LACTASE-PHLORIZIN HYDROLASE COMPLEX FROM MONKEY SMALL INTESTINE: STIMULATION OF PHLORIZIN HYDROLASE ACTIVITY BY ORGANIC ACIDS

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SUMMARY

Lactase-phlorizin hydrolase complex from monkey small intestine reveals a new phlorizin hydrolase activity at pH 3.3 in the presence of certain organic acids in addition to the normal activity at optimum pH 5.4. The highest stimulation (10-15 fold) was obtained with tartaric acid. Lactase activity at pH 5.3 is unaffected but its activity at pH 3.3 is inhibited by organic acids. Tartaric acid-stimulated phlorizin hydrolase activity is inhibited by a number of organic acids which have no effect on the unstimulated enzyme. Pyruvic acid inhibits the unstimulated activity as well. SO_4^{2-} and Cl^- ions are potent inhibitors of the tartaric acid-stimulated phlorizin hydrolase.

Phlorizin, a naturally occuring β-glucoside of plant origin is known for its profound effect on glucose transport. Phlorizin hydrolase catalyzing the hydrolysis of phlorizin into phloretin and glucose is present in the intestinal mucosa of a number of animals (1-3). Although earlier studies with hamster intestine indicated that phlorizin hydrolase was different from lactase (1), subsequent work in the rat (4) and in the human (2) has shown that it might be an oligo-enzyme complex with two catalytic sites, one for the hydrolysis of phlorizin and the other for both phlorizin and lactose.

During our studies on purified lactase-phlorizin hydrolase from monkey intestine, a very prominent phlorizin hydrolase activity peaking at about pH 3.4 was observed in the presence of certain organic acids in addition to the enzyme activity with an optimum at pH 5.4 reported earlier (1-4). The results on the further investigation of this novel phenomenon are reported in this communication.

MATERIALS AND METHODS

Enzyme preparation: A papain solubilized preparation

of the particulate fraction from the mucosal homogenates of monkey small intestine (5,6) was further purified by fractionation with ammonium sulfate, Sepharose 4B, Sephadex G-200 and DEAE-Sephadex A-50 chromatography. The final preparation was nearly 200-fold purified over the starting pellet fraction with a specific activity (units/mg protein) ranging between 5-6 for lactase.

Assay of enzymes: Unless otherwise stated, phlorizin hydrolase was assayed by incubating at 30°C for 20 min., a reaction mixture (0.4 ml) containing phlorizin (2 μmoles), potassium acetate buffer, pH 3.3 (20 μmoles) and enzyme. The reaction was stopped by heating in a boiling water bath for 1 min. and an aliquot (0.2 ml) of the mixture was passed through a column of Dowex-1 to remove interfering phloretin and phlorizin (7). Glucose in the breakthrough fraction was determined using Tris-glucose oxidase reagent (TGO) of Dahlqvist (8) by the following method, a modification of that of Lloyd and Whelan (9), who standardized addition of HCl to increase the sensitivity of the reaction. The TGO reagent in a volume of 100 ml contained 75 ml of 2M Tris-HCl, pH 7.0 containing 27% glycerol, 30 mg. of glucose oxidase (type II, Sigma), 0.9 mg. peroxidase (type I, Sigma), 1.2 ml of 20% Tritonx100 (Rohm and Haas) and 18 mg. of o-dianisidine (Sigma). The reagent (0.5 ml) was added to the breakthrough fraction containing glucose (1.0 ml) and incubated for 1 hr. at 37°C and 10N HCl (1.0 ml) was then added and the color read in a Klett-Summerson colorimeter using No.54 filter. This procedure was quite sensitive (0.005-0.12 μmole of glucose) and was suitable for the assay of even small levels of phlorizin hydrolase. Lactase and cellobiase were assayed as described earlier (5).

Protein: Protein was determined by the method of Lowry et al. (10) using crystalline bovine serum albumin as standard.

Enzyme unit: One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 μmole of substrate/min. Specific activity is expressed as Units/mg protein.

RESULTS

pH Activity profile of lactase and phlorizin hydrolase in acetate and citrate buffers: The pH activity profile of lactase and phlorizin hydrolase is as shown in Fig. 1A & B. In acetate buffer, lactase shows a pronounced peak at pH 5.6 (Fig. 1A) while phlorizin hydrolase has a broad optimum in the pH range, 5.2-5.6 (Fig. 1B). In citrate buffer, there is a dramatic change in the pH profile of phlorizin hydrolase (Fig. 1B) with a sharp increase in activity in the region of pH 3.3-3.5, while the activity at pH 5.2-5.6 remained unaltered. There was essentially no change in the pH profile of lactase in citrate buffer.

Effect of citric acid concentration on phlorizin hydrolase, lactase and cellobiase at different temperatures: The effect of different concentrations of citric acid on phlorizin hydrolase at 25°C, 30°C and 37°C is shown in Fig. 2. The enzyme is maximally stimulated at a concentration of 50 mM citric acid at each of these temperatures but the highest stimulation was at 30°C. At 100 mM citric acid, there was a reduction in activity at 30°C and 37°C but not at 25°C. On the other hand, lactase was inhibited at all levels of citric acid to the same extent both at 30°C and 37°C. Cellobiase activity which is known to be associated with lactase (5,6) ran parallel to that of lactase.

A number of common chelating agents (EDTA, Neocuproine, Cupferron, sodium diethyldithiocarbamate, 2,2'-dipyridyl, o-phenanthroline and sodium azide) at a concentration of 0.5 mM showed neither stimulation nor inhibition of phlorizin hydrolase activity suggesting that citric acid exerts a direct effect on the enzyme activity and not by chelating an inhibitory heavy metal ion.

Effect of various organic acids on phlorizin hydrolase: The effect of various organic acids, all at 10 mM on phlorizin hydrolase activity at pH 3.3 is shown in Table 1 (Expt. 1). Tartaric acid gave the highest stimulation followed in order by fumaric, citric, cis-aconitic, iso-citric and ascorbic acids. Not included in the table are, oxaloacetic, maleic, lactic and oxalic acids, glycine and sorbitol with marginal

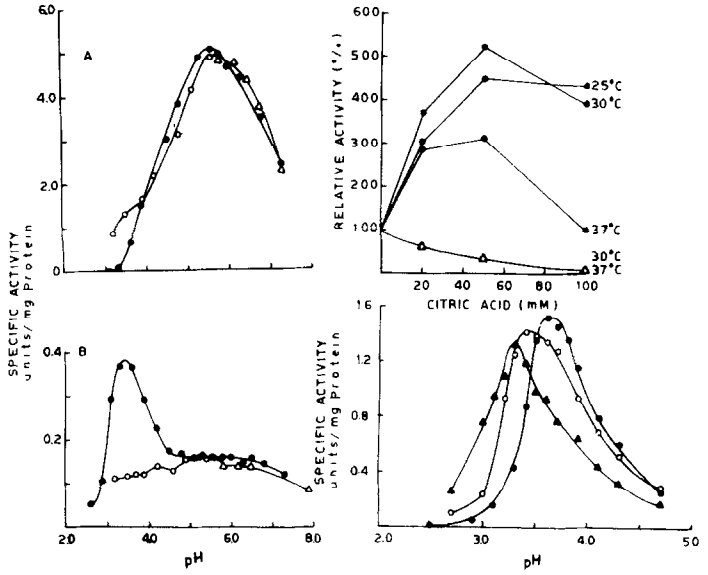

Fig. 1 (left): pH-activity profile of lactase (A) and phlorizin hydrolase (B); potassium acetate buffer, 0.05M (open circles); sodium citrate buffer, 0.05M (closed circles); potassium phosphate buffer, 0.05M (open triangles).

Fig. 2 (top right): Effect of citric acid concentration on enzyme activity. Phlorizin hydrolase (closed circles), lactase (open circles) and cellobiase (open triangles). Standard assay conditions except that lactase and cellobiase were also assayed at pH 3.3.

Fig. 3 (bottom right): Effect of tartaric acid concentration on the pH-activity profile of phlorizin hydrolase. Concentration of tartaric acid: 0.02M (closed triangles), 0.04M (open circles) and 0.06M (closed circles). The pH values given in this figure are actual values of simulated reaction mixtures measured with a sensitive pH meter (Beckman, Research Model).

stimulation, and α-oxoglutaric, succinic, malic, malonic, formic, glutamic and aspartic acids and threonine and pentaerythritol all of which had no effect on the enzyme activity. Pyruvic acid was the only compound showing a strong inhibitory action.

As seen in Expt. 2 (Table 1) tartaric acid stimulated phlorizin hydrolase activity is also inhibited by pyruvic acid and by some organic acids which do not have any effect on the unstimulated enzyme. Surprisingly SO_4^{2-} ions show a pronounced

Table 1. Effect of organic acids on phlorizin hydrolase

Expt. 1		Expt. 2	
Organic acid (10mM)	Relative activity (%)	Additions to the tartaric acid (20mM) activated enzyme	Relative activity (%)
None	100	None	100
L-Tartaric acid	500	Pyruvic acid	4
Fumaric acid	286	Na_2SO_4	12
Citric acid	242	Malic acid	45
cis-Aconitic acid	236	α-Oxoglutaric acid	50
iso-Citric acid	171	Oxaloacetic acid	50
L-Ascorbic acid	160	Oxalic acid	53
Pyruvic acid	50	Maleic acid	56
		NaCl	70

Standard assay conditions were used for both the Expts., with the pH of the mixture adjusted to pH 3.3.

Expt. 1: The activity of the control was 0.082 unit/mg protein and is taken as 100.

Expt. 2: The specific activity of the enzyme in the presence of 20mM tartaric acid was 1.16 units/mg protein and is taken as 100. In its absence the activity was 0.12 unit/mg protein and the relative activity was 10%. The concentration was 10mM for all the compounds tested. The acids used were from Sigma Chemical Co. or Calbiochem, U.S.A.

inhibition of tartaric acid stimulated activity. Cl^- ions, and formic and malonic acids (not given in table) are moderately inhibitory.

Influence of tartaric acid concentration on the optimum pH of phlorizin hydrolase: Since tartaric acid gave the highest stimulation of phlorizin hydrolase activity, further studies were carried out with this system. As with citric acid, higher concentrations of tartaric acid (>25 mM) were inhibitory when the activity was assayed at pH 3.3. However, on different occasions, the degree of stimulation by tartaric acid at pH 3.3 showed discrepancies which prompted us to investigate the effect of concentration of tartaric acid on the pH

optimum. It can be seen from Fig. 3 that there is a slight but definite shift in the optimum pH of phlorizin hydrolase as the concentration of tartaric acid is varied. Thus, the optimum pH was found to be 3.3, 3.4 and 3.6 at a tartaric acid concentration of 0.02M, 0.04M and 0.06M respectively.

DISCUSSION

Highly purified lactase preparations from the small intestine of many animal species also exhibit phlorizin hydrolase activity (11-13). Although differences have been noted between these two enzyme activities in relation to development (3,14) and to the rate of heat inactivation (2,13), it has not been possible to actually separate the two activities. Lactase-phlorizin hydrolase complex may thus be similar to maltase-sucrase-isomaltase and maltase-glucoamylase oligo-enzyme complex (for a review see Ref. 15) of the intestine. In the case of the monkey, there was no change in the ratio of the two activities during purification and also the rates of heat inactivation at 55°C were identical (11). The results presented in this paper show that in addition to the activity at pH 5.2-5.6, a second phlorizin hydrolase activity becomes manifest at pH 3.3-3.6 in the presence of certain organic acids of which tartaric acid was the most active. Although phlorizin inhibits lactase activity at pH 5.4 (7) and at pH 3.3 (16), it is interesting to note that lactase activity is not inhibited by citric acid at pH 5.4 (Fig. 1A) but is inhibited at pH 3.3 (Fig. 2). It has also been observed that tartaric acid stimulated phlorizin hydrolase activity at pH 3.3 is inhibited by a number of organic acids and by anions like SO_4^{2-} and Cl^-. The stimulation by tartaric acid is completely inhibited by SO_4^{2-} ions whereas pyruvic acid inhibits the unstimulated activity as well.

Dietary tartaric and ascorbic acids, and some of the citric acid cycle intermediates profoundly modify the enzyme activity and this effect may have a regulatory role. The enzyme in the activated state also appears to be extremely labile. Reliably linear relationships with enzyme concentration and with time can be obtained over a short incubation time (10 min.) and at lower temperatures (25°C), only in the presence

of added crystalline bovine serum albumin (1 mg/ml). Under these carefully chosen conditions, a 15-fold stimulation of phlorizin hydrolase activity can be attained with 20 mM tartaric acid. In view of the pronounced effects of organic acids and anions like SO_4^{2-} and Cl^- and the extreme lability of the activated complex, many of the earlier results published on phlorizin hydrolase may require a reinvestigation.

Lactase-phlorizin hydrolase thus offers a unique situation to study the mechanism of activation of this enzyme by organic acids. Only tartaric, fumaric, citric, cis-aconitic and iso-citric acids but not the structurally closely related acids have a stimulatory effect suggesting a high degree of specificity. Currently, studies pertaining to the nature of binding of the activator and the number of active sites involved in the hydrolysis of phlorizin and lactose are being carried out.

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