Phosphatase Activities of Rat Intestinal Enterocytes and Their Relation to Diverse Luminal pH, with Special References to the Possible Localization of Phytase along the Brush Border Membrane*

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Summary. Phosphatase activities associated with the intestinal brush border membrane (BBM) of the rat were examined histochemically in relation to the characteristic environment of the intestine, where luminal pH fluctuates drastically between alkaline and acid pH ranges. Special attention was given to intestinal alkaline phosphatase (IALP) and phytase on the BBM. Whole body fresh-frozen sections of young rats and their rapidly frozen and freeze-substituted small intestines, embedded in Technovit 7100, were processed for the histochemical demonstration of phosphatase activity at three different pH values (9.2, 7.3, and 5.2), representing the deviation of luminal pH *in vivo*. Either an azo-dye method or lead-salt method was employed using naphthol AS-MX phosphate and ATP as substrate, respectively. With the azo-dye method, intense phosphatase reactions were demonstrated along the BBM at all three pH ranges. Phosphatase reactions of the BBM at pH 9.2 and 7.3 were abolished by L(+)-phenylalanine, heat pre-treatment, and EDTA chelation although some reaction remained at pH 7.3 after the treatment with EDTA or L(+)-phenylalanine. Phosphatase reactions of the BBM at pH 5.2 were resistant to L(+)-phenylalanine, L(+)-tartrate, PCMB and EDTA chelation, implying that the characteristics of the enzyme responsible for phosphohydrolysis at acid pH values differed from those at higher pH values. The lead-salt method in which ATP was used as substrate revealed intense reactions – which were dependent on Mg^{++} and stimulated by Ca^{++} and resistant to L(+)-phenylalanine – to be localized along the BBM at alkaline and neutral pH values, but not at acid pH values. *In vitro* experiments showed progressive hydrolysis of naphthol AS-MX phosphate by purified phytase at pH

5.2, in a dose-dependent manner, and suggested the possible involvement of phytase in the phosphatase reactions of the BBM at acid pH.

These data indicate that the phosphatase reactions at alkaline and neutral pH values, associated with the BBM of the rat intestine, represent IALP and Mg^{++}/Ca^{++} -ATPase, while those at acid pH appear to correspond to phytase activity, something which has not been demonstrated by histochemical methods despite the availability of extensive data based on biochemical analyses.

The membrane-bound enzymes and the transporters of intestinal enterocytes have been known to play important roles in the digestion, absorption and trans-cellular transport of various ions and molecules (FUJITA, 1988; UESATO, 1988). The intestinal luminal membrane lies in a peculiar environment of the digestive tract, where the pH fluctuates and the substrate for brush border enzymes are thought to be supplied through food intake. The pH profile of the luminal surface of the small intestine is known to vary from acid to alkaline values (WATSON et al., 1972; PETERLIK, 1985), depending on the balance of secretion of gastric acid (approx. pH 1), pancreatic juice (approx. pH 7.0-8.5), and enterocytes' excretes (approx. pH 7.0-8.5) (DEMAND et al., 1968; LEWIS, 1973). Notably in the duodenum, luminal pH has been reported to fluctuate from 3.5 to 8 (PETERLIK, 1985). Moreover, due to the presence of a 600-700 μm thick microclimate layer on the surface of microvilli

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associated with the peptide transporter 1 (PEPT1) and Na^+/H^+ -antiporter (SHIMADA and HOSHI, 1987; BOURQUE et al., 1994), which has a pH gradient ranging from 7.3 (the luminal bulk phase) to 6.0 (the deepest region) *in vitro* (LUCAS, 1984; SHIMADA, 1987), the surface pH of microvilli is estimated to be at least 1 unit pH diminution of gradient compared with the luminal pH (SHIMADA, 1987).

On the brush border membrane (BBM) of intestinal enterocytes, the presence of various enzymes such as alkaline phosphatase (ALPase) (HUGON and BORGERS, 1966; ACHLER et al., 1989; ZHANG et al., 1996), Ca^{++} -ATPase (RUSSEL, 1972), glycosidases (sucrase-isomaltase complex, maltase, lactase and trehalase) (MILLER and CRANE, 1961; DAHLQVIST and NORDSTRÖM, 1966; NEWCOMER and MCGILL, 1966; ASP et al., 1975; SEMENZA, 1981), amino peptidases (amino peptidase A, amino peptidase M, dipeptidyl amino peptidase IV, dipeptidyl carboxypeptidase and glutamyl transpeptidase) (FUJITA et al., 1972; KIM, et al., 1974; SKOVBJERG, 1981), and phytase (BITAR and REINHOLD, 1972) has been confirmed. On the basolateral membrane (BLM), Na^+/K^+ -ATPase (STIRING, 1972; GLYNN, 1985; SACHS et al., 1982), $\text{Mg}^{++}/\text{Ca}^{++}$ -ATPase (WASSERMAN and CHANDLER, 1985), and adenylate cyclase (FUJITA, 1988) have been shown to be located. The functional significance of most of these enzymes associated with the plasma membrane of enterocytes has been clarified either by physiological or histochemical evaluation, or by both. However, in regard to the phosphatases on the BBM of enterocytes, the actual roles of these enzymes have remained to be clarified, particularly those for intestinal ALPase (IALP) and phytase.

IALP is one of the isozymes of alkaline phosphatase [EC 3.1.3.1] whose kinetics of enzyme activity under physiological conditions have not been elucidated *in vivo*. This is partly due to the fact that, in histochemistry, ALPase reactions are generally detected at alkaline pH defined as optimal by biochemical standards, whereby maximal hydrolytic reactions are yielded. In the small intestine, however, it is unlikely that the *in vivo* value of luminal pH coincides with the biochemically optimal "high" pH value under normal conditions. In this context, the proposal based on biochemical data that IALP has the potential to serve as a phosphohydrolase under neutral pH (HIRANO et al., 1985) needs to be explored.

Biochemical properties of phytase [EC 3.1.3.8 or 3.1.3.26] and its function associated with digestion of the diet have been extensively studied, mainly among poultry species (VOHRA et al., 1965; BITAR and REINHOLD, 1972; MAGA, 1982; YANG et al., 1991) and a correlation between phytase and IALP has been

suggested (BITAR and REINHOLD, 1972; WILLIAMS et al., 1985). As regards the localization of phytase, however, it is intriguing to realize that, although it has been biochemically determined to be enriched in the BBM fraction of intestinal mucosa, no histochemical analysis of this enzyme exists in the literature.

In this experiment, we therefore sought to demonstrate the histochemical localization of phosphatase activities in the intestinal epithelium of the rat, in relation to diverse luminal pH values, fluctuating from acid to alkaline ranges, with an investigatory emphasis on IALP and phytase on the BBM.

MATERIALS AND METHODS

Tissue preparation

Whole body sectioning of fresh-frozen animals

Two-week-old ($n=5$) Wistar rats were anesthetized by ether inhalation followed by an intraperitoneal injection of 8% chloral hydrate solution (400 mg/kg B.W.). Then they were subjected to whole body freeze sectioning according to the procedures described by KAWAMOTO and SHIMIZU. (2000). Briefly, the animals were frozen by immersion in hexane kept at -94°C in a cooling apparatus (Neocool Bath, Yamato Kagaku, Tokyo, Japan). The frozen animals were immersed in 5% carboxymethyl cellulose (CMC) gel and further frozen in cold hexane. Frozen CMC blocks of the animals were cut with a cryomicrotome (CM 3500, Leica Microsystems, Wetzlar, Germany) with a disposable type carbide tungsten steel blade (Leica Microsystems, Nußloch, Germany), and 5- μm thick frozen sections supported by a polyvinylidene chloride film (Asahikasei Kogyo, Tokyo, Japan) were obtained. The frozen whole body sections were either freeze-dried in a freezer or stored at -80°C until use.

Rapid freeze, freeze-substitution

Adult Wistar rats (300 g body weight, $n=5$) were used as experimental animals. Under anesthesia (same as described above), the duodenum was excised immediately after killing the animals by phlebotomy, and routinely processed for rapid freezing after being cut into small pieces by immersion in liquid propane cooled with liquid nitrogen. The frozen pieces of the duodenum were freeze-substituted with absolute ethanol at -80°C for 4 days, gradually brought to room temperature, and then embedded in Technovit 7100 (Heraeus Kulzer GmbH & Co.KG., Werrheim, Germany). Two- μm thick sections were cut with glass

Fig. 1. Histochemical demonstration of phosphatase activity on 5 μ m thick whole body fresh-frozen sections at pH 9.2 (a, b), pH 7.3 (c, d), and pH 5.2 (e, f), by the azo-dye method using naphthol AS-MX phosphate as a substrate. At higher magnifications, intense phosphatase reactions are shown to be located along the luminal surface of the villi of the small intestine at all three pH ranges (b, d, f). a, c, e: $\times 1.1$; b, d, f: $\times 136$

knives or a Histoknife (Diatome, Bienne, Switzerland) attached to the ultramicrotome (Leica Ultra Cut, Leica Aktiengesellschaft, Vienna, Austria), and processed for enzyme histochemistry under various conditions.

Animal protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and all experiments were properly carried out under the Guidelines for Animal Experimentation at Tokyo Medical and Dental University.

Enzyme histochemistry

Azo-dye method

Incubation by the azo-dye method (BURSTONE, 1962) was performed at room temperature for 60 min using

a medium comprising 1.5 mM naphthol AS-MX phosphate as the substrate and 0.5 mM Fast Red Violet LB salt as the capture agent, in 0.1 M Tris-HCl buffer (pH 9.2 or 7.3) or 0.1 M acetate buffer (pH 5.2), supplemented with 3 mM MgSO_4 .

Lead-salt method: ATP as substrate

The incubation medium contained either 0.2 M acetate buffer (pH 5.2), 0.2 M Tris-maleate buffer (pH 7.3), or 0.05 M Tris-maleate buffer (pH 9.2), and 3 mM ATP as the substrate, 3 mM MgCl_2 , 3 mM CaCl_2 , 8% glucose and 3 mM PbNO_3 . After incubation for 60 min at room temperature, the sections were thoroughly rinsed in distilled water and treated with 1% ammonium sulfide solution to visualize the sites of lead precipitation.

Enzyme inhibition

To inhibit ALPase activities, some sections adhering to glass slide were heated at 65°C for 60 min (JENSEN et al., 1968) in 0.01 M PBS (pH 7.2), or immersed in 5% EDTA for 1 day at 4°C to eliminate metal ions prior to incubation. L(+)-phenylalanine (10 mM), a specific inhibitor of IALP (FISHMAN et al., 1962), 20 mM L(+)-tartrate, a specific inhibitor of high Mw acid phosphatase (ACPase), 1 mM PCMB, an inhibitor of low Mw ACPase, and 0.15 mM ouabain, a specific inhibitor of Na⁺/K⁺-ATPase, were respectively added to the incubation media. To determine the Mg⁺⁺ and/or Ca⁺⁺ dependency, some EDTA pre-treated specimens were reactivated by immersion in the medium supplemented with 50 mM MgSO₄ or 50 mM CaCl₂, or both of these salts in 0.1 M Tris-maleate buffer (pH 7.3) for 3 days at 4°C.

In vitro evaluation of phytase activity

Tube incubation

Phytase (purified from *aspergillus ficuum*) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 0.2 M acetate buffer (pH 5.2) at a concentration gradient ranging from 0.0034 to 1.73 unit/ml. To test whether phytase has the potential to hydrolyze the synthetic substrate used for the azo-dye phosphatase detection method, 0.5 mM naphthol AS-MX phosphate was added to the respective phytase solutions with 1.5 mM Fast Red Violet LB salt and 3 mM MgSO₄, and incubated for 30 min at 37°C.

Blotting paper incubation

Phytase was dissolved in 0.2 M acetate buffer (pH 5.2) at different concentrations as described for tube incubation. Each phytase solution was blotted on round-shaped pieces of filter paper (Toyo Roshi, Tokyo, Japan), weighing 4.5 mg per piece. Each piece of paper was calculated to contain 0.0019–1.92 munit/mg substratum of phytase. The dried pieces of paper, which contained phytase, were incubated in the azo-dye medium comprising 0.5 mM naphthol AS-MX phosphate as the substrate, 1.5 mM Fast Red Violet LB salt, and 3 mM MgSO₄ in 0.2 M acetate buffer (pH 5.2) for 60 min at 37°C. Another set of phytase-containing papers were incubated by the lead-salt method, using a medium containing phytic acid (phytate) as the substrate. The incubation medium contained 3 mM phytic acid, 8% glucose, 3 mM MgSO₄, and 3 mM PbNO₃ in 0.2 M acetate buffer (pH 5.2). After incubation for 60 min at 37°C in the phytate medium, the pieces of paper were immersed in 1%

ammonium sulfide solution for visualization of lead deposition. They were thoroughly rinsed in distilled water, and dried for observation.

Quantification of reaction products in blotted paper

The images of two sets of pieces of paper incubated either by the azo-dye method or lead-salt method for phytase reactions were captured by a flat bed-type image scanner (Epson GT-7600UF, Epson, Tokyo, Japan), and stored as gray-scale images. The relative intensities of phytase reactions in each paper were then quantified as a gray-scale density using computer software digital densitometer function (Photoshop ver. 5.0, Adobe, California, USA).

RESULTS

Whole body fresh-frozen sections

Strong enzyme reactions were localized throughout the whole body frozen sections of fresh-frozen rats at alkaline, acid, and neutral pH values. The whole body search of frozen sections revealed essentially identical distributions of phosphatase reactions at alkaline and neutral pH values at the tissue and organ levels. Especially in the small intestine, intense enzyme activities were observed along the luminal brush border of enterocytes at the three pH ranges. (Fig. 1a–e)

The Technovit sections of rapidly frozen and freeze-substituted intestine

With the alkaline incubation medium, strong enzyme reactions were observed along the brush border membrane (BBM) of intestinal enterocytes. Weaker but significant reactions were also localized along the basolateral membrane (BLM), in the Golgi regions, and in the small vesicular structures in the apical cytoplasm. The localization pattern of enzyme reactions was identical in both azo-dye and lead-salt methods in which naphthol AS-MX phosphate (Fig. 2a) and ATP (Fig. 3a) were used as substrates, respectively. At neutral pH, the localization patterns of enzyme reactions were similar to those shown at alkaline pH by both substrates (Figs. 2e, 3b). At acid pH, the use of naphthol AS-MX phosphate resulted in intense reactions on the BBM although no enzymatic reactions were detectable along the BLM of enterocytes (Fig. 2i). On the other hand, neither the BBM nor BLM showed enzymatic reactions when ATP was used as the substrate (Fig. 3c)

Inhibition experiments

At alkaline pH, EDTA chelation and heat pre-treatment respectively abolished all enzymatic reactions associated with the BBM and BLM of enterocytes either with naphthol AS-MX phosphate (Fig. 2c, d) or ATP as substrate. Supplementation of L(+)-phenylalanine strongly inhibited the BBM reaction detected by naphthol AS-MX phosphate (Fig. 2b), although it remained when ATP was used as the substrate (Table 1).

At neutral pH, heat pre-treatment abolished all enzymatic reactions in the enterocytes (Fig. 2h), whereas L(+)-phenylalanine treatment and EDTA chelation respectively caused a significant decline in enzymatic reactions, with some activity remaining along the BBM (Fig. 2f, g), by naphthol AS-MX phosphate as the substrate. ATP hydrolytic activity in the BBM at neutral pH was also resistant to L(+)-phenylalanine (Fig. 3d) and ouabain (Fig. 3e). Ion dependency tests revealed that the ATP hydrolytic activity in the BBM is dependent on Mg^{++} and stimulated by Ca^{++} (data not shown).

At acid pH, heat pre-treatment again totally inhibited the enzyme reactions in the enterocytes, when naphthol AS-MX phosphate was used as the substrate (Fig. 2l). EDTA chelation also abolished

enzyme reactions except those in the connective tissue core of villi (Fig. 2k). The BBM reaction at acid pH was not affected by L(+)-phenylalanine (Fig. 2j). Weak but significant enzymatic reactions remained along the BBM under the influence of L(+)-tartrate (Fig. 2m) and PCMB (Fig. 2n). Under all the conditions tested, no sign of enzymatic reactions was detected in the enterocytes at acid pH, when ATP was used as the substrate (Table 1).

Tube and blot paper analyses of phytase activity

In both the tube and blot paper incubation experiments, phytase hydrolyzed naphthol AS-MX phosphate instantaneously. Red reactions of various intensities were revealed in a dose-dependent manner at pH 5.2, when the substrate solution was applied to the enzyme-containing solutions or to the pieces of enzyme-containing blot paper (Fig. 4a, b). On the other hand, there were no visible reactions when phytic acid, a natural substrate of phytase, was used as the substrate and incubated by the lead-salt method at pH 5.2 (Fig. 4c). However, densitometry analysis of the blot paper barely indicated any sign of hydrolysis of phytate in a dose-dependent manner (Fig. 5).

When the color intensity of the blot paper in both

Table 1. Effects of pH and inhibitors on phosphatase activation in the intestinal enterocytes

pH	Inhibition	Naphthol AS-MX phosphate		ATP	
		BBM	BLM	BBM	BLM
9.2	None	+++	++	+++	+++
	EDTA	—	—	—	—
	Heat	—	—	—	—
	L(+)-phenylalanine	—	—	++	—
7.3	None	+++	+++	+++	+++
	EDTA	++	+	—	—
	Heat	—	—	—	—
	L(+)-phenylalanine	+	—	++	—
5.2	None	++	—	—	—
	EDTA	+	—	—	—
	Heat	—	—	—	—
	L(+)-phenylalanine	++	—	—	—
	L(+)-tartrate	+	—		
	PCMB	+	—

Fig. 4. Tube and blot paper analysis of phytase activity at pH 5.2 using naphthol AS-MX phosphate (a, b) and phytic acid (c) as substrates, demonstrated respectively by the azo-dye method and lead-salt method. Phytase appears to hydrolyze naphthol AS-MX phosphate effectively at acid pH in a dose-dependent manner (a, b), whereas no visible sign of hydrolysis of phytase is noted (c).

Fig. 5. Diagrammatic representation of the relative intensities of hydrolytic reaction products of naphthol AS-MX phosphate (♦) and phytate (▲) by phytase at pH 5.2.

experiments was converted into gray scale data and subjected to densitometry, the intensity of the reactions incubated with naphthol AS-MX phosphate as the substrate exceeded that with phytic acid, when the concentration of phytase in the blot paper was beyond approx. 0.2 munit/mg weight substratum (Fig. 5).

DISCUSSION

Enzyme activity on the BBM at acid pH

Phytase (myo-inositol hexakisphosphatase) [EC 3.1.3.8 or 26] is known to be present in the small intestinal mucosa of various vertebrates including the rat, chicken, calf, pig, and human (BITAR and REINHOLD, 1972). Its function has been considered to the hydrolyze phytic acid (phytate: inositol hexakisphosphate) that is contained in many types of foodstuffs such as plants, seeds, and pollen. It has been reported that phytic acid forms insoluble complexes with a variety of nutritionally essential metals such as calcium, zinc, magnesium, and iron, and inhibits metal uptake (VOHRA, et al. 1965; MAGA, 1982). Phytate hydrolysis is thus important to facilitate the uptake of inositol derivatives and inorganic phosphate, and prevent a deficiency of metals (YANG et al., 1991).

Though it depends on the species of animals, the optimal pH of phytase, determined by using phytate as a substrate, has been reported to range from pH 5.4 to pH 8.3, and the enzyme activity of phytase has been detected in the BBM fraction of intestinal mucosa by biochemical assays operated at acid pH (BITAR and REINHOLD, 1972; YANG et al., 1991; ENGELN et al., 1994). Despite an abundance of biochemical data, the activity of phytase on the BBM has never been demonstrated by histochemical methods, most likely owing to extremely low concentrations of this enzyme in the BBM of the small intestine, about one thousandth of that of IALP (MAENZ and CLASSEN, 1998). In this study, the BBM showed phosphatase activities at all three (alkaline, acid, and neutral) pH values. Since the enzyme activity at acid pH value was different from those detected at alkaline and neutral pH values with respect to the substrate specificity and the sensitivity to L(+)-phenylalanine, it is implicated that the phosphatase reactions on the BBM of rat intestinal enterocytes at acid pH represent the activity of a phosphatase different from those responsible for the reactions detected at alkaline and neutral pH values. In previous histochemical studies, LANDSVERK (1980) localized the

enzyme activity on the BBM at acid pH by using β -glycerophosphate as a substrate, and suggested its significance as a fluoride-resistant acid phosphatase. IWANAGA et al. (1994) also demonstrated phosphatase activity on the BBM at acid pH by the azo-dye method. However, in our observations, since the enzyme reaction of the BBM at acid pH was resistant to L(+)-tartrate and sensitive to EDTA chelation when detected by naphthol AS-MX phosphate as a substrate, the profiles of this enzyme appeared different from those of acid phosphatase [EC 3.1.3.2].

In *in vitro* study, we have confirmed that phytase has the potential to hydrolyze naphthol AS-MX phosphate at acid pH and makes an abundance of visible complexes with a capture agent comparable to histochemical reaction products. Based on this evidence, we suggest that phytase is the likeliest candidate for the enzyme responsible for the phosphatase reactions detected on the BBM at acid pH. It is reported that the concentration of phytase in the BBM of the rat intestine is 0.36 munit/mg protein (YANG et al., 1991). In our blot paper analysis, visible reaction products of phytase activity could be demonstrated when the enzyme concentration was in the vicinity of *in vivo* value of phytase (0.36 munit/mg). It is therefore strongly suggested that the reactions demonstrated on the BBM at acid pH by the azo-dye method most likely represent the hydrolytic activity of phytase. Intense enzymatic reactions detected in the blot paper analysis of phytase using naphthol AS-MX phosphate as the substrate, and the lack of detectable hydrolysis of phytate under similar conditions (Fig. 4) may be explained by the difference in the velocity of the reactions in both systems. Further studies are needed to test the hypothesis.

Phosphatase activities on the BBM at neutral and alkaline pH values

In the enterocytes of duodenal mucosa, phosphatase reactions and inhibition patterns at alkaline and neutral pH values have shown to be identical in both pH ranges with both substrates tested in this experiment, though a slight difference in sensitivity for EDTA chelation and L(+)-phenylalanine has been noted. It is thus obvious that, besides acid phosphatase (phytase), the BBM contains heat sensitive phosphatase(s) that are reactive at both neutral and alkaline pH values. They are totally inhibited at alkaline pH but only partially inhibited at neutral pH by L(+)-phenylalanine, a specific inhibitor of IALP, and by EDTA chelation that chelates metal ions. These and the immunolocalization patterns of IALP in the intestinal mucosa (ACHLER et al., 1989; ZHANG

et al., 1996) suggest an intimate correlation between IALP and phosphatase reactions demonstrated on the BBM at both alkaline and neutral pH values, and also indicate a potential of IALP to serve as a phosphohydrolase at neutral pH.

The enzymatic reaction remaining on the BBM after EDTA chelation or L(+)-phenylalanine treatment at neutral pH (Fig. 2f, g) may either represent the trace activity of phytase or indicate the existence of another phosphohydrolase that can hydrolyze naphthol AS-MX phosphate at neutral pH. In fact, phytase has shown to hydrolyze naphthol AS-MX phosphate at neutral pH in our *in vitro* tube experiment (data not shown).

As summarized in Table 1, ATP and naphthol AS-MX phosphate resulted in the identical localization of phosphohydrolytic reactions on the BBM at neutral pH, with or without inhibitors for IALP. Mg^{++}/Ca^{++} -ATPase is therefore suggested to be one of the potential candidates for the enzyme responsible for the reactions remaining on the BBM at neutral pH (Fig. 3b, d, e).

In summary, we have investigated the phosphatase activities in the intestinal enterocytes at three different pH values from acid to alkaline, through neutral pH, and depicted the candidate enzymes which represent the phosphohydrolytic activities at the respective pH values. In this experiment, a possible phytase activity has been demonstrated on the BBM of the rat intestine, which is activated at acid pH. We have also demonstrated histochemical evidence suggesting that IALP serves as a phosphohydrolase not only at alkaline pH but also at neutral pH range in the intestinal enterocytes. The presence of various phosphatases of different pH optimums in the enterocytes appears to be suited to the specific environment of the intestinal lumen where pH fluctuates to a great extent dependent on the luminal content.

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