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LACTASE-PHLORIZIN HYDROLASE COMPLEX FROM MONKEY SMALL INTESTINE

PURIFICATION, PROPERTIES AND EVIDENCE FOR TWO CATALYTIC SITES*

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Summary

Lactase-phlorizin hydrolase (EC 3.2.1.-) has been purified from the monkey small intestine by gel filtration and ion-exchange chromatographic procedures and the properties of the purified enzyme complex have been studied. Lactose was the most active substrate. Cellobiose and other synthetic hetero-β-glycosides were hydrolysed at a very much reduced rate. The rate of hydrolysis of phlorizin was about 2.5% that of lactose. Lactase and phlorizin hydrolase activities were indistinguishable by heat inactivation experiments. The purified enzyme complex also hydrolysed cerebrosides. Lactose hydrolysis was competitively inhibited by phlorizin as well as by the brain cerebroside. However, there was no mutual inhibition between phlorizin and the brain cerebroside. It is suggested that the native enzyme complex might have two catalytic sites, a phlorizin site and a cerebroside site but both hydrolysing lactose.

Introduction

The lactase-phlorizin hydrolase complex has been purified to a very high degree from the small intestine of baby rats [1]. Partial purification of this complex has been reported in the hamster [2] and in the rabbit [3]. The lactase activity of the purified complex is relatively unstable to heat. Utilizing

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this property, the specificity of the two enzymatic sites has been studied [2,4]. Leese and Semenza [5] have identified the phlorizin hydrolase site of the complex with the glycosyl ceramidase activity earlier studied by Brady et al. [6]. They have also envisaged that the normal physiological role of phlorizin hydrolase might be the hydrolysis of cerebrosides whose presence in milk is known [7].

In this paper, a method for the isolation of lactase-phlorizin hydrolase from adult monkey small intestine is described. Data to be presented also reveal that, unlike in the case of rat [4], rabbit [3], hamster [2] and human [8], the lactase and phlorizin hydrolase activities exhibit identical heat inactivation rates, and that the lactase site rather than the phlorizin hydrolase site is involved in the hydrolysis of cerebrosides.

Materials and Methods

The chemicals (other than mentioned in ref. 9) and enzymes were purchased commercially as indicated: phlorizin (British Drug House Ltd., England); PEI-cellulose (Bio Rad Labs, U.S.A.); *N*-palmitoyl dihydroglucosyl ceramide and *N*-palmitoyl dihydro galactosyl ceramide (Miles Laboratories, U.S.A.); cerebroside isolated from buffalo brain and papain (ultracentrifugally homogenous) from Biochemicals Unit, V.P. Chest Institute, New Delhi; Aquacide II (M_r = 250 000) from Calbiochem., U.S.A.; other sugars and enzymes (Sigma Chem. Co., U.S.A.). Sodium cholate was prepared from cholic acid (Sigma) by titrating the acid with sodium hydroxide and crystallizing the salt by the addition of acetone.

Assay of enzymes activities

Lactase: During purification, lactase was assayed using 0.05 M lactose [9] and 0.1 M potassium acetate buffer, pH 5.6 by the Tris/glucose oxidase procedure of Dahlqvist [10] using 0.5 M (final) Tris buffer in the reagent [11]. However, it was subsequently found that the maximal velocity was attained only at 0.15 M lactose and this concentration was used for the kinetic studies.

Phlorizin hydrolase: Phlorizin hydrolase was measured by incubating at 37 °C a reaction mixture of 0.4 ml containing 5 mM phlorizin and 0.1 M potassium acetate buffer, pH 5.6. The liberated glucose was determined by the modified Tris/glucose oxidase procedure after passing the assay mixture through Dowex-1 columns as described earlier [12,13].

Glycosyl ceramidase

The enzyme activity was assayed essentially by the method of Leese and Semenza [5] with slight modifications as given below.

The reaction mixture (total vol. 0.2 ml) contained a cerebroside (12.5 mM), sodium cholate (0.02 M), bovine serum albumin (200 μg), potassium acetate buffer, pH 5.6 (0.1 M) and the enzyme. After incubation at 37 °C (up to 1 h), 0.1 ml each of $ZnSO_4$ (5%) and $Ba(OH)_2$ (0.15 M) was added [14] followed by 0.2 ml H_2O and the contents mixed well. The tubes were then centrifuged and the sugars in the supernatant fraction were determined. Galactose was directly determined by the reducing sugar method [15], and after

column separation, glucose was assayed by the modified Tris/glucose oxidase procedure [13]. The cerebrosides were first dissolved in a small volume of a mixture of chloroform : methanol (2 : 1, v/v) and the tubes were kept at 37 °C for 2—3 h to evaporate the solvent completely prior to the addition of other constituents of the reaction mixture. The concentration of brain cerebroside in the reaction mixture was calculated using a mean molecular weight of 799 suggested for the naturally occurring monohexosyl ceramides by Forstner and Wherret [16].

Enzyme unit

One unit of enzyme activity is defined as the amount of enzyme hydrolysing 1 μmol of the substrate/min; the specific activity is expressed as units/mg protein.

Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

Disc electrophoresis

Disc electrophoresis was carried out according to the general method of Davis [18] but using 4% acrylamide and employing a current of 2 mA per tube for 90 min. The gels were stained for protein with Amidoschwarz (0.5% in acetic acid, 7% v/v).

Animals

Adult monkeys (4—6 animals) of either sex belonging to *Macaca radiata* species were killed by Nembutal anaesthesia. The small intestines from pyloric to ileocaecal end were removed and washed thoroughly with ice-cold KCl (1.15%). They were then cut open longitudinally and the mucosa scraped with a blunt knife.

Results

Purification of lactase-phlorizin hydrolase complex

The procedure was essentially similar to earlier reports [1,9] and is briefly outlined below. A 20% homogenate prepared from 150 g of monkey intestinal mucosa was centrifuged (12 000 × *g*; 30 min) and the pellet washed twice with 0.01 M potassium phosphate buffer, pH 7.0. The pellet was then treated with papain [9] and the dialyzed 100 000 × *g* supernatant fraction was fractionated as follows. The fraction precipitating between 40—80% saturation of ammonium sulfate was applied on a Sepharose 4B column (2.2 cm × 100 cm; bed vol. 400 ml) and eluted with 0.01 M potassium phosphate buffer, pH 7.0 and the fractions between 258 ml—300 ml were pooled from each of three separate column operations. The pooled Sepharose 4B fraction was concentrated with Aquacide and applied on a Sephadex G200 column (2.3 cm × 132 cm; bed vol. 580 ml) and eluted with 0.01 M potassium phosphate buffer, pH 7.0. The fractions between 180 ml—216 ml were pooled and applied on a DEAE-Sephadex column (1 cm × 14 cm; bed vol. 15 ml), washed with 10 bed vols. of 0.01 M potassium phosphate buffer pH 7.0 and the enzyme eluted with 0.05 M buffer. The active fractions between 12 ml—72 ml were pooled. In all these

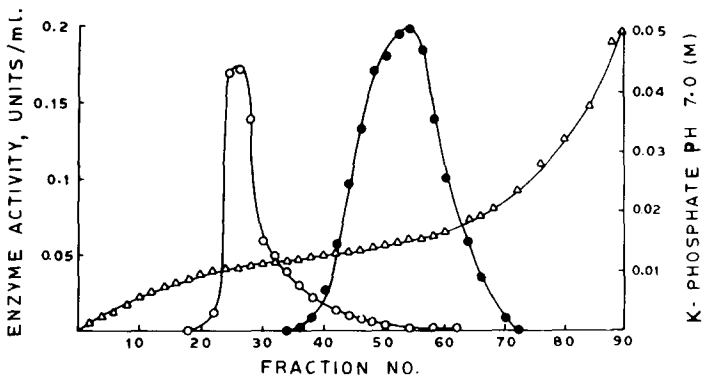

Fig. 1. PEI-cellulose chromatography. The pooled lactase-phlorizin hydrolase fraction from the DEAE-Sephadex column was used. For other details see the text. ●———●, lactase; ○———○, maltase; △———△, concentration of potassium phosphate in the eluant.

fractionations lactase, phlorizin hydrolase and cellobiase activities showed identical elution patterns but the preparation at this stage was still contaminated with maltase activity and showed two protein bands in disc electrophoresis. However, it was possible to eliminate the maltase activity by PEI-cellulose chromatography as detailed below.

PEI-cellulose was soaked in a large excess of potassium phosphate buffer, pH 7.0 and the supernatant was decanted off. The resin was successively washed with dilute buffers (0.1—0.01 M) and finally with 1 mM potassium phosphate buffer, pH 7.0. The resin was packed in a column (0.85 cm × 11 cm; bed vol. 5.0 ml) and washed with equilibrating potassium phosphate buffer, pH 7.0 (1 mM). The DEAE-Sephadex fraction was dialysed against 1 mM potassium phosphate, pH 7.0 for 24 h and an aliquot (containing 5 mg of total protein) was loaded on to the column and then eluted using a previously standardized compound gradient employing a Varigrad (Technicon) apparatus. For this, the first four chambers contained 20 ml each of 1 mM , 20 mM, 1 mM and 50 mM potassium phosphate, pH 7.0, respectively. The shape of the gradient obtained was determined in a separate experiment by keeping 0.0024, 0.048, 0.0024 and 0.12 mM glucose in the first four chambers respectively and determining the glucose in the effluent by the modified Tris/glucose oxidase procedure. The flow rate was maintained at about 15 ml per h and 1 ml fractions were collected. The elution profile showing the separation of lactase and maltase is illustrated in Fig. 1.

A summary of the purification steps is given in Table I. The enzyme with a specific activity of 16.1 (for lactase) was purified 475-fold over the crude pellet fraction with an overall recovery of 20%. Phlorizin hydrolase was also purified to the same degree since the lactase to phlorizin hydrolase ratio ranging 33—35, remains constant during purification.

Properties of the purified enzyme

K_M and V values: Within limits, the rate of hydrolysis of lactose was linear with time (up to 1 h) and enzyme concentration (up to 0.8 μg). The K_m

TABLE I

PURIFICATION OF LACTASE-PHLORIZIN HYDROLASE COMPLEX FROM MONKEY SMALL INTESTINE

Fraction	Total protein mg	Lactase*		Phlorizin hydrolase		Lactase/ phlorizin hydrolase
		Total activity units	Specific activity (units/mg protein)	Total activity units	Specific activity (munits/mg protein)	
Pellet	4450	150	0.034	4.6	1.0	34
Papain extract	795	147	0.185	4.2	5.3	35
Ammonium sulphate	510	132	0.261	4.05	7.9	33
Sepharose 4B	163	122	0.751	3.56	23.0	32.6
Sephadex G-200	43	106	2.46	3.12	72.0	34.2
DEAE-Sephadex	15	76	5.05	2.28	150.0	33.6
PEI-Cellulose	1.8	29	16.1	0.81	450.0	35.0

* Assays were performed with 0.05 M lactose and hence the values do not represent optimal rates as observed with 0.15 M lactose.

and V values for lactose, cellobiose and phlorizin as substrates are given in Table II. The ratio of V/K_M for lactose, and cellobiose is almost the same (Table II) indicating that the catalytic efficiency of the enzyme with respect to these substrates is the same.

The pH activity profile for lactase in acetate and citrate buffer systems was essentially the same with a pronounced peak at pH 5.6. However, as already reported [13], there was an anamolous phlorizin hydrolase peak in the acidic pH range in citrate buffer.

Heat inactivation of lactase and phlorizin hydrolase

Lactase and phlorizin hydrolase activities have been distinguished by heat inactivation experiments in the infant rat [4] in human [8], in hamster [2] and in rabbits [3]. In the monkey, contrary to these results both lactase and phlorizin hydrolase are identically heat inactivated at 55°C.

Substrate specificity

The relative rates of various substrates hydrolysed by the purified lactase-

TABLE II

V/K_M RATIO FOR LACTOSE, CELLOBIOSE AND PHLORIZIN

The data for lactose and cellobiose were obtained using the PEI fraction and for phlorizin the DEAE-Sephadex fraction. The assays were carried out at pH 5.6.

Substrate	V*	K_M (mM)	V/K_M
Lactose	31.2	21.8	1.43
Cellobiose	3.84	2.78	1.38
Phlorizin	0.330	0.4	0.83

* μmol of substrate hydrolysed/min mg protein; values from the double-reciprocal plot.

phlorizin hydrolase are given in Table III. Lactose was the most active substrate with a value of 21.7 μmol/min/mg protein and is comparable with the value reported in the case of infant rat [1]. Cellobiose and a number of synthetic aryl β-galactosides and β-glucosides are hydrolysed at a much lower rate (7.25—16.0% of the rate of lactose hydrolysis). Phlorizin and salicin were hydrolysed extremely slowly (1.6—2.34%). Under the assay conditions, phenyl β-glucoside and phenyl β-galactoside were not hydrolysed at all.

Hydrolysis of glycolipids and identification of two catalytic sites

Among the glycolipids, the naturally occurring brain cerebroside which is essentially a galactocerobroside [19] and the synthetic *N*-palmitoyldihydroglucosylceramide were hydrolysed to the extent of 1.65% and 1.5% of lactose hydrolysis at pH 5.6 (Table III), but there was no detectable hydrolysis of *N*-palmitoyl-dihydrogalactosylceramide.

In order to delineate whether or not a single catalytic site is involved in the hydrolysis of all these substrates by the lactase-phlorizin hydrolase complex, mixed substrate incubations were carried out using the DEAE-Sephadex fraction as the enzyme source. Three sets of experiments were done at pH 5.6 using mixtures of lactose and phlorizin, lactose and brain cerebroside, and phlorizin and brain cerebroside. Lactose hydrolysis is competitively inhibited by phlorizin (K_i 0.18 mM, Fig. 2) and by brain cerebroside (K_i 1.11 mM, Fig. 3). However, there is no mutual inhibition between phlorizin and brain cerebroside (Table IV). Sodium cholate [6] and bovine serum albumin [5] which are together required for maximal hydrolysis of cerebrosides do not have any effect on phlorizin hydrolysis (Table IV). A mixture of sodium cholate and bovine serum albumin, however, inhibits lactose hydrolysis in a competitive manner but the degree of inhibition was much lower than when cerebroside was also present (Fig. 3).

TABLE III

SUBSTRATE SPECIFICITY OF THE PURIFIED ENZYME

Substrate	Enzyme activity*
Lactose (150 mM)	100
Cellobiose (50 mM)	16
o-Nitrophenyl-β-galactoside (10 mM)	15.4
o-Nitrophenyl-β-glucoside (10 mM)	13.0
p-Nitrophenyl-β-glucoside (10 mM)	12.7
p-Nitrophenyl-β-galactoside (10 mM)	9.3
β-Methyl glucoside (50 mM)	7.25
Phlorizin (5 mM)	2.34
Salicin (10 mM)	1.6
Phenyl-β-galactoside (10 mM)	0
Phenyl-β-glucoside (10 mM)	0

* The rate of lactose hydrolysis was 21.7 μmol/min/mg protein and was taken as 100%. Concentration of the substrates is indicated in parenthesis.

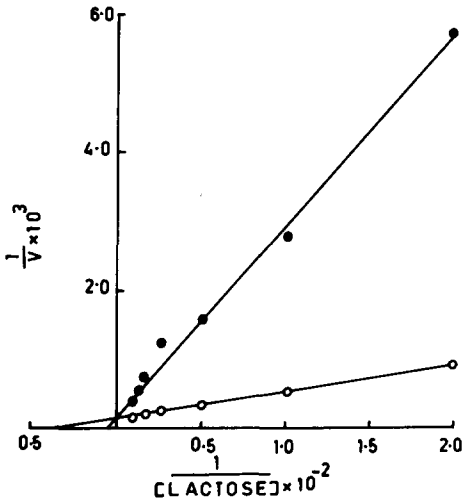

Fig. 2. Inhibition of lactose hydrolysis by phlorizin. DEAE-Sephadex fraction was used as enzyme. V, expressed as nmol lactose hydrolysed/min/mg protein. ○———○, in the absence of phlorizin; ●———●, in the presence of phlorizin (1 mM).

Purity of the enzyme preparation

The disc electrophoresis pattern obtained for the PEI fraction is shown in Fig. 4. There is a single protein band which is slightly diffuse at the moving front. The protein failed to penetrate a 7.5% gel and stayed at the origin. This supports the Sepharose 4B data indicating that lactase-phlorizin hydrolase might be a large molecule. Electrophoretic patterns obtained for Sepharose 4B, Sephadex G-2000, and DEAE-Sephadex fractions, when compared with that of PEI fraction revelaed that the lactase-phlorizin hydrolase complex shows the lowest mobility and all other fast moving bands disappear during purification.

TABLE IV

LACK OF INHIBITION OF PHLORIZIN HYDROLYSIS BY BRAIN CEREBROSIDE

The assay was done at pH 5.6. The liberated glucose was determined by the modified Tris/glucose oxidase procedure after column treatment (see text).

Additions to the phlorizin hydrolase reaction mixture	V (nmol glucose/min/mg protein) Concn of phlorizin (mM)	
	0.25	2.0
None	19.4	70
Sodium-cholate (0.02 M +		
Bovine serum albumin (0.4 mg/ml)	19.4	70
+ brain cerebroside (5 mM)	20.2	70
+ brain cerebroside (12.5 mM)	19.4	69

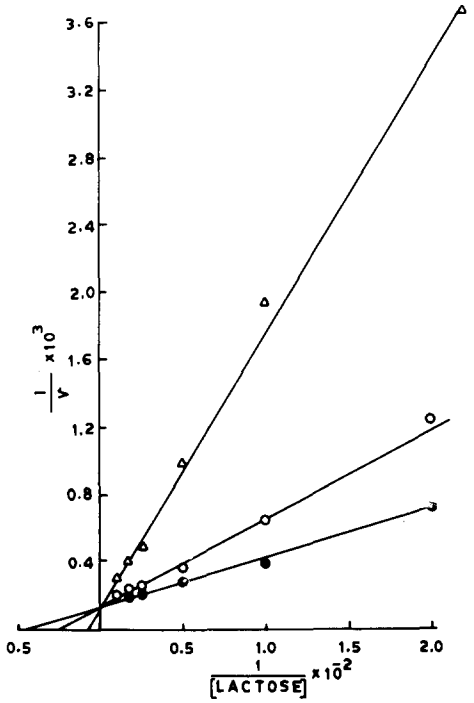

Fig. 3. Inhibition of lactose hydrolysis by the brain cerebroside. DEAE-Sephadex fraction was used as the enzyme. V, expressed as nmol lactose hydrolysed/min/mg protein. ●———●, in the absence of inhibitor; ○———○, in the presence of sodium cholate (0.02 M) and bovine serum albumin (1 mg/ml of reaction mixture); △———△, in the presence of sodium cholate, bovine serum albumin and the brain cerebroside (5 mM).

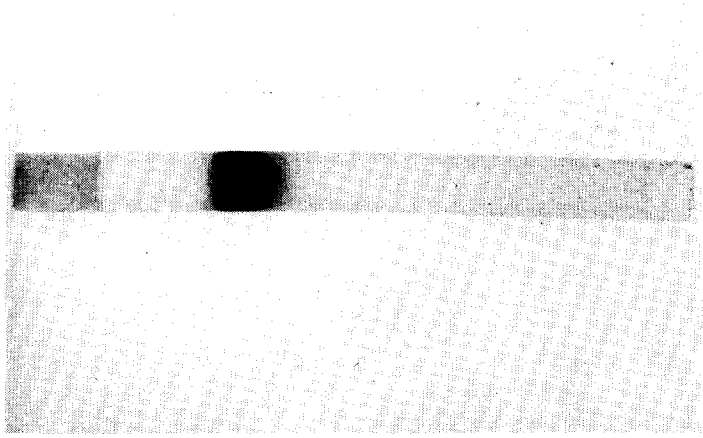

Fig. 4. Disc electrophoresis of the PEI-cellulose fraction. Approximately 10 μg protein was used. The anode is at the right hand side of the gel.

Discussion

The lactase-phlorizin hydrolase complex has been isolated in an essentially pure form from the adult monkey intestine. Earlier, lactase has been purified to homogeneity only in rat among the other adult species [20]. In the present work, lactase and phlorizin hydrolase maintain a constant ratio during the purification and show strikingly identical elution profiles. The lactase-phlorizin hydrolase complex is probably a glycoprotein as indicated by its strong interaction with concanavalin A (Ramaswamy, S. and Radhakrishnan, A.N., unpublished observation).

Apart from lactose and phlorizin, the purified lactase-phlorizin hydrolase complex hydrolyses a variety of β-glycosides including some of the naturally occurring ones present in the milk [2,4]. On the basis of heat inactivation experiments, it has been shown that in the infant rat, the heat labile lactase site hydrolyses all the substrates except phlorizin which is hydrolysed by a separate heat stable site [4]. In the hamster, however, the heat stable phlorizin hydrolase shows some activity against the synthetic β-glucosides also [2]. On the contrary, lactase and phlorizin hydrolase activities in the monkey are identically heat inactivated and cannot be distinguished by this method.

Leese and Semenza [5] observed that the lactase-phlorizin hydrolase from infant rat exhibits a glycosyl ceramidase activity resembling the one reported earlier in the intestine [6], in properties like substrate and inhibitor specificities, K_M values and optimum pH. The glycosyl ceramidase activity was stable to heat as is phlorizin hydrolase and phlorizin inhibited the hydrolysis of galactosyl ceramide competitively. The rate of hydrolysis of glucosyl-*N*-palmitoyl ceramide and galactosyl-*N*-palmitoyl ceramide was the same but glucosyl-*N*-stearoyl ceramide and glucosyl-*N*-lignoceryl ceramide were hydrolysed at a much reduced rate. It was inferred that the phlorizin hydrolase site and not the lactase site was involved in the hydrolysis of glycosylceramides and the rate of hydrolysis decreased with increase in the chain length of the fatty acid residue present in the glycolipid [5].

On the other hand, the results in the monkey were found to be different. Lactose hydrolysis was inhibited competitively by both phlorizin and brain cerebroside, but there was no mutual inhibition between phlorizin and brain cerebroside. This provides a modest basis for assuming the presence of two catalytic sites in the native enzyme complex, one for the hydrolysis of lactose and phlorizin and the other for the hydrolysis of lactose and cerebroside. Supporting these results is the observation that in the adult guinea pig intestine which lacks in phlorizin hydrolase activity but exhibits lactase activity [21], the brain cerebroside is hydrolysed (Ramaswamy, S. and Radhakrishnan, A.N., unpublished observation). The presence of two enzymatic sites on the lactasephlorizin hydrolase complex in the monkey is reminiscent of the brush border maltase-sucrase-isomaltase complex in the rabbit intestine [22] where two sites with maltase-sucrase and maltase-isomaltase activity have been recognized. It has been suggested that the physiological role of phlorizin hydrolase in the infant rat may be in the hydrolysis of dietary cerebrosides. However, in the monkey, the observed results suggest that the lactose site rather than the phlorizin site may be responsible for the hydrolysis of cerebrosides. It is thus possi-

ble that there may be a species variation in this regard and it leaves the role of phlorizin hydrolase still an open question.

Purified lactase-phlorizin hydrolase from the monkey intestine hydrolysed brain cerebrosides and *N*-palmitoyl glucosylceramide but not *N*-palmitoyl galactosylceramide. Brain cerebroside is essentially a galactosylceramide with longer chain fatty acids predominantly of 24 carbons belonging to both saturated and unsaturated groups with or without hydroxylation [23]. Hydrolysis of this glycolipid but not its synthetic analogue with palmitic acid is rather curious and may be related to the fatty acid composition of the individual glycolipid.

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