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Localization and characterization of rat small intestinal aminopeptidase P and its role in prolyl peptide digestion

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The distribution of aminopeptidase P was examined in rat small intestinal epithelial cells using the substrate Gly-Pro-Hyp. Soluble activity accounted for 65% of the total activity in these cells while 27% was membrane bound and localized to the brush border membrane fraction. Soluble and brush border membrane associated activities differed with respect to their pH optima, kinetic constants, and thermostability indicating that they are due to different enzymes. Both activities, however, exhibited similar sensitivity to several types of inhibitors. Brush border membrane aminopeptidase P was readily inhibited by metal chelating agents and was reactivated by addition of increasing concentrations of Mn^{2+}. The mode of membrane association was investigated by detergent solubilization and enzymatic release of aminopeptidase P. A phosphatidylinositol-specific phospholipase C was the most effective in releasing aminopeptidase P, suggesting that the enzyme is anchored via a glycosyl-phosphatidylinositol moiety similar to alkaline phosphatase. These results indicate that aminopeptidase P is a major intestinal brush border membrane enzyme and probably plays an important role in conjunction with other intestinal prolyl peptidases in the digestion of proline containing peptides and proteins. (J. Nutr. Biochem. 6: 104–110, 1995.)

Keywords: intestine; brush border membrane; peptidase; protein digestion

Introduction

Proteins that are relatively rich in proline such as collagen, gliadin, and α-casein are important components of a normal diet. Although many dietary proteins are efficiently hydrolyzed by the digestive peptidases of gastric and pancreatic origin, prolyl peptide bonds are generally not hydrolyzed by these proteases. Thus, proline-containing peptides may escape the action of the gastric and pancreatic enzymes and be relatively intact upon reaching the lumen of the small intestine.

Previous studies from our laboratory have shown that the mammalian small intestine contains several brush border membrane peptidases that are unique in their ability to hydrolyze peptide bonds involving proline.[1–3] These enzymes include dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), angiotensin-converting enzyme (ACE, EC 3.4.15.1), and carboxypeptidase P (CPP, EC 3.4.17.-). The specificity of these enzymes is complimentary to one another, and studies have shown that they can act in a sequential, concerted manner to efficiently hydrolyze prolyl peptides.[4,5] In addition, studies have shown that a proline-specific aminopeptidase (aminopeptidase P) is present in bacteria,[6] and an enzyme with similar specificity is found in the membrane fractions of lung,[7–9] and kidney.[10,11] Soluble forms of the enzyme have been reported in brain tissue and human leucocytes.[12,13] Studies[14,15] have also indicated that this enzyme is present in the small intestine and localized to the brush border membrane fraction where it may play an important role in the digestion of dietary prolyl peptides. Although aminopeptidase P has been well characterized from several tissues, relatively little is known about the intestinal

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variety of the enzyme. Therefore, the following studies were undertaken in an effort to localize and characterize intestinal aminopeptidase P and to examine its possible role in protein digestion.

Methods and materials

The tripeptide Gly-Pro-Hyp was purchased from Bachem Biosciences, Inc. (Philadelphia, PA USA) and phosphatidylinositol-specific phospholipase C (*Bacillus cereus*) was obtained from Boehringer Manheim Biochemicals. All chemicals used in this study were of reagent grade quality.

Isolation of brush border membranes

Male Wistar rats (≈300 g) (Simonsen Labs, Gilroy, CA USA) were routinely maintained on a standard laboratory chow diet. Rats were fasted overnight, sacrificed, and the entire small intestine was removed. The contents of the intestine were rinsed out with cold saline, and mucosal scrapings were prepared. Cell homogenates and brush border membranes were prepared from the mucosal scrapings by the method of Kessler et al.[16] In some experiments the entire small intestine was measured and divided into six equal length segments. Brush border membranes were prepared from each segment by the procedure described above. Cytoplasmic (soluble) and total membrane fractions of intestinal mucosal cells were obtained by centrifuging samples of total cell homogenates at 100,000*g* for 1 hr.

Enzyme assays

Aminopeptidase N (APN, EC 3.4.11.2), DPP IV, and alkaline phosphatase (AL-P, EC 3.1.3.1) were assayed as described previously.[17–19] Aminopeptidase P activity was routinely monitored using a modified version of a previously described procedure.[10] Briefly, enzyme preparations were initially preincubated for 1 hr at 37°C in an equal volume of "activating" buffer (24 mM sodium barbiturate, 25 mM sodium acetate, 108 mM sodium chloride, 20 mg/ml of bovine serum albumin, and 2 mM $MnCl_2$, pH 8.0). Aliquots of the activated enzyme preparation were then incubated with 17 mM Gly-Pro-Hyp in activation buffer for 1 hr at 37°C. The enzyme reaction was terminated by addition of an equal volume of 6% sulfosalicylic acid. Precipitated proteins were removed by centrifugation, and the supernatant was filtered with an Amicon Centrifree MPS-1 micropartition system. Hydrolysis of Gly-Pro-Hyp was monitored by measuring the amount of free glycine in the filtrate using an automated Beckman 119 CL amino acid analyzer.

pH optimum, thermostability, and kinetic constants

The pH optimum for hydrolysis of Gly-Pro-Hyp was determined in the range of pH 6.8 to 9.4 using barbiturate buffer and the standard assay procedure described above. The heat stability of enzyme preparations was assessed after preincubation at 50 and 60°C for 20 and 40 min prior to assay. For the determination of kinetic constants (*Km* and *V*), the concentration of substrate (Gly-Pro-Hyp) was varied from 1 to 40 mM. A computer graphing program was used to analyze the results and generate an Eadie–Hofstee plot (*V* vs. *V/S*) from which *Km* and *V* values were determined.

Effect of inhibitors and cations

The effect of various inhibitors on aminopeptidase P activity was determined by preincubating enzyme preparations with the individual inhibitor at room temperature for 30 min prior to assay. The same concentration of inhibitor was present during the assay. To test the effect of various cations on aminopeptidase P activity, brush border membranes were washed with 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, by sedimentation. The membranes were resuspended, activated with Mn^{2+} free barbital buffer, and treated with 1 mM EDTA at room temperature for 30 min. Treated brush border membranes were then diluted (1:5) in barbital buffer containing increasing concentrations (0, 0.5, 1, 2, 4, and 8 mM) of various cations (Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}) and assayed. Using this procedure, EDTA-treated brush border membranes had approximately 5% of the aminopeptidase P activity of untreated controls.

Solubilization of brush border membranes

The detergents CHAPS (20 mM), deoxycholate (10 mM), Triton X-100 (5.9 mM), and NP-40 (6.1 mM) were added to aliquots of brush border membranes and incubated on ice for 1 hr. They were then centrifuged at 27,000*g* for 30 min, and the enzyme activity in the supernatant and pellet fractions were determined. Purified phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was a gift from Dr. Martin Low. PI-PLC (5 U/mL) was incubated with brush border membranes for 1 hr at 37°C, centrifuged as described above, and the distribution of enzyme activity was determined in the supernatant and pellet fractions.

Determination of molecular weight

A column (1.5 × 95 cm) was packed with Ultrogel ACA 34 and equilibrated in 50 mM Tris-HCl, 0.15 M NaCl, 2 mM $MnCl_2$, pH 8.0. The excluded (V_o) and included (V_i) volumes were determined with blue dextran and leucine, respectively. The following molecular weight standards were used to calibrate the column: apoferritin (443,000), β-amylase (200,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000). Brush border membrane aminopeptidase P was solubilized with PI-PLC (*Bacillus cereus*) and chromatographed. Enzyme activity was measured in the various fractions to determine the elution volume.

Results

Assay of aminopeptidase P

Maximum aminopeptidase P activity was achieved after activating the brush border membranes for 1 hr at 37°C in buffer containing Mn^{2+} and BSA. This phenomenon displayed a time dependence (*Figure 1A*) and was positively influenced by increasing concentrations of BSA in the activating buffer (*Figure 1B*). Following activation, the specific activity of aminopeptidase P was approximately doubled from 600 to 1100 nmoles/min/mg of protein.

As shown in *Figure 2*, membrane associated aminopeptidase P had a pH optimum of 8.0 to 8.2 while the activity in the soluble fraction (described below) had an optimum pH of 7.4. At pH 8.0, the activity of the soluble fraction was 20% of that at pH 7.4.

Subcellular distribution of Gly-Pro-Hyp hydrolyzing activity

Using Gly-Pro-Hyp as a substrate, enzyme activity was present in both the soluble and membrane fraction of intes-

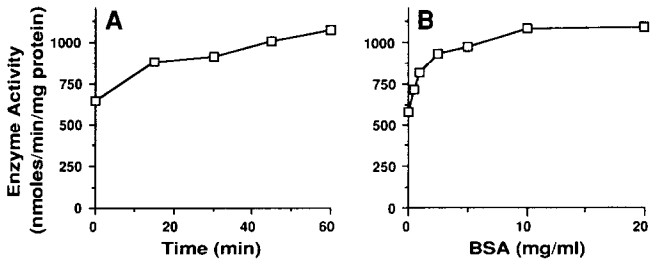

Figure 1 Effect of preincubation time and bovine serum albumin (BSA) on brush border membrane aminopeptidase P activity. (A) Time. Samples of brush border membrane were incubated with BSA (20 mg/mL) for varying periods of time (0–60 min) at 37°C before assay. (B) BSA. Brush border membranes were incubated (1 hr, 37°C) with increasing concentrations of BSA (0 to 20 mg/mL) prior to assay. The same concentration of BSA was present in the assay mixture.

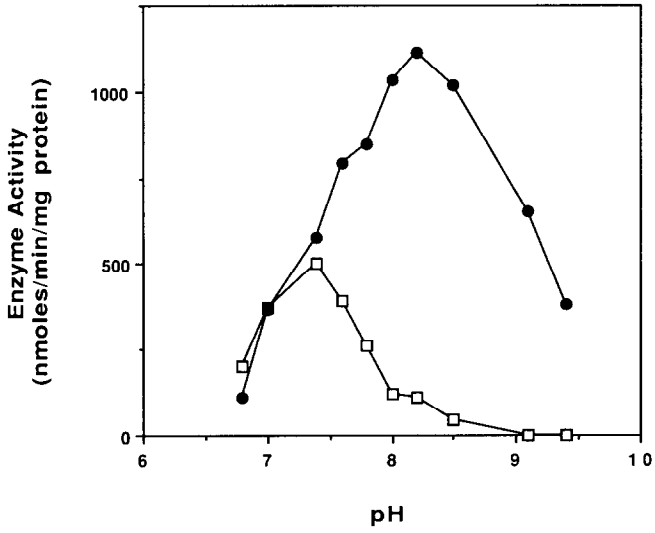

Figure 2 pH optimum for hydrolysis of Gly-Pro-Hyp in intestinal brush border membrane and cytosol fractions. Brush border membrane (●–●); cytosol (□–□).

tinal epithelial cells (*Table 1*). One third of the total activity was membrane associated when assayed at pH 8.0.

Levels of APN, DPP IV, and AL-P were used as a criteria for the purity of intestinal brush border membranes. As shown in *Table 2*, the specific activity of these enzymes was enriched 12 to 13 fold over that in the homogenate fraction. By comparison, aminopeptidase P activity was enriched 9 fold in the brush border membranes.

Kinetic constants and thermostability

The kinetic constants for brush border membrane and cytosol activity were determined at their respective pH optima with Gly-Pro-Hyp (1 to 40 mM). From Eadie–Hofstee plots, the *Km* and *V* values were estimated to be 40.3 mM and 3483 nmol/min/mg of protein, respectively, for the membrane associated enzyme. The same parameters for the soluble activity were 6.9 mM and 518 nmol/min/mg of protein. The membrane associated activity was relatively stable during incubation at elevated temperatures (50 and 60°C)

Table 1 Distribution of Gly-Pro-Hyp hydrolyzing activity in rat intestinal mucosal cells

Fraction	Total Activity (nmol/min)	Percent
Homogenate	906 ± 57	100
Cytosol	592 ± 43	65
Total membrane	247 ± 30	27

Results are the mean value ± SD of four experiments.

Table 2 Aminopeptidase P activity in brush border membranes of rat small intestine

Enzyme	Specific Activity (nmol/min/mg of protein) Homogenate	Brush Broder Membrane	Enrichment (fold)
1. AP-P	127. ± 8	1,146 ± 65	9.0
2. DPP IV	88 ± 6	1,072 ± 50	12.2
3. APN	89 ± 4	1,149 ± 65	12.9
4. AL-P*	1 ± 0.1	13 ± 0.6	13.0

Results are the mean value ± SD of six experiments. AP-P, aminopeptidase P; DPP IV, dipeptidyl peptidase IV; APN, aminopeptidase N; AL-P, alkaline phosphatase.
*AL-P activity is expressed as μmol.

whereas the soluble activity was unstable under these conditions (*Figure 3*).

Effect of inhibitors and metal ions

Figure 4 shows the effect of a variety of inhibitors on aminopeptidase P activity in the soluble and membrane fractions. It was apparent that the inhibition profiles in these two fractions closely paralleled one another. Metal chelating agents such as EDTA and 1,10-phenanthroline were among the most effective inhibitors as was bestatin, a microbial derived aminopeptidase inhibitor. In addition, 50% inhibition of both activities was observed with phenylmethylsulfonyl fluoride (2 mM). Inhibitors of trypsin-like enzymes, sulfhydryl proteases, and angiotensin-converting enzyme (captopril) were without effect.

When brush border membranes were washed and pretreated with EDTA, aminopeptidase P activity was reduced to approximately 5% of untreated control membranes. Addition of Mn^{2+} restored approximately 90% of the enzymatic activity present in untreated controls at a concentration of 1 to 2 mM (*Figure 5*). A slight increase in activity was observed with Co^{2+} while Zn^{2+} and Mg^{2+} had no effect.

Mode of membrane attachment

The mode of attachment of membrane associated aminopeptidase P was investigated by solubilizing the enzyme with several detergents or by enzymatic (PI-PLC) means. The results were compared with profiles obtained for other brush border membrane enzymes such as APN, DPP IV, and

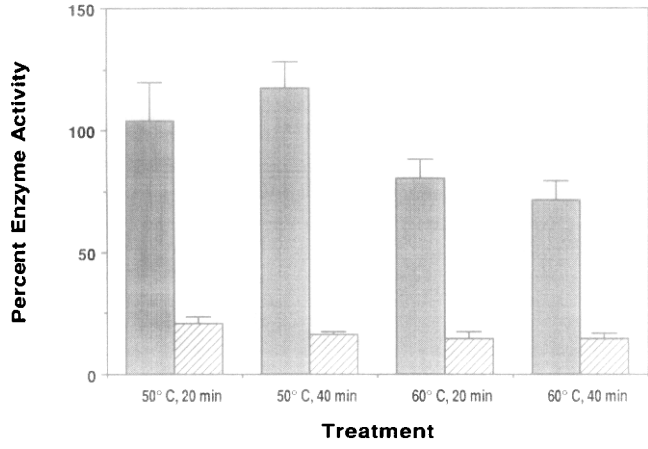

Figure 3 Thermostability of aminopeptidase P activity in brush border membrane and cytosol fractions. Aminopeptidase P activity was measured after incubating the fractions at 50 and 60°C for 20 and 40 min. Brush border membrane (▨); cytosol (▨). Results are the mean value ± SD of four experiments.

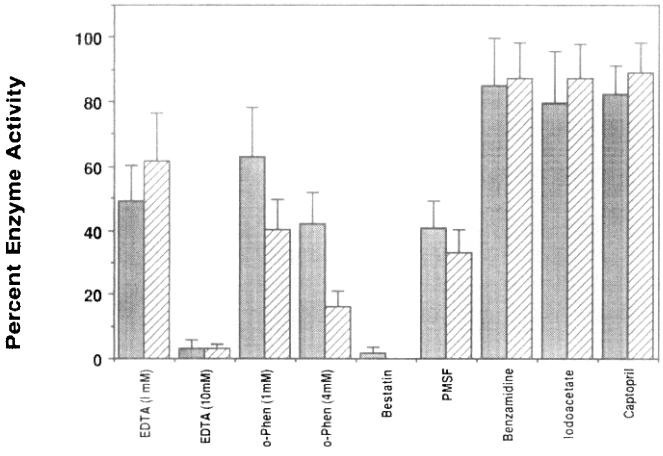

Figure 4 Effect of inhibitors on Gly-Pro-Hyp hydrolyzing activity in brush border membrane and cytosol fractions. Inhibitors were pre-incubated with the fractions for 30 min at room temperature prior to assay. The inhibitor concentration was 1 mM except where noted otherwise. Brush border membrane (□); cytosol (▨); o-Phen (1-10 phenanthroline). Results are the mean value ± SD of four experiments.

AL-P (*Figure 6*). These data show that aminopeptidase P was most effectively solubilized by PI-PLC while detergents tended to be less effective. This pattern of solubilization was very similar to that observed for alkaline phosphatase. In contrast, aminopeptidase N and dipeptidyl peptidase IV activities were readily solubilized by detergents such as Triton X-100 and NP-40 while PI-PLC was not effective.

Thermostability of solubilized aminopeptidase P

Brush border membrane associated aminopeptidase P after solubilization with either detergent (CHAPS) or PI-PLC was stable to heat treatment at 50°C for 40 min (data not shown). This result was similar to that obtained with the membrane associated enzyme (*Figure 3*).

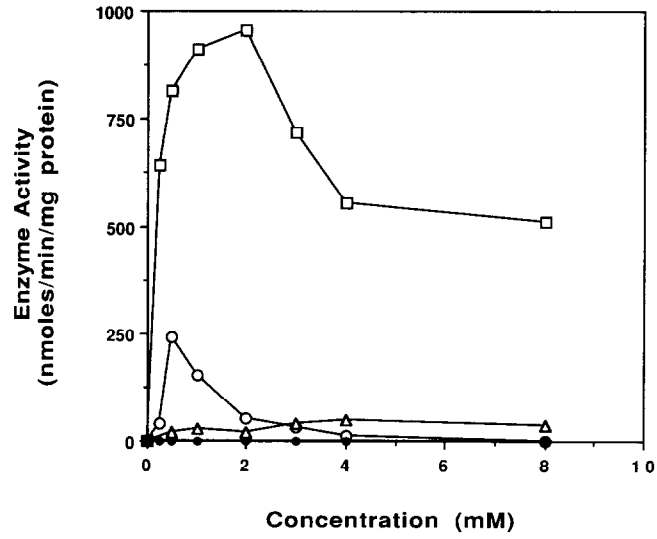

Figure 5 Reactivation of brush border membrane aminopeptidase P activity by various cations following treatment with EDTA. Mn^{2+}, (□–□); Co^{2+}, (○–○); Mg^{2+}, (●–●); Zn^{2+}, (△–△).

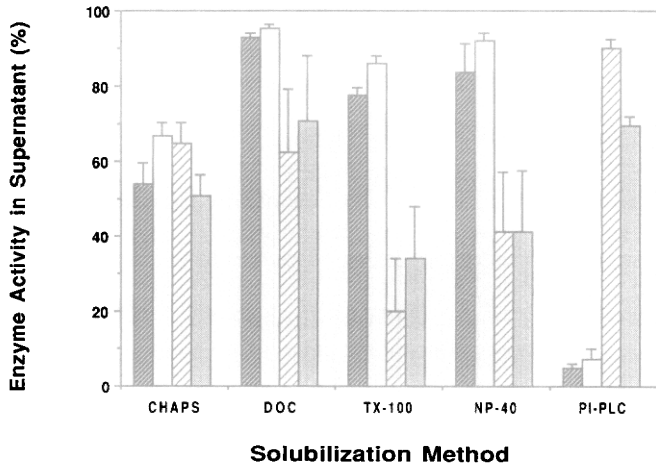

Figure 6 Solubilization of brush border membrane associated enzymes by detergents and phosphatidylinositol-specific phospholipase C. Brush border membranes were incubated with various detergents for 1 hr on ice or with enzyme (PI-PLC) for 1 hr at 37°C. Samples were then centrifuged at 27,000*g* for 30 min and the amount of total enzyme activity present in the pellet and supernatant fractions was determined. CHAPS, 3-[3-chloramidopropyl) dimethylammonio]-1-propane sulphonate; DOC, deoxycholate; NP-40, Nonidet P-40; PI-PLC, phosphatidylinositol-specific phospholipase C; TX-100, Triton X-100. Aminopeptidase N, (▨); dipeptidyl peptidase IV, (□); alkaline phosphatase, (▨); aminopeptidase P, (▨). Results are the mean value ± SD of four experiments.

Determination of molecular weight

Membrane associated aminopeptidase P was solubilized with PI-PLC and chromatographed on a gel filtration (Ultrogel ACA-34) column. The enzyme eluted as a single peak of activity. When compared with the elution profile of known molecular weight standard (data not shown), the molecular weight of the PI-PLC solubilized enzyme was estimated to be 220,000.

Distribution of aminopeptidase P along the intestine

The distribution profile of aminopeptidase P activity along the longitudinal axis of the small intestine was examined in brush border membrane and cytosol fractions (*Figure 7*). These data show that brush border membrane activity was highest in the middle regions and the profile was similar to that observed for APN. The soluble activity was also highest in the middle regions of the intestine, however, no APN activity was present in these fractions.

Discussion

In the present study we have shown that aminopeptidase P is present in rat intestinal mucosal cells and is localized to the brush border membrane. The enzyme is distinct from pancreatic proteases and other known brush border membrane peptidases hydrolyzing the amino-terminal glycine from the aminopeptidase P substrate Gly-Pro-Hyp used by other investigators.[10,11,20,21] We chose this substrate over others that are available because of its relative specificity for aminopeptidase P. In addition important dietary proteins such as collagen contain relatively high amounts of these three amino acids, often in repeated sequences. Thus it was felt that Gly-Pro-Hyp could provide insight into the role intestinal aminopeptidase P plays in the digestion of these types of dietary prolyl peptides. Using this substrate it was evident that there were substantial amounts of enzymatic activity able to remove the amino-terminal glycine in both the soluble and membrane fractions of intestinal mucosal cells. In other tissues soluble or membrane associated forms of aminopeptidase P have been described.[7–11,14,15] Therefore, we carried out a number of studies to determine what the prevalent form is in intestinal mucosal cells. These studies show that the activity in the brush border membrane and soluble fraction of intestinal epithelial cells are clearly different with respect to their pH optima, kinetic constants, and heat stability profiles. In addition, when the membrane associated activity was solubilized with detergent or PI-PLC, it retained the heat stability characteristics of the membrane associated form.

Taken together, the results of this study indicate that aminopeptidase P is responsible for hydrolyzing Gly-Pro-Hyp in the brush border membrane fraction of intestinal mucosal cells whereas the soluble activity is most likely due to a different enzyme. It is well known that the soluble fraction of intestinal mucosal cells contains several peptidases that complete the hydrolysis of the di- and tripeptides absorbed intact from the intestinal lumen.[22,23] These currently include the two proline dipeptidases prolinase (EC 3.3.13.8) and prolidase (EC 3.4.13.9) and an aminotripeptidase (EC 3.4.11.4).[22,23] Since relatively little is known about some of these enzymes, it is possible that one or several cytolasmic peptidases may be responsible for removing glycine from the substrate (Gly-Pro-Hyp) used in this study.

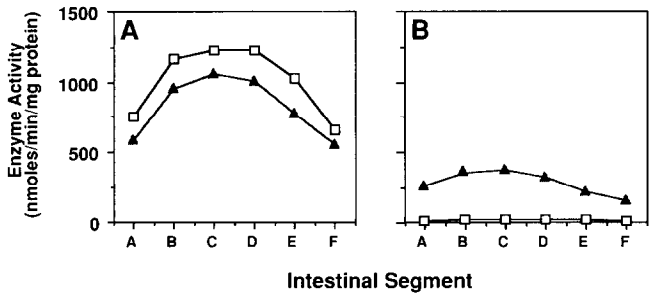

Figure 7 Distribution of Gly-Pro-Hyp hydrolyzing activity in brush border membrane and cytosol fractions along the longitudinal axis of rat small intestine. The entire rat small intestine was divided into six equal length segments. Brush border membranes and cytosol fractions were prepared from each segment. (A, proximal segment; F, distal segment). (A) Brush border membrane; (B) cytosol; aminopeptidase P, (▲–▲); aminopeptidase N (□–□).

Intestinal brush border membrane aminopeptidase P is a metalloprotein since chelating agents such as EDTA and 1,10-phenanthroline were among the most effective inhibitors. After pretreatment of brush border membranes with EDTA, Mn^{2+} was able to completely restore aminopeptidase P activity while other cations were much less effective. These results are consistent with other studies showing that Mn^{2+} is important in the catalysis of these types of substrates.[11,13,21] Bestatin, a known inhibitor of aminopeptidases N and A, was also an effective inhibitor of aminopeptidase P. Previous studies have indicated that the enzyme may not be very sensitive to this inhibitor.[11,12]

The distrubution profile along the longitudinal axis of the small intestine revealed that aminopeptidase P was most prevalent in the middle regions gradually decreasing toward the proximal and distal portions. This pattern is similar to that observed for intestinal APN, CPP, and neutral endopeptidase.[3,24] Other prolyl peptidases such as intestinal ACE has a sharp proximal-distal gradient of activity while DPP IV has a more distal distribution.[25]

Brush border membrane associated aminopeptidase P was not readily solubilized from the membrane fraction with various detergents. This was particularly evident when Triton X-100 and NP-40 were used. These two detergents readily solubilized other membrane peptidases such as APN and DPP IV, which are integral membrane proteins. We also observed (data not shown) that treatment with various proteases such as papain or trypsin did not yield a soluble form of the enzyme as was the case for APN and DPP IV. These data suggest that intestinal aminopeptidase P is anchored to the brush border membrane differently from APN and DPP IV, which have an amino-terminal hydrophobic (type II) membrane anchor. It was also evident from these studies that aminopeptidase P exhibited characteristics that were similar to AL-P, which is attached to the membrane via a glycosyl-phosphatidylinositol moiety.[26] The fact that phosphatidylinositol-specific phospholipase C effectively solubilized these two enzymes strongly supports the conclusion that intestinal aminopeptidase P is bound to the membrane through a glycosyl-phosphatidylinositol moiety. This type of membrane anchor has been found for aminopeptidase P in other tissues.[8,27] Thus along with microsomal dipeptidase (EC 3.4.13.19)[28] and carboxypeptidase M (EC 3.4.17.12),[29] aminopeptidase P is the only other brush border membrane peptidase described to date that has this type of anchor. Trehalase, a brush border membrane carbohydrase, is also anchored to the membrane in this manner.[30] The PI-PLC solubilized form of the enzyme had an

apparent molecular weight of 220,000 as determined by gel filtration. This is similar to that reported for the pig kidney enzyme (280,000), which consists of two identical subunits.[11] Other studies have reported molecular weights of 143,000 and 360,000 for the undenatured enzyme from brain cytosol[12] and lung membranes,[8] respectively.

Like the original description of the kidney form of the enzyme,[10,20] maximal activity was achieved following a preincubation procedure in the presence of bovine serum albumin and Mn^{2+}. The reasons for this activation effect are unclear but may be important in stabilizing the enzyme[20] and incorporating Mn^{2+} into aminopeptidase P molecules that have lost the metal ion from their active site during brush border membrane preparation. A kinetic study of activated aminopeptidase P shows that the Km for Gly-Pro-Hyp is 40 mM with a *V* of 3600. This *Km* value is much higher than reported for the lung enzyme (0.34 mM) with the same substrate.[7] At the present time the reason for this difference is unknown. The soluble enzymatic activity had lower *Km* and *V* values again indicating that the enzymes in the two subcellular compartments are different.

Using the normal assay protocol, the final concentration of Gly-Pro-Hyp was 17 mM. Therefore, these were nonsaturating conditions with respect to the high *Km* value observed for Gly-Pro-Hyp in this study. Nevertheless, a specific activity of 1,100 nmol/min/mg of protein was routinely observed in the brush border membrane fraction under these conditions (*Table 2*). This value is comparable to that observed for other peptidases such as APN and DPP IV, suggesting that aminopeptidase P is also a major intestinal brush border membrane peptidase.

In the lung and kidney, aminopeptidase P is thought to be important in the hydrolysis of bradykinin, thereby playing a role in the metabolism of vasoactive peptides.[7–9,12,13,21,31–33] Its importance in the degradation of other prolyl peptides of endogenous origin has also been implicated in a recent description of aminopeptidase P deficiency.[34] In two individuals elevated levels of urinary prolyl peptides, particularly Gly-Pro-Hyp-Gly, were observed and correlated with a lack of aminopeptidase P-like activity.

In the small intestine, the brush border membrane localization of aminopeptidase P and high activity suggests a digestive function for the enzyme. Its proposed role in the hydrolysis of dietary prolyl peptides is graphically illustrated in *Figure 8*. Proline oligopeptides, which are relatively resistant to gastric and pancreatic proteases, are effectively degraded by the four intestinal peptidases shown here. Each enzyme has been shown to have high activity with prolyl peptides. Depending on the sequence of the prolyl peptide, degradation can occur from either the carboxy- or amino-terminal end. At the amino terminus, aminopeptidases N and A sequentially remove the exposed amino acids but are unable to hydrolyze the C-Pro peptide bond. Aminopeptidase P, however, can efficiently hydrolyze this bond, releasing the C amino acid. In an alternative hydrolytic mechanism, the C-Pro dipeptide may also be removed by DPP IV. At the C-terminal end, angiotensin-converting enzyme and carboxypeptidase P have been previously shown to sequentially degrade prolyl peptides in a concerted fashion.[4] The end result of the combined hydrolytic action of these four enzymes is to reduce prolyl oligopeptides to a mixture of single amino acids and di- and tripeptides. In this form they can be efficiently absorbed by the various transport carriers of the enterocyte. Final hydrolysis is accomplished by the soluble peptidases present in these cells.

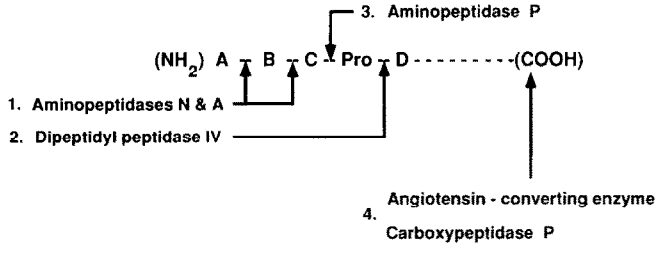

Figure 8 Proposed mechanism of hydrolysis of prolyl peptides by intestinal brush border membrane peptidases.

Acknowledgments

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