. On Bio-Gel A-0.5m gel filtration, α -galactosidase and α -*N*-acetylgalactosaminidase were eluted before CGase, and β -hexosaminidase, β -galactosidase and α -fucosidase were co-eluted with CGase. These contaminating exoglycosidases were not retained by the octyl-Sepharose column. Therefore octyl-Sepharose chromatography not only removed β -hexosaminidase, α -fucosidase and most of the β -galactosidase

Table 1. Purification of CGase from 660 g of gutted earthworm (*L. terrestris*)

Procedures	Total protein (mg)	Total units (nmol/min)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Water extract	29930	1326	0.0443	1	100
Protamine sulphate	13005	1318	0.101	2.28	99.4
(NH ₄) ₂ SO ₄ (0–60 %)	5483	660	0.120	2.71	49.8
Bio-Gel A-0.5m	2055	816	0.397	8.96	61.5
Octyl-Sepharose	56.1	643	11.46	258.69	48.5
PAP-S-Gal-agarose I	10.88	137	12.59	284.20	10.3
PAP-S-Gal-agarose II	6.93	345	49.78	1123.70	26.0

Fig. 2. (a) Octyl-Sepharose chromatography of earthworm CGase and (b) thin-layer chromatogram showing CGase activity in the column eluate

(a) The preparation (1028 g of protein, 408 units) derived from Bio-Gel A-0.5m gel filtration was applied on to an octyl-Sepharose column (2.5 cm × 18 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.0. After the column had been washed with the same buffer to remove unadsorbed proteins, CGase was eluted from the column with water (as indicated by the arrow); 10 ml fractions were collected. The black bar shows the active fractions. (b) To check the CGase activity, 5 µl of each fraction was incubated with G_{M1} at 37 °C for 16 h under the assay conditions described in the Experimental section. Cer is the standard ceramide.

from CGase, but also greatly increased the purity of the enzyme (from 9-fold to 260-fold). As shown in Fig. 2, CGase was detached from the octyl-Sepharose by elution with water. In contrast, leech CGase was not eluted from an identical column by water. Only by using octyl-glucoside could we remove the leech enzyme from the octyl-Sepharose column [4]. Although the CGase obtained from this step was suitable for releasing intact glycan chains from various glycosphingolipids, this preparation still contained some β-galactosidase activity. Gel filtration, ion-exchange chromatography and isoelectrofocusing all failed to

Fig. 3. (a) PAP-S-Gal-agarose affinity chromatography of earthworm CGase and (b) thin-layer chromatogram showing CGase activity in the column eluate

(a) The enzyme from octyl-Sepharose column (28 mg of protein, 322 units) was loaded on a PAP-S-Gal-agarose column (1 cm × 15 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 8.0. The column was washed with the equilibrating buffer and then eluted with 50 mM-sodium acetate buffer, pH 4.0, as indicated by the arrow. The black bars show the active fractions. (b) To check the CGase activity, 5 µl of each fraction was incubated with G_{M1} at 37 °C for 3 h under the assay conditions described in the Experimental section. Cer is the standard ceramide.

remove this contaminant. Only PAP-S-Gal-agarose affinity chromatography could remove β-galactosidase from CGase. In this step, β-galactosidase was retained by the column, whereas CGase was eluted behind the unadsorbed protein peak (Fig. 3). The final preparation of earthworm CGase (PAP-S-Gal-agarose II) gave three protein bands when examined by native PAGE. CGase activity was found to be associated with only one of these protein bands.

Fig. 4. Effect of pH on the hydrolysis of G_{M1} by CGases isolated from the earthworm (□) and the leech (◆).

G_{M1} (30 nmol) was incubated with 0.2 unit of either earthworm CGase or leech CGase in 200 μ l of the following buffers containing 1 μ g of sodium cholate/ μ l: 50 mM-sodium acetate, pH 2.5–6.0 and 50 mM-sodium phosphate, pH 6.0–8.0. The incubations were carried out at 37 °C for 20 min. The detailed assay conditions for measuring the enzyme activity are described in the Experimental section.

Table 2. Hydrolysis of various glycosphingolipids by earthworm CGase

Glycosphingolipids	Relative activity*	
	Earthworm CGase (%)	Leech CGase† (%)
GgOse ₄ Cer	116	185
G_{M1}	100	100
G_{D1a}	79	41
G_{T1b}	70	22
GgOse ₃ Cer	150	197
G_{M2}	108	115
G_{M3}	61	35
nLcOse ₄ Cer	45	38
LcOse ₃ Cer	64	31
GbOse ₅ Cer	20	46
GbOse ₄ Cer	30	50
GbOse ₃ Cer	69	67
LacCer	5	19

* Activity relative to hydrolysis of G_{M1} ; average of two determinations.

† Data taken from ref. [4].

General properties of earthworm CGase

Incubation of 0.6 unit of the purified enzyme with various *p*-nitrophenylglycosides, glycolipids and 4-methylumbelliferyl-NeuAc at 37 °C for 16 h failed to detect any exoglycosidase activities, such as α - and β -glucosidases, α - and β -galactosidases, α - and β -mannosidases, α -*N*-acetylglucosaminidase, α -*N*-acetyl-galactosaminidase, α -*L*-fucosidase and neuraminidase. Although 0.36 munit of β -hexosaminidase was detected under these conditions with *p*-nitrophenyl β -GlcNAc as substrate, this amount of β -hexosaminidase did not alter the released intact glycan chain from GbOse₄Cer. The purified enzyme was also free of sphingomyelinase and proteinase activities as determined with sphingomyelin (see the Experimental section) and Azocoll [20] respectively as substrates.

As measured by Bio-Gel A-0.5m gel filtration, earthworm CGase was found to have a molecular mass of 43.7 kDa, which

is much smaller than that of the leech enzyme (330 kDa) estimated under the same conditions [4]. Earthworm CGase appears to be hydrophobic in nature, for it can bind to octyl-Sepharose. It seems to be less hydrophobic than leech CGase, since it was eluted from the octyl-Sepharose column by water, not by 1% octylglucoside as in the case of leech CGase [4]. With G_{M1} as substrate, earthworm CGase was found to be stable between pH 3.5 and 8.5. The pI of earthworm CGase was determined to be between pH 4.6 and 5.2, which is similar to that of the leech enzyme (pH 4.7–4.9) [4]. The optimal pH of the enzyme was found to be between pH 3.5 and 4.0, which is significantly more acidic than the optimal pH of the leech enzyme. As shown in Fig. 4, the leech enzyme exhibits the highest activity at pH 5.0, whereas at this pH the activity of the earthworm enzyme is less than 50% of that at pH 4.0.

The effect of metal ions on earthworm CGase was studied and the results compared with those of leech CGase. Similarly to the leech enzyme, 1 mM-Hg²⁺ almost completely abolished earthworm CGase activity. Ba²⁺, Ca²⁺, Co²⁺, Mg²⁺ and Mn²⁺ at 5 mM did not affect its activity appreciably. Earthworm CGase was found to be more resistant to inhibition by Zn²⁺, Cu²⁺ and Ag⁺ than the leech enzyme. Whereas Zn²⁺ at 5 mM did not have much effect on earthworm CGase, it inhibited 60% of leech CGase activity [4]. In the presence of 1 mM-Cu²⁺ or -Ag⁺, earthworm CGase retained about 85% of activity, whereas leech CGase lost more than 60% of its activity [4]. On increasing the concentration of Cu²⁺ and Ag⁺ to 5 mM, the concentration that completely inactivated leech CGase [4], earthworm CGase still retained 47% and 20% of its activity respectively.

Similarly to leech CGase, a detergent is required for earthworm CGase to carry out hydrolysis of glycolipids. Of various bile salts tested, sodium cholate was most effective in stimulating hydrolysis of G_{M1} by both earthworm and leech CGase. When G_{M1} was used as substrate, the optimal concentration of sodium cholate was determined to be 1.0–1.5 μ g/ μ l for earthworm CGase. At a concentration of 1.0 μ g/ μ l, the next most effective bile salt for the earthworm enzyme was sodium deoxycholate which, however, had only very slight stimulatory activity toward leech CGase [4]. Surprisingly, sodium taurodeoxycholate (1 μ g/ μ l), the second most effective stimulator of the leech enzyme, had no stimulatory effect on the earthworm enzyme. Triton X-100 at 0.2% was about 80% as effective as sodium cholate in stimulating the hydrolysis of G_{M1} by earthworm CGase.

Hydrolysis of various glycosphingolipids by earthworm CGase

Similarly to leech CGase, earthworm CGase released intact glycan chains from various glycosphingolipids in which the glycan chain is linked to the ceramide through a β -glucosyl linkage. DiGalCer, G_{M4} , GalCer and GlcCer were not hydrolysed. When analysed by a double-reciprocal plot [24] under standard conditions with G_{M1} as substrate, the K_m for earthworm CGase was found to be 16.7 μ M, which is comparable with that of leech CGase (15.4 μ M) [4]. This indicates that the two CGases have a similar affinity for G_{M1} . Table 2 summarizes the substrate specificity of earthworm CGase. The specificity of the leech enzyme [4] is also included for comparison. The results show that the globo series of glycosphingolipids are slightly better substrates for leech CGase than for earthworm CGase, whereas the neolacto series of glycosphingolipids are better substrates for the earthworm enzyme than the leech enzyme. Of all the glycosphingolipids tested, the ganglio series appear to be the best substrates for both enzymes. However, there is an interesting difference between these two enzymes in the hydrolysis of gangliosides that have the same oligosaccharide backbone but differ in the number of sialic acid residues. As shown in Table 2, the rate of hydrolysis of glycosphingolipids with Ggose₄Cer backbone by leech enzyme

decreases in the following order: $GgOse_4Cer > G_{M1} > G_{D1a} > G_{T1b}$. From $GgOse_4Cer$ to G_{T1b} , addition of each sialic acid resulted in a 50 % decrease in the rate of hydrolysis [4]. Similarly, from $GgOse_3Cer$ to G_{M2} the rate of hydrolysis fell by one-half on addition of a sialic acid residue. With earthworm CGase no such relationship was observed.

Hydrolysis of synthetic β -lactosides by earthworm CGase

Like leech CGase, earthworm CGase did not release lactose from *p*-nitrophenyl- β -lactoside, benzyl- β -lactoside and phytlyl- β -lactoside. Both leech and earthworm CGases hydrolysed LacCer, alkyl-lactosides and lactosyldialkylglycerol. This indicates that the structural requirement of the lipid moiety is not absolute for the enzyme reaction. There are some subtle differences between the two enzymes: (1) although both enzymes cleave alkyl- β -lactosides, earthworm CGase requires a minimal chain length of eight carbon atoms in the alkyl groups, whereas that for leech CGase is only four carbon atoms; (2) both enzymes hydrolyse OTE-lactoside, which has 20 carbon atoms in its aglycone portion; however, unlike leech CGase, the earthworm enzyme was not able to hydrolyse CETE-lactoside, which has only six carbon atoms in its aglycone moiety. These results indicate that earthworm CGase prefers more hydrophobic substrates than does leech CGase.

Although there are some minor differences in general properties and substrate specificity between earthworm CGase and leech CGase, they catalyse similar reactions. Owing to the ability of both enzymes to hydrolyse not only glycosphingolipids but also alkyl-lactosides and lactosyldialkylglycerol, CGase should be regarded as a glycolipid glycanase or a glycolipid glycan-detaching enzyme [25]. It should be pointed out that the lipid moiety of a glycolipid greatly affects the rate of its hydrolysis by CGase. Thus the same glycosphingolipid isolated from different sources may be hydrolysed by CGase at different rates because of the heterogeneity in the ceramide moiety.

Comparing the starting material of leeches and gutted earthworms, the specific activity of leech CGase was about five times that of earthworm CGase. As earthworms are widely distributed in nature and much more readily available than leeches, CGase suitable for structural analysis of glycolipids can easily be isolated from earthworms by the procedures developed in this study.

This work was supported by grant DMB-8617033 from the National Science Foundation and grant NS-09626 from the National Institutes of Health.

REFERENCES

1. Li, S.-C., DeGasperi, R., Muldrey, J. E. & Li, Y.-T. (1986) *Biochem. Biophys. Res. Commun.* **141**, 346–352
2. Ito, M. & Yamagata, T. (1986) *J. Biol. Chem.* **261**, 14278–14282
3. Li, Y.-T., Ishikawa, Y. & Li, S.-C. (1987) *Biochem. Biophys. Res. Commun.* **149**, 167–172
4. Zhou, B., Li, S.-C., Laine, R. A., Huang, R. T. C. & Li, Y.-T. (1989) *J. Biol. Chem.* **264**, 12272–12277
5. Ito, M. & Yamagata, T. (1986) *J. Biol. Chem.* **264**, 9510–9519
6. Hansson, G. C., Li, Y.-T. & Karlsson, H. (1989) *Biochemistry* **28**, 6672–6678
7. Shimamura, M., Hayase, T., Ito, M., Rasilo, M.-L. & Yamagata, T. (1988) *J. Biol. Chem.* **263**, 12124–12128
8. McKibbin, J. M. (1969) *Biochemistry* **8**, 679–685
9. Esselman, W. J., Laine, R. A. & Sweeley, C. C. (1972) *Methods Enzymol.* **28**, 140–156
10. Svennerholm, L. (1972) *Methods Carbohydr. Chem.* **6**, 464–474
11. Li, S.-C., Chien, J.-L., Wan, C.-C. & Li, Y.-T. (1978) *Biochem. J.* **173**, 697–699
12. Li, Y.-T., Li, S.-C. & Dawson, G. (1972) *Biochim. Biophys. Acta* **260**, 88–92
13. Svennerholm, L., Månsson, J.-E. & Li, Y.-T. (1973) *J. Biol. Chem.* **248**, 740–742
14. Renou, J.-P., Giziewicz, J. B., Smith, I. C. P. & Jarrell, H. C. (1989) *Biochemistry* **28**, 1804–1814
15. Nakamura, K. & Handa, S. (1984) *Anal. Biochem.* **142**, 406–410
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. T. (1951) *J. Biol. Chem.* **193**, 265–275
17. Kitamikado, M., Ito, M. & Li, Y.-T. (1981) *J. Biol. Chem.* **256**, 3906–3909
18. Harris, G. & MacWilliams, I. C. (1954) *Chem. Ind. (London)* **249**
19. Ando, S., Chang, N.-C. & Yu, R. K. (1978) *Anal. Biochem.* **89**, 437–450
20. Moore, G. L. (1969) *Anal. Biochem.* **32**, 122–127
21. Li, Y.-T. & Li, S.-C. (1972) *Methods Enzymol.* **28**, 702–713
22. Potier, M., Mameli, L., Belisle, M., Dallaire, L. & Melancon, S. B. (1979) *Anal. Biochem.* **94**, 287–296
23. Egen, N. B., Thormann, W., Twitty, G. E. & Bier, M. (1984) in *Electrophoresis* (Hirai, H., ed.), pp. 547–550, Walter de Gruyter, Berlin
24. Dixon, M. & Webb, E. C. (1979) *Enzymes*, 3rd edn., pp. 55–68, Academic Press, New York
25. Li, Y.-T. (1989) *Glycoconjugate J.* **6**, 372

Received 11 December 1991; accepted 20 January 1992