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# Characterization of brush border membrane-bound alkaline phosphatase activity in different segments of the porcine small intestine

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*This study was conducted to characterize enterocyte apical membrane-bound alkaline phosphatase activity in different segments of the porcine small intestine. Duodenal, jejunal, and distal ileal segments were isolated from three 26-kg pigs and enterocyte brush border membrane, enriched between 19- and 24-fold in sucrase specific activity, was prepared by $Mg^{2+}$ precipitation and differential centrifugation. With P-nitrophenyl phosphate as substrate, the optimum pH for porcine brush border membrane-bound alkaline phosphatase activity was defined to be 10.5 for all three segments. At the optimal pH, the kinetics of membrane-bound alkaline phosphatase were determined for the three intestinal segments. The affinity of this enzyme ($K_m$, mM) in the jejunum (0.64 ± 0.07) was four times greater than that in the duodenum (2.75 ± 0.59) and the distal ileum (2.71 ± 1.14). These results indicate that different isomers of membrane-bound alkaline phosphatase might have been expressed in different segments of porcine small intestine. The maximal specific activity ($V_{max}$, μmol/mg protein · min) of this enzyme was highest in the duodenal (7.74 ± 0.95), intermediate in the jejunal (4.31 ± 0.18), and lowest in the distal ileal (3.53 ± 0.84) brush border membrane. Therefore, the maximal specific activity of brush border membrane-bound alkaline phosphatase along the intestinal longitudinal axis in growing pigs decreases from the duodenum toward the distal ileum.* (J. Nutr. Biochem. 10:299–305, 1999) 

**Keywords:** small intestine; brush border membrane; alkaline phosphatase; pigs

## Introduction

Intestinal alkaline phosphatase (*EC* 3.1.3.1) is present in both soluble and membrane-bound forms in suckling animals,[1–3] whereas this enzyme is found predominantly on the apical surface of the differentiated enterocyte in postweaned animals.[1,2] Studies in several animal species have shown that this enzyme is attached to the apical membrane through a covalent phosphatidylinositolglycan linkage.[4,5] Both a direct Golgi-to-apical membrane pathway and an indirect pathway via basolateral membrane have been suggested as mechanisms of targeting the enzyme molecules to the apical membrane.[6] More recently, intestinal alkaline phosphatase has been reported to be associated with a surfactant-like particle and secreted into the lamina propria, lymphatic capillaries, serum, and small intestinal lumen in response to fat intake.[7,8]

As one of the most abundant intestinal brush border membrane-bound proteins, several functions associated with the intestinal alkaline phosphatase have been suggested. This enzyme is responsible for hydrolyzing phosphoric ester bonds of organic compounds,[9] plays a role in fat absorption,[7,8,10] and is likely involved in intestinal transcellular nutrient transport.[11,12] There are marked species differences in the intestinal alkaline phosphatase[13] and the comparative biochemical and physiologic aspects have been investigated in several animal species including rodents,[1,14,15] avian and bovine species,[15] and humans.[15] The intestinal alkaline phosphatase genes also have been cloned

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and characterized in rats,[16,17] bovine species,[18] dogs,[19] and humans.[20,21]

However, there is a scarcity of information about the porcine intestinal alkaline phosphatase, such as maximal pH and enzyme distribution along the small intestinal longitudinal axis. Therefore, this study was conducted to characterize the activity and distribution of intestinal apical membrane-associated alkaline phosphatase activity in different segments of the small intestine in growing pigs.

## Materials and methods

### *Chemicals*

Bio-Rad dye reagent was purchased from Bio-Rad laboratories (Richmond, CA USA). Hepes, Trizma · base, Trizma · HCl, phenylmethysulfonyl fluoride (PMSF), bovine serum albumin (fraction V), D- glucose determination kit, *P*-nitrophenyl phosphate, and other chemicals were from Sigma Chemical Co. (St. Louis, MO USA).

### *Animals and mucosa collection*

The intestinal mucosal samples used in these studies were collected from three 26-kg pigs obtained from the Purdue University Swine Research Center (a cross of Yorkshire-Landrace dams and Hampshire-Duroc sires). While pigs were under anesthesia, the small intestine, traversed at 5 cm from both the pyloric and the ileocecal sphincters, was surgically removed. A 80-cm duodenal segment was dissected at 5 cm posterior to the pyloric sphincter. A 100-cm jejunal segment was traversed at 250 cm posterior to the pyloric sphincter. A 80-cm ileal segment was dissected at 5 cm anterior to the ileocecal sphincter. The isolated intestinal segments were immediately flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF, pH 7.4) and divided into 15-cm segments. The mucosa was collected by scraping the luminal surface firmly with a spatula. The mucosal scrapings were pooled within the same pig, divided, and placed in screw-capped plastic tubes. Each tube, which contained approximately 15 g of tissue, was tightly capped and stored at −70°C. The experimental protocol was approved by Purdue University Animal Care and Use Committee.

### *Preparation of intestinal brush border membrane*

Porcine intestinal brush border membranes were prepared by $Mg^{2+}$ precipitation and differential centrifugation according to an established procedure.[22] All tissue preparation and centrifugation were conducted at 4°C. Specifically, approximately 15 g of mucosal scraping was thawed in ice-cold homogenizing buffer (50 mM D-mannitol and 0.1 mM PMSF, pH 7.4, adjusted with 0.50 M Hepes buffer) at a ratio of 20 mL of homogenizing buffer per gram of mucosal scrapings. The thawed tissue and homogenizing buffer mixtures were divided into six 45-mL plastic tubes and the contents of each tube were homogenized with a polytron homogenizer. The resulting homogenate was pooled and centrifuged in a Sorvall SS-34 rotor at $2{,}000 \times g$ for 15 minutes. After removing the top foamy layer and discarding the pellet, supernatant was mixed with 1 M $MgCl_2$ to a final concentration of 10 mM $MgCl_2$, stirred for 15 minutes, and then centrifuged at $2{,}400 \times g$ for 15 minutes. The top foamy layer was discarded and the resultant supernatant was centrifuged at $19{,}000 \times g$ for 30 minutes to pellet the crude brush border membrane. The supernatant was discarded and the crude brush border membrane pellets were suspended in suitable amount of a buffer (300 mM D-mannitol, pH 7.4, adjusted with 0.50 M Hepes buffer) and centrifuged at $39{,}000 \times g$ for 30 minutes to generate the final brush border membrane pellets, which were again re-suspended in the same buffer for enzyme assay.

### *Protein and enzyme assays*

Protein concentration was determined according to the Bradford method[23] using the Bio-Rad protein dye reagent and bovine serum albumin (fraction V) as the standard.

Sucrase (*EC* 3.2.1.48) was assayed according to the procedure of Dahlgvist.[24] In the sucrase activity assay, incubations were conducted in a final volume of 50 μL containing mucosal homogenate sample (12.5 μg protein) or brush border membrane suspension sample (6.3 μg protein), 28.0 mM sucrose, and 50.0 mM sodium maleate, pH 6.0, at 37°C for 20 minutes. The end product of this enzyme reaction, D-glucose, was measured with the Sigma D-glucose determination kit (HK).

$Na^+/K^+$-ATPase (*EC* 3.6.1.3) was assayed by the method of Schwartz *et al*.[25] In the $Na^+/K^+$-ATPase activity assay, samples were incubated in a final volume of 1 mL containing 12.5 μg membrane protein, or 25.0 μg mucosal homogenate protein, 5.0 mM $MgCl_2$, 100.0 mM NaCl, 20.0 mM KCl, and 6.0 mM $Na_2ATP$, pH 7.4, at 37°C for 20 minutes in the presence or absence of 5 mM ouabain. Inorganic phosphate was measured by the method of Heinoen and Lahti.[26]

Alkaline phosphatase (*EC* 3.1.3.1) was assayed according to established procedures.[27,28] Potassium fluoride (2.0 mM) was used in all alkaline phosphatase assays to inhibit possible acid phosphatase activity.[28]

For the determination of optimum pH of brush border membrane-bound alkaline phosphatase activity in different segments of porcine small intestine, incubations were conducted at 37°C for 10 minutes in a final volume of 1 mL containing 10 μg protein of duodenal, jejunal, or ileal brush border membrane suspension, 2.0 mM KF, 4.0 mM $MgCl_2$, and 2.0 mM *P*-nitrophenyl phosphate, pH ranging from 4.0 to 11.0. The buffers used were sodium acetate (pH 4–6), Trizma · HCl and Trizma · base (pH 6.5–9.0), and $NaHCO_3$/NaOH (pH 9.5–11.0).

To approximate the linearity of the alkaline phosphatase reaction, incubations were carried out at 37°C for designated time periods (0, 10, 20, 30, 40, 50, and 60 minutes) in a final volume of 1 mL containing jejunal brush border membrane suspension (10.0 μg protein), 2.0 mM KF, 4.0 mM $MgCl_2$, and *P*-nitrophenyl phosphate (0.2 and 2.0 mM), pH 10.5.

In the inhibition experiments, alkaline phosphatase activity was determined at 37°C for 10 minutes in a final volume of 1 mL containing jejunal brush border membrane suspension (10.0 μg protein), 2.0 mM inhibitors (D-mannitol, $Na_2EDTA$, sodium phytate, $ZnCl_2$, L-phenylalanine, and L-leucine), 2.0 mM KF, 4.0 mM $MgCl_2$, and 2.0 mM *P*-nitrophenyl phosphate, pH 10.5.

To determine the kinetics of membrane-bound alkaline phosphatase in the three segments of porcine small intestine, the enzyme assays were carried out at 37°C for 10 minutes in a final volume of 1 mL containing 10 μg protein of duodenal, jejunal, or ileal brush border membrane suspension, 2.0 mM KF, 4.0 mM $MgCl_2$, and *P*-nitrophenyl phosphate (0–4.0 mM), pH 10.5.

In the above alkaline phosphatase activity assays, incubations were stopped by adding 1 mL of 0.50 M NaOH into the culture tubes, and the end product of the enzyme reaction, *P*-nitrophenol, was measured in 0.25 M NaOH at 400 nm.

### *Calculations and statistical analyses*

The kinetic parameters of alkaline phosphatase were analyzed according to Michaelis-Menten equation using the *Fig. P* curve fitting program (*Fig. P*, 1993, Biosoft, Cambridge, UK). Comparison of kinetic parameters was conducted using the two-tailed Student's *t*-test.[29]

**Table 1** Specific activity and enrichments* of sucrase in the brush border membrane fractions of different segments of porcine small intestine

| Items | Small intestinal segments | | |
|---|---|---|---|
| | Duodenum | Jejunum | Ileum |
| Specific activity in homogenate | 17.3 ± 0.4 | 22.5 ± 2.3 | 42.4 ± 2.6 |
| Specific activity in brush border | 370.8 ± 3.9 | 528.5 ± 47.1 | 825.7 ± 87.0 |
| Enrichment | 21.5 ± 0.6 | 23.6 ± 0.9 | 19.4 ± 0.9 |

*Values are means ± SE (specific activity: nmol/mg protein · min; $n = 3$ separate membrane preparations from three separate pig intestinal mucosal samples). Enrichment (fold) = ratio of sucrase specific activity (nmol/mg protein · min) in the brush border membrane fraction to that in the mucosal homogenate.

## Results and discussion

Relative to the mucosal homogenate, the final brush border membrane preparation had a 19- to 24-fold enrichment in sucrase specific activity (*Table 1*). The enrichment of specific activity of $Na^+/K^+$-ATPase in the final brush border membrane preparation was less than two folds relative to the mucosal homogenate.

At substrate concentrations of 0.2 and 2.0 mM, the time course of alkaline phosphatase-catalyzed reaction is illustrated in *Figure 1*. The reaction appeared to be linear up to 20 minutes. Therefore, 10-minute incubation was used in the inhibition and kinetic experiments.

To determine the optimal pH for alkaline phosphatase activity, effects of pH on the membrane-bound alkaline phosphatase activity in the duodenal, jejunal, and ileal segments were examined (*Figure 2*). For all the intestinal segments, the optimal pH of the activity of alkaline phosphatase was 10.5. Using the same substrate (*P*-nitrophenyl phosphate), a previous study showed differences in the pH optimum for this enzyme among animal species, with an optimum pH of 9.0 in rats and avian species and 9.5 in bovine species and humans.[15] On the other hand, it has been established that an acidic microclimatic environment (5.3–6.1) exists on the small intestinal mucosal surface area.[30,31] Therefore, under physiologic conditions, intestinal brush border membrane-bound alkaline phosphatase activity should be low.

The effects of 2 mM potential inhibitors on intestinal membrane-bound alkaline phosphatase activity were examined (*Figure 3*). $Na_2EDTA$ and sodium phytate at the concentration of 2 mM demonstrated a trend ($P < 0.20$), causing 18% and 15% decrease of the enzyme activity, respectively (*Figure 3b*). The inhibition was likely due to chelation of $Mg^{2+}$, an essential divalent cation that is required as a cofactor for the enzyme by EDTA and phytic acid. Thus, the magnitude of inhibition is likely dependent on the concentration of the inhibitor relative to the concentration of $Mg^{2+}$. As shown in *Figure 3*, membrane-bound alkaline phosphatase activity was strongly inhibited by $Zn^{2+}$; 79% of its activity was inhibited in the presence of 2 mM $ZnCl_2$. Mammalian alkaline phosphatases are zinc-containing metalloenzymes.[32] The $Zn^{2+}$ concentration required for maximal alkaline phosphatase activity was reported to be within 25 to 50 μM.[15] Nevertheless, higher concentrations of $Zn^{2+}$ (millimolar range) caused reduction in alkaline phosphatase activity as demonstrated in this study and in a previous study in rodents.[15] On the other hand, 2 mM of L-leucine and L-phenylalanine had no effect on the activity of the membrane-bound alkaline phosphatase in this study. Previous studies with rodents indicated that 20

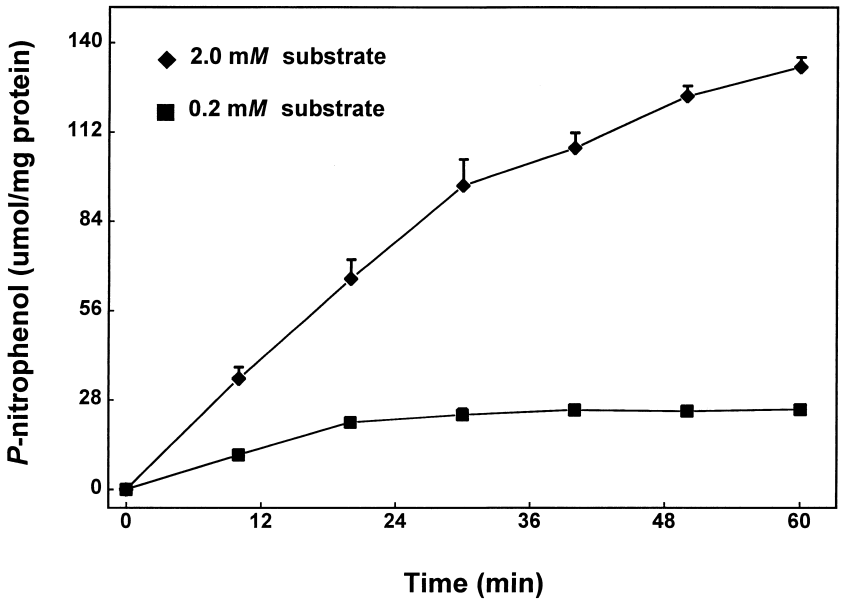


**Figure 1** Time courses of porcine jejunal brush border membrane-bound alkaline phosphatase hydrolyzing 0.2 and 2.0 mM *P*-nitrophenyl phosphate. Each point represents the mean and standard error derived from three separate experiments (each experiment in triplicate).

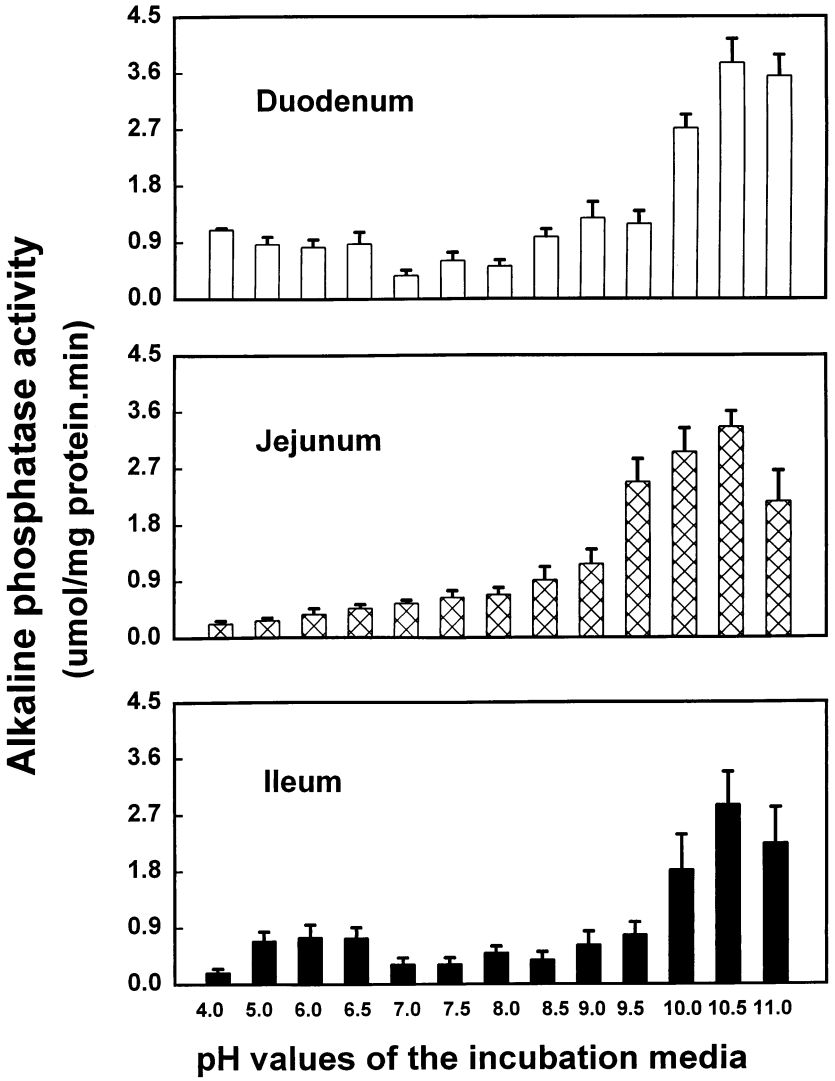


**Figure 2** Effects of pH on enterocyte brush border membrane-associated intestinal alkaline phosphatase activity in different segments of porcine small intestine. Each bar represents the mean and standard error derived from three separate experiments (each experiment in triplicate).

mM or higher concentrations of L-phenylalanine effectively depressed the activity of this enzyme.[14,15]

The kinetics of brush border membrane-bound alkaline phosphatase activity in the duodenal, jejunal, or distal ileal segments are shown in *Figure 4*. There were differences ($P < 0.01$) in the maximal specific activity of this enzyme between the intestinal segments: It was high in the duodenal, intermediate in the jejunal, and low in the distal ileal brush border membrane (*Table 2*). Numeric values of kinetic parameters are specific only to the substrates used. By using different substrates, one should expect to obtain different $K_m$ and $V_{max}$ values for alkaline phosphatase activity. Although we used only *P*-nitrophenyl phosphate, a typical experimental substrate, in our kinetic measurements, the patterns of regional differences in $K_m$ and $V_{max}$ values, as revealed with this substrate, should, in principle, stay unchanged if these are determined with more physiologic substrates. Consistent with these findings, Borowitz and Granrud[33] reported steeper decreasing gradient toward the distal ileum in brush border membrane-bound alkaline phosphatase activity in growing rabbits. Because the $K_m$ value of this enzyme was similar between the duodenum and the distal ileum, the $V_{max}$ gradient between the two segments should reflect the differences in the expression levels of this enzyme molecule; that is, more alkaline phosphatase molecules were associated with the duodenal than with the distal ileal brush border membrane. In the case of the jejunal segment, because the $K_m$ value was different from those in the duodenum and the distal ileum, the $V_{max}$ gradient formed between the duodenum and the distal ileum was likely due to the differences in both enzyme affinity and levels of enzyme expression in different intestinal regions. Therefore, the differences in the density of this enzyme protein molecule and the possible expression of different isoenzymes were perhaps responsible for the regional variation in the maximal specific activity on the intestinal apical membrane.

Affinity of the membrane-bound alkaline phosphatase was similar between the duodenal and the distal ileal segments ($P > 0.01$). However, the affinity of alkaline phosphatase in the jejunum was four times ($P < 0.01$) higher than that in the duodenal and the ileal segments. Two possible mechanisms may explain the distinctly different enzyme affinity values between the intestinal segments.

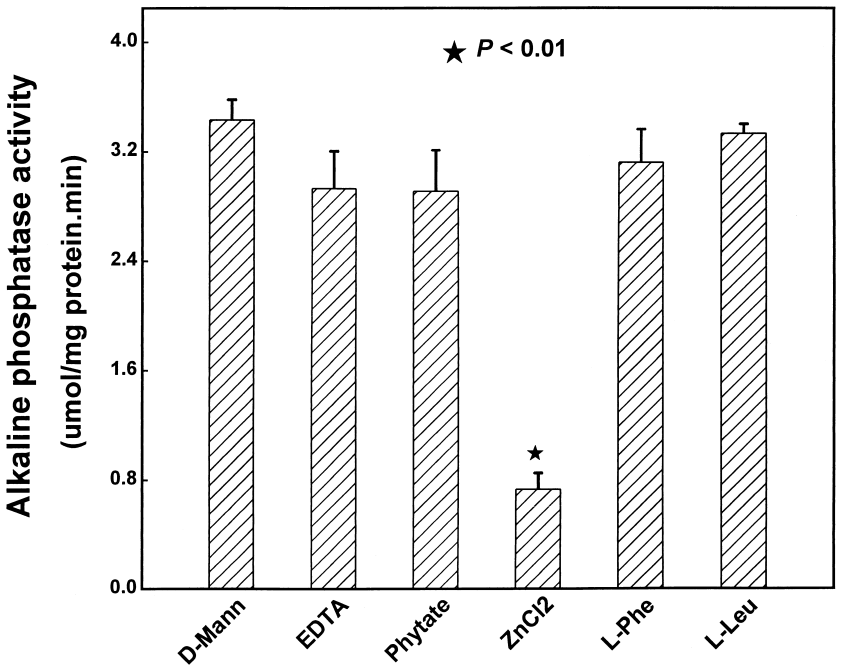


**Figure 3** Effects of 2 mM various potential inhibitors on the jejunal brush-border membrane-associated alkaline phosphatase activity. Each bar represents the mean and standard error derived from three separate experiments (each experiment in triplicate).

Membrane-bound alkaline phosphatase is a glycoprotein that is posttranslationally glycosylated in the Golgi apparatus. Regional differences in glycosyltransferase activity, as demonstrated in previous studies,[34] might have resulted in regional variation in the glycosylation of the membrane-bound alkaline phosphatase protein molecules, which, in turn, could affect the enzyme affinity. Alternatively, several studies have demonstrated that multiple forms of alkaline phosphatase, (i.e., isoenzymes) are expressed in the small intestine of rodents,[3,4,35] bovine species,[36,37] and humans.[38] Two distinct intestinal alkaline phosphatase mRNA transcripts origination from the same gene also were identified to be responsible for the two isoforms of the enzyme in rats and humans.[4,35,38] In light of the dramatic regional differences in the enzyme affinity values, we speculate that two distinct isoenzymes of alkaline phosphatase also might have been expressed on the brush border membrane along the longitudinal axis of the porcine small intestine, one in the jejunal region and the other in the duodenal and the distal ileal regions. To further confirm and characterize the biochemical aspects of the possible isomers of alkaline phosphatase in different regions of the porcine small intestine, it is essential to isolate and purify the enzyme molecules and compare their isoelectric points, electrophoretic mobility nature, amino acid sequences, molecular weights, and so forth. These will be the focus of our continued effort in this research area in the future.

In summary, the optimum pH for porcine small intestinal membrane-bound alkaline phosphatase activity was at 10.5. The maximal specific activity of membrane-bound alkaline phosphatase is distributed in decreasing gradients along the small intestinal longitudinal axis. Our kinetic data suggest

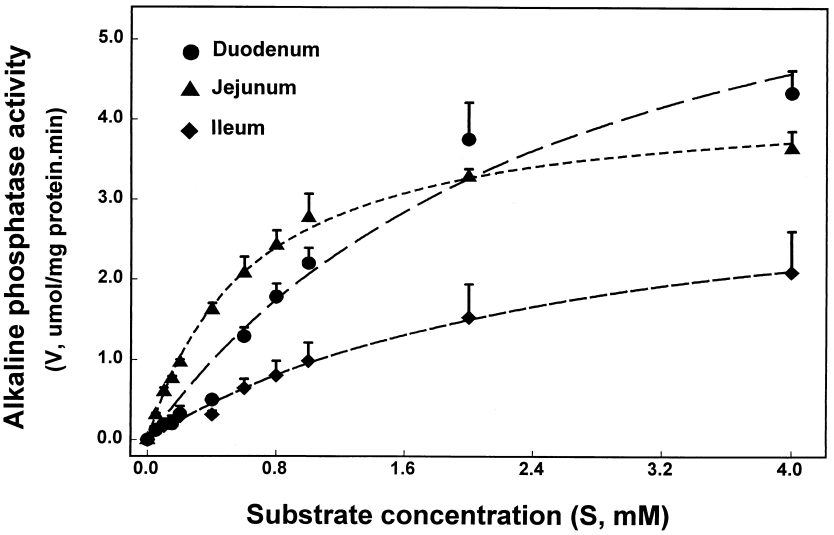


**Figure 4** Kinetics of porcine small intestinal brush border membrane-bound alkaline phosphatase activity in hydrolyzing *P*-nitrophenyl phosphate. Each point represents the mean and standard error derived from three separate kinetic experiments (each experiment in triplicate).

**Table 2** The Michaelis constant ($K_m$) and the maximal enzyme activity ($V_{max}$) of brush border membrane-bound alkaline phosphatase in different segments of porcine small intestine (parameters ± SE)

| Kinetic parameters | Small intestinal segments | | |
|---|---|---|---|
| | Duodenum | Jejunum | Ileum |
| $K_m$, mM | 2.75 ± 0.59[a] | 0.64 ± 0.07[b] | 2.71 ± 1.14[a] |
| $V_{max}$, μmol/mg protein · min | 7.74 ± 0.95[a] | 4.31 ± 0.18[b] | 3.53 ± 0.84[c] |

$K_m$: Michaelis constant of the enzyme affinity derived from three separate kinetic experiments ($P < 0.05$, $n = 31$).
$V_{max}$: the maximal enzyme activity derived from three separate kinetic experiments ($P < 0.05$, $n = 31$).
[a,b,c]Means in the same row with different superscript letters differ ($P < 0.01$).

that two isomers of membrane-bound alkaline phosphatase are likely expressed in different regions of porcine small intestine.

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