

Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane

MISAKO AITO-INOUE,^a DALE LACKEYRAM,^b MING Z. FAN,^b KENJI SATO^a and YOSHINORI MINE^{c*}

^a Department of Food Sciences and Nutritional Health, Kyoto Prefectural University, 1-5 Nakaragi-cho, Shimogamo, Kyoto 606-8522, Japan

^b Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

^c Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Abstract: The transcellular transport of oligopeptides across intestinal epithelial cells has attracted considerable interest in investigations into how biologically active peptides express diverse physiological functions in the body. It has been postulated that the tripeptide, Gly-Pro-Hyp, which is frequently found in collagen sequences, exhibits bioactivity. However, the mechanism of uptake of dietary di- and tripeptides by intestinal epithelial cells is not well understood. In this study, we used porcine brush-border membrane (BBM) vesicles to assess Gly-Pro-Hyp uptake, because these vesicles can structurally and functionally mimic *in vivo* conditions of human intestinal apical membranes. The present study demonstrated the time-dependent degradation of this tripeptide into the free-form Gly and a dipeptide, Pro-Hyp, on the apical side of the BBM vesicles. In parallel with the hydrolysis of the tripeptide, the dipeptide Pro-Hyp was identified in the BBM intravesicular space environment. We found that the transcellular transport of Pro-Hyp across the BBM was inhibited by the addition of a competitive substrate (Gly-Pro) for peptide transporter (PEPT1) and was pH-dependent. These results indicate that Gly-Pro-Hyp can be partially hydrolyzed by the brush-border membrane-bound aminopeptidase N to remove Gly, and that the resulting Pro-Hyp is, in part, transported into the small intestinal epithelial cells via the H⁺-coupled PEPT1. Gly-Pro-Hyp cannot cross the epithelial apical membrane in an intact form, and Pro-Hyp is highly resistant to hydrolysis by intestinal mucosal apical proteases. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: porcine brush-border membrane; bioactive peptide; Pro-Hyp; aminopeptidase N; collagen; HPLC; PEPT1

INTRODUCTION

Small intestinal epithelial cells are the primary site of absorption of nutrients such as glucose and amino acids. Absorption through the intestine requires such molecules to cross two distinct membranes: (i) take up by the epithelial cell from the lumen across the brush-border membrane (BBM), (ii) followed by transfer to the blood across the basolateral membrane [1]. Pancreatic proteases, small intestinal brush-border proteases, and peptidases digest proteins of dietary or endogenous origin to release short-chain peptides and free amino acids. Over the past two decades it has been found that hydrolysis of various food proteins results in several physiologically active peptides. These peptides include opioid, hypotensive, mineral-absorption stimulating, anticoagulative, and immunomodulatory types [2].

It has been demonstrated that di- and tripeptides are taken up into the intestine and renal epithelial cells by H⁺-coupled peptide transporters (PEPT1 and PEPT2) across the BBM, which have recently been cloned and extensively characterized [3,4]. Only PEPT1 is expressed in the small intestine, and is localized to the BBM [5].

PEPT1 operates as an electrogenic protein/peptide symporter with the ability to transport essentially every possible di- and tripeptide. This transport process is enantioselective, involving a variable proton-to-substructure stoichiometry in the uptake of neutral and mono- or polyvalently charged peptides [6]. However, the mechanism for transepithelial oligopeptide transport in the intestinal tract is not yet fully understood. Satake *et al.* have demonstrated that PEPT1-mediated transport is not involved in the transepithelial transport of a bioactive tripeptide, Val-Pro-Pro, with paracellular diffusion instead being the main mechanism [7]. Adson *et al.* reported that the transports of D-Phe-Gly, D-Phe₂-Gly, and D-Phe₃-Gly were predominantly paracellular [8]. A similar transcytotic mechanism of oligopeptides has been reported, indicating that the intestinal epithelium has another route specific for long-chain oligopeptides [9,10].

Gelatin-based food derivatives obtained from animals (including fish) have been attracting attention as health-food ingredients in Asian countries. In particular, collagen-based peptides represent functional peptides that exhibit various physiological activities. Bone mineral density was shown to be increased by the oral ingestion of gelatin [11]. The collagen-based dipeptide Pro-Hyp and tripeptide Gly-Pro-Hyp exert chemotactic effects on fibroblast peripheral blood neutrophils [12,13] and monocytes [14] in cell culture

* Correspondence to: Y. Mine, Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1; e-mail: ymine@uoguelph.ca

systems. Gly-Pro-Hyp was found to produce platelet aggregation [15]. We previously found, using HPLC, that Pro-Hyp was the major collagen-derived peptide in the peripheral serum and plasma of human volunteers following the oral ingestion of gelatin hydrolysates, with small amounts of other di- and tripeptides – Ala-Hyp, Ala-Hyp-Gly, Gly-Pro-Hyp, Lue-Hyp, Ile-Hyp, and Phe-Hyp – also being detected [16]. The collagen triple helix consists of a repeating Gly-X-Y sequence. In theory there are more than 400 possible Gly-X-Y triplets, but analysis of sequences from fibrillar and nonfibrillar collagens shows that only some of these triplets are found in significant quantities. The nonrandom prevalence of Gly-X-Y triplets makes it possible to experimentally assess the stability of much of the collagen sequence by studying a limited set of host–guest peptides, where individual Gly-X-Y triplets constitute the guest and the Gly-Pro-Hyp tripeptide constitutes the host. A designed set of host–guest peptides contains the most common nonpolar and charged triplets found in collagen [17]. On the basis of this background, we assumed that when the tripeptide Gly-Pro-Hyp comes into contact with the apical enterocyte membrane, it can be hydrolyzed into the dipeptide Pro-Hyp, which can be taken up into the blood circulation. However, the mechanism for transcellular Pro-Hyp transport in the intestinal tract has not been reported.

In the present study, we investigated a mechanism for the transcellular transport of Gly-Pro-Hyp in porcine intestinal BBM vesicles.

MATERIALS AND METHODS

Reagents

Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Whitby, ON, Canada). Triethylamine (TEA; sequence grade) was purchased from Pierce (Rockford, IL, USA). Protein dye reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Gly-Pro-Hyp, Pro-Hyp, and Gly-Pro were purchased from Bachem (Bubendorf, Switzerland). Phenyl isothiocyanate (PITC), bovine serum albumin, D-mannitol, trizma-HCl, HEPES, aprotinin, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Na- ρ -tosyl-L-lysine chloromethyl ketone (TLCK), tosyl phenylalanyl chloromethyl ketone (TPCK), ouabain, Na₂ATP, ρ -nitrophenyl phosphate, potassium thiocyanate (KSCN), and other chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).

Animals and Preparation of Mucosal Scraping

Proximal jejunal mucosal samples were collected from six Yorkshire grower pigs aged 70–75 days and weighing about 35–40 kg obtained from the University of Guelph Arken Swine Research Station (Guelph, ON, Canada). Pigs were anesthetized by inhalation of 5% isoflurane (Aerrane, Anaquest, WI, USA) via a facial mask. The abdomen was immediately opened and the entire small intestine – from 30 cm posterior to

the pyloric sphincter to 60 cm anterior to the ileocecal sphincter – was removed and then immediately flushed twice with ice-cold phosphate buffered saline containing 0.2 mM PMSF and 0.5 mM DTT (pH 7.4). The jejunal mucosal samples were taken from the midpoint of the jejunal segment. All samples were pulverized to be homogeneous using a set of mortar and pestle under liquid N₂ and were kept at –83 °C until use. The experimental protocol had been approved by Animal Care and Use Committee of the University of Guelph.

Preparation of BBM Vesicles

Intestinal BBM vesicles were prepared using the magnesium chloride precipitation and differential centrifugation according to an established procedure [18]. The BBM vesicles prepared from our previously established procedures were primarily 'out-side out' with very little or no contaminations from the basolateral membrane and other subcellular membrane fractions [18,19]. In addition, the BBM vesicles prepared by this procedure were associated with an intravesicular space of 1.1–1.6 μ l/mg membrane protein [19]. Briefly, about 12 g of the proximal jejunal mucosa was thawed in ice-cold homogenate buffer (50 mM D-mannitol, 10 mM HEPES, 1 mM Na₂EDTA, 2 μ g/ml TPCK, 2 μ g/ml TLCK, 2 μ g/ml leupeptin hemisulfate, 2 μ g/ml aprotinin, 2 μ g/ml peptain A, and 0.2 mM PMSF at pH 7.4) at a ratio of 20 ml of homogenate buffer per gram of the proximal jejunal mucosa and homogenized (Polytron homogenizer, Brinkman Instruments, Inc., Mississauga, ON). The resulting homogenate was pooled and then centrifuged at 2000 *g* for 15 min. The supernatant, obtained by removing the top foam layer and discarding the pellets, was mixed with 1 M of MgCl₂ solution (to produce 10 mM MgCl₂), stirred for 15 min, and centrifuged at 2400 *g* for 15 min. After the top foam layer was discarded, the supernatant was centrifuged at 19000 *g* for 30 min to generate crude BBM pellets which were then resuspended in a suitable amount of a vesicle resuspension buffer (300 mM D-mannitol and 50 mM HEPES at pH 7.4). The final membrane vesicle suspension was assayed for protein content and diluted to an appropriate level for subsequent *in vitro* transport investigations.

Protein Assays

Proteins were determined according to the Bradford method [20] using the Bio-Rad Laboratories protein dye reagent and bovine serum albumin as standard.

Transport Studies by BBM Vesicles

In vitro transport experiments were performed according to the method described previously [18] with a slight modification. In a routine assay, 100 μ l of a BBM vesicle suspension (4.0 mg/ml protein) was added to a corresponding uptake buffer (10 mM D-mannitol, 150 mM KSCN, 10 mM HEPES at pH 5.5 or 7.4) and mixed with 100 μ l of Gly-Pro-Hyp substrate (1 μ M) in a 1.0 ml centrifuge tube. The mixture was incubated at 37 °C, for 0, 0.5, 1, 2, 4, or 8 h. After incubations, the tubes were centrifuged at 15000 *g* for 10 min, and the resulting supernatant was kept for further analysis. The pellets were washed three times with 100 μ l of the same buffer.

Solubilization of BBM Vesicles

Washed BBM vesicle pellets were suspended in 200 μ l of Milli-Q water, and they were solubilized by mixing them with a solvent comprising chloroform-methanol (2:1, v/v). After 30 min of mixing, the water phase containing peptides and free amino acids was removed and used in subsequent HPLC analyzes.

Solid-phase Extraction of Peptides

The supernatant or aqueous layer of pellets was cleaned using a previously described method [21]. A strong cation exchanger (AG 50W-X8, Bio-Rad Laboratories) was washed with 50% methanol and packed into a spin column (5 mm \times 5 mm i.d., Pierce). The column was successively washed with 50% methanol and equilibrated with 200 μ l of the vesicle resuspension buffer (300 mM D-mannitol and 50 mM HEPES at pH 7.4). Elution was performed by centrifugation at 15 000 g for 3 min. After incubations, the supernatants and pellets were loaded onto the spin column, which was washed with 200 μ l of the vesicle resuspension buffer, 2.0 M of ammonium hydroxide containing 50% methanol, and then again with the vesicle resuspension buffer. Bound peptides were eluted six times with 200 μ l of 7.5 M of ammonium hydroxide containing 50% methanol.

Derivatization of Peptides with PITC and HPLC Analysis

Aliquots of the peptide fractions after solid-phase extraction were dried in tubes under vacuum. The peptides in the tubes were derivatized with PITC according to the method of Bidlingmeyer *et al.* [22] with a slight modification. Ten microliters of an alkaline solution containing methanol, TEA, and water at a ratio of 7:2:1 (v/v/v) was added to the tube and then dried under vacuum. The dried samples were reacted with 20 μ l of a derivatizing solution containing methanol, TEA, water, and PITC at a ratio of 7:1:1:1 (v/v/v) for 20 min at 25 $^{\circ}$ C. The excess reagent was removed by vacuum. The resultant phenylthiocarbonyl peptides were dissolved in 200 μ l of 5 mM sodium phosphate buffer (pH 7.4) containing 10% (v/v) acetonitrile, and the solution was filtered through a 0.45 μ m filter.

After derivatization, peptides were separated by an HPLC system equipped with a binary pump (1525, Waters, Milford, MA, USA) in combination with a dual-wavelength absorbance detector (2487, Waters) and an autosampler (717 Plus, Waters) under software control (Breeze, Waters). The peptides solution (20 μ l) was injected into a column (250 mm \times 4 mm i.d., Supersphere RP-18 (e), Merck, Darmstadt, Germany) equilibrated with 0.15 M ammonium acetate buffer (pH 6.0) containing 5% (v/v) acetonitrile at 0.8 ml/min. Elution was performed using 60% (v/v) acetonitrile as described previously [21] with the following gradient profile: 0–5 min, 0%; 5.1–25 min, from 10 to 47.5%; from 25 to 30 min, from 47.5 to 100%; 30–37 min, 100%; and 37.1–40 min, 0%. The column was maintained at 25 $^{\circ}$ C, and the absorbance at 254 nm was monitored. Gly-Pro-Hyp, Pro-Hyp, and Gly were identified by their retention times using standard peptides or amino acids as described previously [21].

All transport measurements were performed in triplicate, and the obtained data were expressed as the amount of

peptides (or amino acid) in nanomoles per milligram of BBM vesicle protein.

Statistical Analysis

Data are given as mean \pm SD values of triplicate measurements. Statistical significance of the data was determined by Student's *t*-test.

RESULTS

Hydrolysis of Gly-Pro-Hyp on BBM Vesicles

Figure 1(A) shows the time dependence of the Gly-Pro-Hyp level when it was incubated on the apical side of the BBM (1 μ M). After incubation at 37 $^{\circ}$ C for an appropriate duration, the supernatant and BBM pellets were separated by centrifugation at 15 000 g for 10 min. After 30 min of incubation at pH 7.4, about 19% of the tripeptide had disappeared from the incubation media without being detected in the intravesicular space environment of the BBM vesicles. In parallel with the hydrolysis of Gly-Pro-Hyp, free Gly was identified and its concentration was increased in a time-dependent manner up to 8 h (Figure 1(B)), which suggested the formation of an *N*-terminal cleavage product by proteolysis of Gly from Gly-Pro-Hyp. Interestingly, free forms of Pro and Hyp were not detected.

Transport of Pro-Hyp through the BBM

The transcellular transport rate of Pro-Hyp was measured in BBM vesicles *in vitro*. Gly-Pro-Hyp was incubated with BBM vesicles and the pellets were isolated by centrifugation as described in 'Materials and Methods' after incubations at 37 $^{\circ}$ C for 0, 0.5, 1, 2, 4, or 8 h, respectively. The identification and quantification of peptides appearing in the BBM vesicles were determined by HPLC after solubilizing the BBM vesicle pellets. Figure 2 shows the amount of Pro-Hyp being up taken in the BBM vesicles in a time-dependent manner. Pro-Hyp was readily detected after 1 h of incubation, and reached a plateau at 4 h of incubation. Free Gly was also detected in the BBM intravesicular space environment after solubilizing the vesicles, whereas Gly-Pro-Hyp was not detected in the intravesicular space.

Effect of Gly-Pro on the Transcellular Transport of Pro-Hyp across the BBM

The flux of Gly-Pro-Hyp into the BBM was measured in the presence of 10 mM of Gly-Pro. Gly-Pro is a good substrate for the PEPT1, which has a low K_m value [23], and is fairly resistant to brush-border peptidases. Gly-Pro has therefore often been used to analyze the functioning of PEPTs [24,25]. The flux of Pro-Hyp was

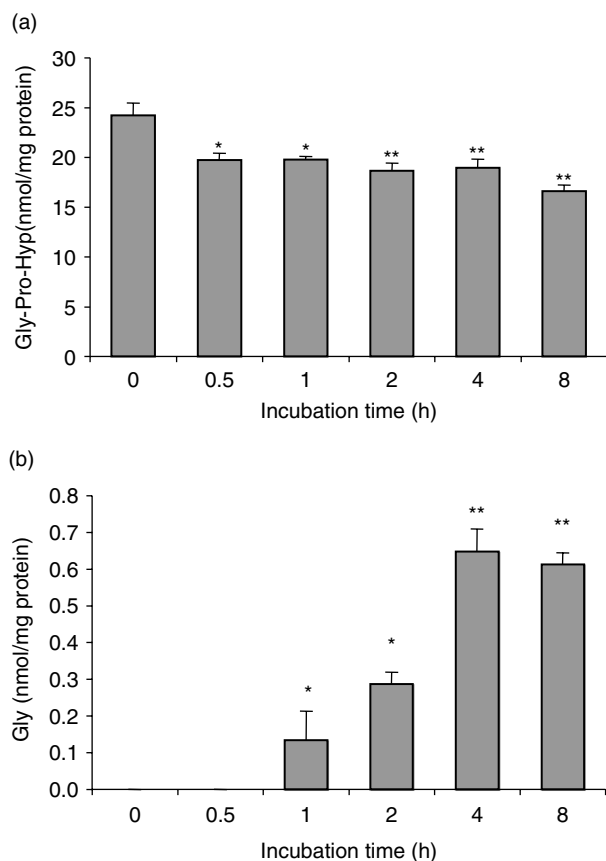


Figure 1 Time-dependent hydrolysis of the tripeptide Gly-Pro-Hyp (A) and formation of free Gly (B) on the apical side of porcine brush-border membrane (BBM). The incubation buffer had a pH of 5.5, whereas the BBM vesicles were preloaded and resuspended in a buffer with pH of 7.4. Each bar indicates the mean and SD ($n = 3$). Values with symbols differ significantly at $P < 0.05$ (*) and $P < 0.01$ (**).

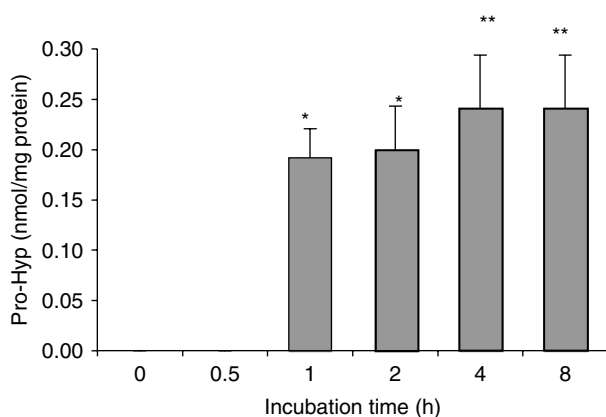


Figure 2 Transcellular transport of the dipeptide Pro-Hyp across the porcine BBM in the BBM vesicles as a function of time. The incubation buffer had a pH of 5.5, whereas the BBM vesicles were preloaded and resuspended in a buffer with pH of 7.4. Each bar indicates the mean and SD ($n = 3$). Values with symbols differ significantly at $P < 0.05$ (*) and $P < 0.01$ (**).

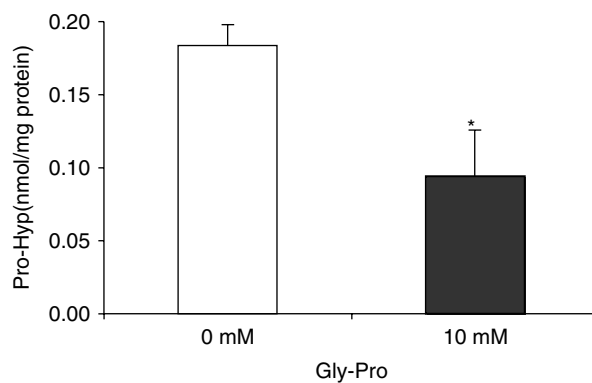


Figure 3 Effect of Gly-Pro on the transcellular transport of Pro-Hyp across the porcine BBM examined in BBM vesicles. The incubation buffers had a pH at 5.5, whereas the BBM vesicles were preloaded and resuspended in a buffer with pH at 7.4. Each bar indicates the mean and SD ($n = 3$). Values with symbols differ significantly at $P < 0.05$ (*).

partially inhibited in the presence of 10 mM of Gly-Pro (Figure 3), which suggests that the PEPT1 played a significant role in the transcellular transport of Pro-Hyp across the BBM.

pH-dependent Transport of Pro-Hyp across the BBM

Transport experiments in BBMs are often conducted using a pH gradient in which the pH values on the apical and basolateral sides are maintained at 5.5–6.0 and 7.4, respectively [26]. This approach attempts to mimic the acidic microclimate of the small intestine [6]. The transport rate for dipeptide at apical and basolateral pH values of 5.5 and 7.4, respectively, was compared with the ratio obtained in the absence of a pH gradient (pH 7.4 on both sides). Reducing the apical pH to 5.5 significantly increased the transport rate during the first 30 min of incubation, but not after 1 h of incubation (Table 1), which suggests that proton-coupled PEPT1-mediated uptake of Pro-Hyp is involved in the transport of dipeptides across the BBM.

Table 1 Transcellular transport of the dipeptide Pro-Hyp across the porcine brush-border membrane (BBM) in BBM vesicles when applying a buffer pH at 7.4 or 5.5 on the apical side of the extravascular environment

Uptake (nmol/mg protein)			
Apical pH	0.0 h	0.5 h	1.0 h
5.5	0.0	0.18 ± 0.02^a	0.23 ± 0.04
7.4	0.0	0.0	0.19 ± 0.08

^aThe pH of the intravesicular environment was kept at 7.4. Each bar indicates mean \pm SD ($n = 3$). Value with symbols is significantly different at $P < 0.05$.

DISCUSSION

The small intestinal mucosa is a layer of epithelia compartmentalized into villi and crypt regions [27]. The villi bear mature cells that arise from multipotent stem cells, and they undergo proliferation and differentiation as they migrate from the crypt compartment [27]. Enterocytes constitute up to 90% of epithelial cells in the crypt and >95% of villus cells [27]. Thus, growth of the small intestinal mucosa is dominated by the proliferation and differentiation of enterocytes. Caco-2 cells derived from a human intestinal adenocarcinoma have been found to provide a useful cell culture model of the small intestinal epithelium. The caco-2 cell line exhibits spontaneous enterocyte-like differentiation under standard culture conditions, showing morphological polarity and expressing brush-border proteases and transporters, including PEPT1, transcytotic activity, and tight junctions [28]. Caco-2 cells have been used for studying the transport of hexose, amino acids, and di- and tripeptides [29]. However, the activities of PEPT1 and transcytosis in Caco-2 cells are lower than those of epithelial cells isolated from animal intestines. Because porcine BBM vesicles can structurally and functionally mimic the biology of the human intestinal apical membrane, we prepared apical membrane vesicles by magnesium chloride precipitation and differential centrifugation according to our previously established procedures [18]. Investigations of amino acids have been largely conducted with porcine BBM [18].

The transepithelial transport of oligopeptides across intestinal epithelial cells has attracted considerable interest in investigations into how biologically active peptides express diverse physiological functions in the body. There are three possible mechanisms for the intestinal transport of oligopeptides: (i) PEPT1-mediated transcellular transport for di- and tripeptides [6], (ii) a transcytotic route, which is known to be used for the transport of macromolecules such as proteins [10], and (iii) intracellular passive transport for peptide absorption [8]. However, the role of these pathways in intestinal oligopeptide absorption is not yet fully understood, with the mechanism for oligopeptide transport across the intestinal epithelial apical membrane still being obscure.

The present study demonstrated the time-dependent hydrolysis of a tripeptide, Gly-Pro-Hyp, into the free-form Gly and a dipeptide, Pro-Hyp, on the apical side of the BBM vesicles. In parallel with the hydrolysis of Gly-Pro-Hyp, Pro-Hyp was identified in the intravesicular space environment. The porcine intestinal BBM contains many intestinal enzymes, including alkaline phosphatase, aminopeptidase N, and sucrase [30]. These enzymes are present in both bound and cytosolic (soluble) forms, and are predominantly bound on the apical surface of the mature enterocyte [30]. These results suggest that Gly-Pro-Hyp was first cleaved to Gly and Pro-Hyp by the

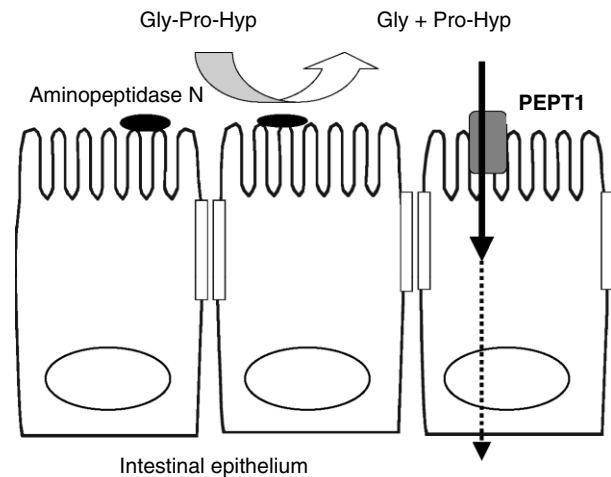


Figure 4 Model of the metabolism and transport of Gly-Pro-Hyp in the intestinal epithelium. This tripeptide is partially hydrolyzed by aminopeptidase N on the apical membrane, and its resulting dipeptide Pro-Hyp is, in part, transported into the cell via the peptide transporter PEPT1.

membrane-bound aminopeptidase N, with only Pro-Hyp and Gly being transported across the BBM. As no Na^+ gradient was established between the extra- and intravesicular environment and CSN^- was used to clamp the membrane potential, the uptake of Gly in the BBM vesicles in our present study condition must have occurred via the Na^+ -independent L system and by simple diffusion due to the leaky nature of the vesicles described and discussed in our previous reports [19,31].

The present study also demonstrated that transcellular transport of Pro-Hyp across the BBM was partially inhibited by the addition of a competitor (Gly-Pro) for PEPT1 and was pH-dependent. These results suggest that H^+ -coupled PEPT1-mediated transport is the major pathway for the transcellular transport of Pro-Hyp (Figure 4). Most proteins and oligopeptides are rapidly hydrolyzed to free amino acids and di- or tripeptides. However, some peptide structures are fairly resistant to hydrolysis, and the extent and the speed at which dietary proteins and oligopeptides are broken down into their constituents depend on their compositions. Peptides containing multiple prolyl residues appear to be more resistant to attack by proteases and peptidases. Biologically active peptides containing multiple prolyl residues have also been identified in protein digests, such as those of dietary proteins (mainly milk proteins), which led to the suggestion that peptides released during the digestion of protein in the gut affect body functions by their opioid, immunomodulatory, or angiotensin-converting-enzyme-inhibiting activity [2]. Di- and tripeptides, or tetrapeptides, exhibit a H^+ -coupled peptide co-transport process. The proton or acidic pH gradient

across the intestinal epithelial apical membrane surface is, in part, maintained by the Na^+/H^+ antiporter activity (NHE-3) on the BBM [6]. Bound peptides containing proline residues have pronounced differences in their mesomeric structures [32]. This led to the determination that the *trans* conformation of the peptide bond alone allows transport of a dipeptide. However, the key structural and conformational elements in PEPT1 substrate and how they affect substrate affinity and electrogenic transport are not yet fully understood. On the other hand, the incomplete inhibition of Pro-Hyp uptake by a typical PEPT1 substrate Gly-Pro in our BBM vesicle model, as shown in Figure 3, would suggest other potential routes of transcellular uptake of Pro-Hyp across the BBM. Indeed, a proton-dependent amino acid transporter (PAT1) has been identified [33,34]. The PAT1 has been shown to transport Pro, Pro derivatives, and Pro-containing peptides or drugs [35,36]. In addition, leaking of Pro-Hyp into the BBM vesicles via the simple diffusion might have also contributed to the Pro-Hyp uptake measured with extravesicular pH at 7.4 (no proton gradient) as shown in Table 1, as well as the incomplete inhibition data reported in Figure 3 of this study. The leaky nature of the BBM vesicle model under *in vitro* conditions had been well reported and also discussed in our previous studies in dealing with amino acid transport measurements [30].

In conclusion, the present study has shown that (i) the tripeptide Gly-Pro-Hyp can be partially hydrolyzed by membrane-bound aminopeptidase N on the apical membrane; (ii) the dipeptide Pro-Hyp is transported across the apical membrane of the cell via the PEPT1; (iii) Gly-Pro-Hyp cannot cross epithelial apical membrane in an intact form; and (iv) Pro-Hyp is highly resistant to hydrolysis by intestinal proteases.

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