



Gastrointestinal Digestion and Absorption of Pen j 1, a Major Allergen from Kuruma Prawn, *Penaeus japonicus*

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Tropomyosin had been identified as a major allergen in shrimp. The digestion and absorption of tropomyosin (Pen j 1) from kuruma prawn were investigated by *ex vivo*, *in vitro*, and *in vivo* techniques in order to elucidate the relationship between the allergenicity of the allergen and its gastrointestinal behavior. Pen j 1 transported the Caco-2 monolayer in a dose-dependent manner, and also enhanced the permeability of lucifer yellow, a marker of paracellular transportation, at high concentrations of the allergen. Studies with everted sacs revealed that Pen j 1 was rapidly degraded to small peptides (MW < 3.5 kDa) and amino acids by intestinal proteases and absorbed from enterocytes. Furthermore, Pen j 1 orally administered to rats tended to remain in the stomach rather than in the small intestine, after which the allergen moved to the epithelial cells. These observations suggest that Pen j 1 may be absorbed via the gastric mucosa prior to its digestion in the intestines.

Key words: shrimp allergen; tropomyosin; gastrointestinal digestion and absorption; Caco-2 cell

An increasing number of patients suffer from food allergy in westernized countries, as is the case with other atopic disorders. Eggs, cow's milk, rice, wheat and soybeans had been thought to be major allergenic foodstuffs in Japan; however, recent studies have indicated that the major cause of food allergies is changing as the dietary habit shifts to a western style.¹⁾ It should be particularly noted that such crustaceans as shrimp and crab are primary allergenic foods for schoolchildren and adults. Crustaceans are frequent causes of allergic reactions throughout the world,²⁻⁵⁾ and an allergen in decapods has been isolated and

identified as tropomyosin, a component of muscle protein.⁶⁻⁸⁾ It has subsequently been reported that tropomyosin was a common allergen in such other crustaceans as lobster^{9,10)} and crab¹¹⁾ and in such mollusks as squid¹²⁾ and oyster.¹³⁾

Sensitization to food allergens occurs in the gastrointestinal tract (class 1 food allergy) or as a consequence of an allergic sensitization to inhalant allergens (class 2 food allergy).¹⁴⁾ The major food allergens identified in class 1 are water-soluble glycoproteins with a molecular mass of 10–70 kDa and fairly good stability to heat, acids, and proteases.¹⁵⁾ It has become apparent, as increasing numbers of allergenic proteins are isolated, characterized, and identified, that similar types of animal and plant proteins make up the vast majority of food allergens. As reviewed by Breiteneder and Radauer,¹⁶⁾ plant allergens are predominantly found in the cupin and prolamin superfamilies, and the protein families of the plant defense system. It has also been reported that the allergenicity of some plant allergens with an *N*-linked glycan moiety could be connected with this linkage.¹⁷⁻²⁰⁾ The growing knowledge about allergenic plant proteins has led to the proposition that both the structural and functional properties of these allergenic proteins are the most important factors in understanding their allergenicity. For example, profilins, which play a major role in regulating the polymerization of actin filaments, are highly conserved throughout the plant kingdom and frequently show IgE cross-reactivity between pollen and food.¹⁴⁾ Likewise, tropomyosins, which occur universally in the animal kingdom and constitute thin myofilaments together with actin and troponin, are common allergens not only in crustaceans but also in such invertebrates as house dust mites and cockroaches.²¹⁻²³⁾

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Abbreviations: BSA, bovine serum albumin; CBB, Coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' balanced salt solution; HE, hematoxylin-eosin; HRP, horseradish peroxidase; LY, lucifer yellow; 2-ME, 2-mercaptoethanol; OVA, ovalbumin; PBS, phosphate-buffered saline; PBS/T, phosphate-buffered saline containing 0.05% Tween 20; PR, permeation rate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TEER, transepithelial electrical resistance

Elucidating their amino acid sequences is therefore most important to understand the allergenicity of tropomyosins from different species.

We have reported in a previous paper²⁴⁾ the isolation, characterization and molecular cloning of Pen j 1, a major allergen in kuruma prawn (*Penaeus japonicus*), which is popularly consumed in Japan. We have furthermore demonstrated that IgE-binding epitopes of Pen j 1 are located over the whole sequence of the allergen, and that most IgE antibodies in the sera strongly recognize its C-terminal region.

The development of food hypersensitivity, *i.e.*, food allergy, is related to a failure to induce oral tolerance.²⁵⁾ Individuals are therefore thought to be sensitized by the allergens when antigenic proteins prevented from being digested with enzymes are absorbed in the body *via* the gastrointestinal tract without any uptake by Microfold cells (M cells) to induce oral tolerance. Since the gastrointestinal digestibility and permeability of a food protein may affect the allergenic potential,^{26–29)} these are key factors for understanding the allergenicity of a target protein. Recent studies have demonstrated that food allergens were able to cross the intestinal barrier by the transcellular pathway^{30,31)} or by paracellular transport.^{32,33)} The previous paper²⁴⁾ has shown that Pen j 1 had no cysteine residue on the polypeptide, and this led us to the idea that the allergen would be easily attacked by digestive proteases. However, no study on the gastrointestinal digestibility and permeability of Pen j 1 has been performed. We report in this present paper the digestibility and permeability of Pen j 1 by using *in vitro*, *ex vivo* and *in vivo* assays to elucidate the mechanisms involved in the gastrointestinal transport of the allergen and the subsequent induction of an allergic reaction.

Materials and Methods

Purification of tropomyosin from kuruma prawn. Pen j 1 was prepared from live kuruma prawn, *Penaeus japonicus*, as previously described.²⁴⁾

Digestibility of the allergen in SGF (pepsin) and SIF (trypsin). We examined the digestibility of Pen j 1, ovalbumin (OVA) and bovine serum albumin (BSA) by incubating with pepsin (Sigma Chemical Co., St. Louis, MO, USA) as a standard simulated gastric fluid (SGF) and with trypsin (Roche Diagnostics Co., Indianapolis, IN, USA) as a standard simulated intestinal fluid (SIF). The allergens were incubated with 0.3 µg of pepsin in a 100 mM KCl-HCl (pH 2.0) buffer or with trypsin (0.3 µg) in a 0.1 M Tris-HCl buffer (pH 8.0). The ratio of the proteases to the allergens was about 1:100 (w/w). At intervals of 0, 1, 2, 4, 8, and 24 h, the sample (10 µL) incubated with a protease was taken out, added to an equal aliquot of the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by using 15% gel.

Cell culture. The human colon cell line, Caco-2 (American Type Culture Collection), was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), 44 mM NaHCO₃, 0.9 mM sodium pyruvate (Gibco-BRL), a 1 mM non-essential amino acid solution (Gibco-BRL), 5 U/mL of penicillin (Gibco-BRL), 5 µg/mL of streptomycin (Gibco-BRL) and 0.01 N HCl in 75-cm³ flasks. The cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ in air and subcultured up to 70–80% confluence. The cells were passaged by trypsinizing with trypsin/EDTA (Gibco-BRL) after one week. The cells of passages 40–50 were

then grown in Transwell inserts (a polycarbonate membrane, 6.5 mm diameter and 0.4 µm pore size; Corning Costar, NY, USA) in 12-well plates at a density of 2×10^5 cells/mL. The basolateral (serosal) and apical (mucosal) compartments respectively contained 0.8 mL and 0.2 mL of the culture medium. The culture medium was replaced three times each week. The monolayer became confluent 7 d after seeding, and cells that had been cultured for 21 d were used for the subsequent experiments.

Measurement of the epithelial barrier function. The intestinal barrier function was evaluated by measuring the unidirectional flux of lucifer yellow (LY) in the Caco-2 cell monolayers. Fully differentiated cells on Transwell inserts are impermeable to any LY due to the formation of intercellular tight junctions.^{34–36)} Fluorescent LY was used as a marker for all the experiments to evaluate the paracellular permeability. LY across confluent Caco-2 monolayers was measured after removing the culture medium in the Transwell inserts and adding instead a Hanks' balanced salt solution (HBSS) at pH 7.4. After incubating for 30 min at 37 °C, 0.2 mL of LY (0.1 mM) was added to the apical side of the Transwell inserts, and 0.8 mL of HBSS was added to the basolateral compartment during the incubation of the Caco-2 cell monolayers with various allergens. The fluorescence of LY in each basolateral medium was measured with an F-2000 fluorescence spectrophotometer (Hitachi), respectively using 480 and 530 nm filters for excitation and emission.

Glucose transport and sucrase activity. The glucose transport capacity and sucrase activity were determined to evaluate the enterocytic differentiation of Caco-2 cells grown on Transwell inserts. Caco-2 monolayers, which had been cultured in 12-well plates for 21 d, were gently washed with 500 µL of the HBSS warmed at 37 °C and used for the assays. One hundred microliter of a glucose solution (1.0 mg/mL in HBSS) was added to the apical side of the Transwell insert, and 0.6 mL of HBSS was supplemented to the basolateral side, before incubating at 37 °C for 3 h in a CO₂ incubator. The basolateral solution was collected, and the amount of glucose was determined by the enzymatic method³⁷⁾ with glucose oxidase and peroxidase to measure the rate of glucose transport across the Caco-2 cell monolayers. The sucrase activity was measured in the same manner, except that 100 µL of sucrose solution (1.9 mg/mL in HBSS) was added to the apical side of the Transwell insert.

Transport of allergenic proteins across the Caco-2 monolayers. Caco-2 cells were grown on Transwell inserts for 21 d, and then the inserts were washed 3 times with 37 °C HBSS, incubated for 30 min in a CO₂ incubator, and the test substances added. Purified Pen j 1 together with LY was added to the apical side of the inserts to a final concentration of 0.5–5.0 mg/mL in HBSS, before incubating at 37 °C for 3 h in a CO₂ incubator. The media on both sides of the inserts were collected and stored at –30 °C until needed for analysis by sandwich ELISA. BSA as standard protein and Gly m Bd 30K, which is the major allergen of soybean, were also used in this study to compare the permeability through the Caco-2 monolayers with that of Pen j 1. Gly m Bd 30K was purified from the oil body pad of soybeans as previously described.³⁸⁾

Preparation of the polyclonal antibody against Pen j 1. Nihon white rabbits (female, 2 kg) were used to obtain the antiserum. Purified Pen j 1 (200 µg/rabbit) was subcutaneously injected into the rabbit dorsum with Freund's complete adjuvant (Difco Laboratories, MI, USA). Pen j 1 (100 µg/rabbit) was subsequently administered with Freund's incomplete adjuvant (Difco Laboratories, MI, USA) at intervals of 7 d. The rabbits were killed one week after the last injection by withdrawing blood from the aorta, and the antiserum was then obtained. The polyclonal antibody in the antiserum was fractionated by collecting the precipitate formed with ammonium sulfate between 0% and 50% saturation, and then purified by using an Affi-gel protein A MAPS II kit (Bio-Rad, Hercules, CA, USA). A quantitative determination of Pen j 1 with a sandwich enzyme-linked immunosorbent assay (ELISA) was performed by biotinylating the purified polyclonal antibody (IgG class) with D-biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Tokyo, Japan). The present experiment with the animals was conducted

according to the National Research Council's guide for the care and use of laboratory animals.

Labeling of Pen j 1 with ^{125}I . The lysine residues (ϵ -amino group) of Pen j 1 (75 μg) were radio-iodinated with the ^{125}I -Bolton-Hunter reagent at 500 μCi (GE Healthcare UK, Little Chalfont, Bucks, England) according to the manufacturer's recommendations. The reaction mixture together with cold Pen j 1 (425 μg) was applied to a Sephadex G-25 column (1 $\times$ 25 cm) to remove free ^{125}I and fractionated in 1-mL samples. ^{125}I -Pen j 1 was eluted in fractions 9 and 10, collected and finally had a specific radioactivity of about 20 $\mu\text{Ci}/500 \mu\text{g}$. The number of lysine residues per molecule was 25 for Pen j 1.

Small intestinal absorption of ^{125}I -Pen j 1. Male Sprague-Dawley (SD) rats weighing about 270 g (Charles River Lab., Kanagawa, Japan) were used in this experiment. They were kept in a $23 \pm 2^\circ\text{C}$ air-conditioned room that was lit from 8:00 a.m. to 8:00 p.m. The rats were starved for 16 h, but with free access to water, sacrificed under ether anesthesia, and the small intestines were immediately excised. A portion of the jejunum was excised, rinsed several times with a cold 0.9% NaCl solution to remove endogenous digestive enzymes, and carefully everted over a steel rod. The everted jejunum was cut into 4-cm segments, and each segment was tied with a thread at one end, filled with an oxygenated 0.9% NaCl solution containing 0.1% glucose (solution A) to give a physiological distension, and again tied with a thread at the other end to make a gut sac. These sacs were placed and incubated at 37°C in 5 mL of solution A containing Pen j 1 (1 mg) and 50 μg of ^{125}I -Pen j 1. A gut sac was taken out at given intervals, and separated into the serosal and mucosal solutions and small intestinal tissue. An equal volume of a 10% trichloroacetic acid (TCA) solution was added to an aliquot of the serosal and mucosal solutions collected, and the radioactivity of the TCA-soluble fractions was measured. The radioactive degradation products in each sample were analyzed by SDS-PAGE and Sephacryl S-100 column chromatography (1 $\times$ 30 cm; GE Healthcare UK, Little Chalfont, Bucks, England).

Oral administration of the allergenic proteins. Male SD rats (3 weeks old, $n = 6/\text{group}$) weighing about 45 g were used in this experiment, and were starved before the experiment in the same manner as that just described. Five milligrams of Pen j 1 or OVA (Sigma Chemical Co., St. Louis, MO, USA) in 1 mL of physiological saline was administered to each rat by means of gastric intubation. A celiotomy was quickly performed under anesthesia at the indicated times after the administration, and portal blood samples were collected. Plasma samples were obtained by centrifuging at $2,000 \times g$ for 10 min at 4°C . The gastrointestinal tract was excised and divided into the stomach and small intestine, and the small intestine was further divided into six equal parts. The contents of the each part were collected by washing out with 1 mL of physiological saline containing a protease inhibitor cocktail, and the supernatant was recovered by centrifuging at $2,000 \times g$ for 15 min. The tissue segments were washed six times with 10 mL of saline to remove the compounds adsorbed to the surface of the mucosa, homogenized in 5 vols. of physiological saline containing a protease inhibitor cocktail and centrifuged at $8,000 \times g$ for 30 min. All of the supernatant samples obtained were stored at -30°C until being subjected to SDS-PAGE and sandwich ELISA.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were carried out in basically the same manner as that described previously.²⁴ An anti-Pen j 1 polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Funakoshi Co., Tokyo, Japan) were respectively used as 1st and 2nd antibodies for detecting Pen j 1 and its degradation products. OVA and its degradation products on the membrane were immune-stained with the HRP-conjugated anti-OVA antibody (Funakoshi Co., Tokyo, Japan).

Sandwich ELISA. ELISA plates (96 wells, Falcon) were incubated overnight at 4°C with 50 μL of the anti-Pen j 1 polyclonal antibody (1 $\mu\text{g}/\text{mL}$ of phosphate-buffered saline (PBS)) and then washed three times with PBS containing 0.05% Tween (PBS/T). The wells were blocked with 1% BSA/PBS (100 μL) at 37°C for 1 h. The wells were next incubated with precisely diluted sample solutions at 37°C for 1 h and similarly washed as just described. Pen j 1 and its degradation

products binding to the 1st antibody on the wells were further reacted with the biotinylated anti-Pen j 1 polyclonal antibody and then with HRP-conjugated streptavidin. The immunocomplexes on the wells were detected as previously described.³⁹ The total amount of Pen j 1 and its degradation products in the samples was determined with a calibration curve obtained with purified Pen j 1 as a standard specimen. Anti-OVA (Funakoshi Co., Tokyo, Japan) and HRP-conjugated anti-OVA were respectively used as the 1st and 2nd antibodies to determine OVA and its degradation products. Apart from this exception, the assay procedure was similar to that already described.

Immunolocalization of allergenic proteins in the gastrointestinal organs after the oral administration. The rat stomach and small intestine (jejunum) were fixed with 10% buffered formalin and embedded in paraffin blocks (Fisher Scientific Japan, Tokyo, Japan). Tissue sections of 3 μm thickness were immuno-stained with hematoxylin-eosin (HE) for anti-Pen j 1 or the anti-OVA antibody. Paraffin sections of each specimen were mounted on MAS-coated slides (Matsunami Glass Ind., Osaka, Japan) and deparaffinized with Clear Plus (Falma, Tokyo, Japan).

Immunostaining was performed by rinsing deparaffinized sections for 5 min in PBS, treating with absolute methanol containing 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity, and washing with 10 mM PBS. They were incubated with the blocking solution for 30 min at room temperature and then incubated with the anti-Pen j 1 polyclonal antibody for 1 h at room temperature in a moist chamber. They were then washed 3 times with PBS and treated with HRP-conjugated goat anti-rabbit IgG for 1 h. After washing with PBS, they were reacted for 10 min with 0.25% diaminobenzidine and 0.03% H_2O_2 in a 0.05 M Tris-HCl buffer at pH 7.6, and then washed with PBS. All sections were briefly counterstained with Mayer's hematoxylin (Wako Pure Chemicals Co., Osaka, Japan) and mounted in Para-mount D (Falma, Tokyo, Japan).

Data analyses. Each result is expressed as the mean $\pm$ SE. Statistical evaluations were performed by Student's *t*-test.

Results

Digestibility of the allergens in SGF and SIF

The resistance to digestion of proteases could be a major factor for the development of allergic reactions. Pen j 1 was examined for the digestibility of SGF and SIF by SDS-PAGE (Fig. 1), OVA and BSA being used as reference proteins. As shown in Fig. 1, Pen j 1 was relatively stable in SGF and remained in the peptide form with a molecular mass of 19–16 kDa after 24 h. In the case of OVA, two protein bands corresponding to 45- and 43-kDa proteins were detected after 24 h. BSA was rapidly digested and converted to low-molecular-weight peptides ($<10 \text{ kDa}$) within 1 h, and completely hydrolyzed within 24 h. In the experiments with SIF, however, Pen j 1 was rapidly digested and could not be detected after a 1-h incubation with SIF. OVA and BSA were hardly digested within 24 h. These results suggest that Pen j 1 may be digested more easily in the small intestine than other allergens, but may be relatively stable in the stomach.

Dose-dependent absorption of Pen j 1 in Caco-2 cells

Caco-2 cells grown on a permeable membrane filter in Transwell inserts reached confluence after 7 d. The permeable rate (PR) of LY decreased, reached its lowest level (about 0.1%) after a 2-week cultivation, and maintained this lowest level for up to 3 weeks (Fig. 2A). Moreover, Caco-2 cells grown on a permeable membrane filter for 21 d exhibited high glucose transport capacity (Fig. 2B) and sucrase activity as a marker of differentiation (Fig. 2C). These results indicate that

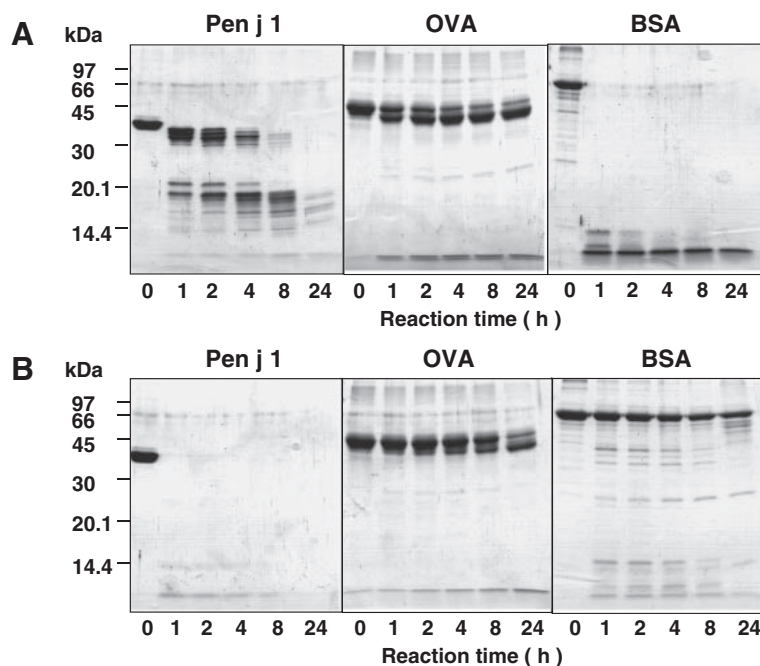


Fig. 1. Digestion of Allergens with SGF (A) and SIF (B).

Pen j 1, OVA and BSA were examined for their digestibility by pepsin and trypsin. They were incubated with each enzyme (an enzyme/substrate weight ratio of 1/100) at 37°C for 0, 1, 2, 4, 8, and 24 h. The proteolytic products were analyzed by SDS-PAGE (15% gel).

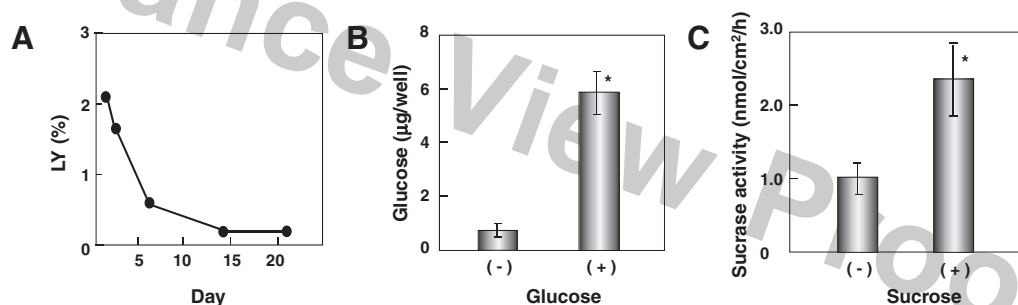


Fig. 2. Proliferation and Differentiation of Caco-2 Cells on the Cell Culture Insert.

A, Change in the amount of transported LY with the proliferation of Caco-2 cells on the cell culture insert. The initial concentration of LY in the apical solution was 100 μM. The concentration (X μM) of LY in the basolateral solution was determined after incubating LY with Caco-2 cells. The values in the figure represent $X/100 \times 100$ (%) and are expressed as the means of the values for 3 wells. B, Transport of glucose across the Caco-2 cell monolayer. The amount of glucose in each basolateral solution was determined after supplementing glucose to the apical side and incubating with Caco-2 cells. Each result is expressed as the mean $\pm$ SE μg/well ($n = 4$). *Values obtained by incubating with glucose are significantly different from those obtained by incubating without glucose ($p < 0.05$). C, Sucrase activity of Caco-2 cells on the cell culture insert. The amount of glucose in each basolateral solution was determined after supplementing sucrose to the apical side and incubating with Caco-2 cells. Each result is expressed as the mean $\pm$ SE nmol/cm²/h ($n = 4$). *Values obtained by incubating with sucrose are significantly different from those obtained by incubating without sucrose ($p < 0.05$).

Caco-2 cells grown on a permeable membrane filter for 21 d formed a confluent monolayer consisting of well-polarized columnar cells displaying tight junctions at their apical aspect and a brush border. We therefore used Caco-2 cells exhibiting characteristics of mature small intestinal enterocytes in this study.

The transportation of Pen j 1 from the apical to basolateral solution was investigated after 3 h of incubation. Pen j 1 (0.5 to 5 mg/mL) supplemented to the apical solution was rapidly transported to the basolateral solution after 1 h of incubation with the cells and then more slowly up to 3 h, the amount of intact Pen j 1 in the basolateral solution remaining at a similar level thereafter (data not shown). Pen j 1 was detected in the basolateral solution by using sandwich ELISA. Amounts of 0.5 and 2 mg/mL of Pen j 1 incubated with the cells on the inserts respectively resulted in the concentrations

of the allergen detected in the basolateral solution being 4.2 ± 1.0 ng/mL (0.0008% of the total) and 52.5 ± 12.5 ng/mL (0.0027% of the total) (Fig. 3A). Pen j 1 was thus transported in a dose-dependent manner, but PR of LY added together with 2 mg/mL of Pen j 1 in the inserts was markedly increased ($1.4 \pm 0.3\%$, $p < 0.01$) when compared with incubating with 0 or 0.5 mg/mL of Pen j 1. Only intact Pen j 1 was detected by immunoblotting in the basolateral solution in this experiment (data not shown). These results suggest that Pen j 1 might have been transported *via* a paracellular route to the Caco-2 monolayer. The high level of PR for LY resulting from incubation with 2 mg/mL of Pen j 1 indicates the formation of a monolayer exhibiting a loose tight junction between cells. Pen j 1 at this high concentration might therefore appear to open the tight junction between cells of the Caco-2 monolayer.

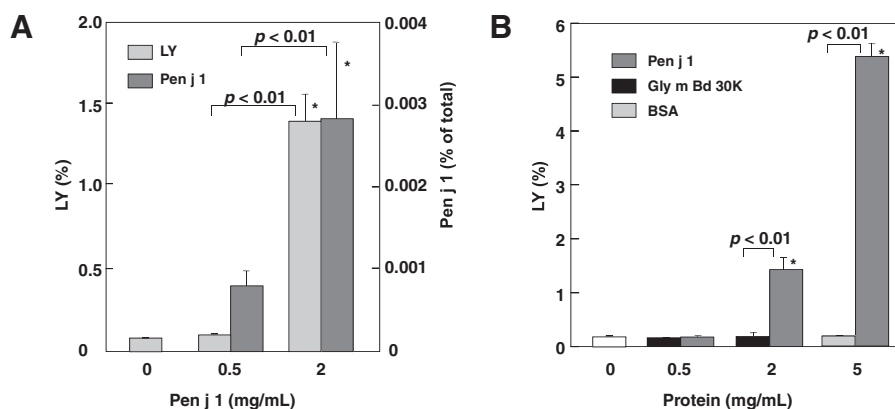


Fig. 3. Pen j 1 Transportation Across the Caco-2 Cell Monolayer and Its Effect on the Barrier Function of the Cells.

A, Pen j 1 and LY were supplemented to the apical side of the same well. The amounts of both substances in the basolateral solution were determined after incubating with Caco-2 cells as described in the text. B, LY and various proteins were supplemented to the apical side of the wells. The amount of LY transported was determined as described in the footnote to Fig. 2. The soybean allergen, Gly m Bd 30 K, and BSA were used as proteins other than Pen j 1. Each result is expressed as the mean $\pm$ SE% ($n = 5$). *Values are significantly different from those under the other incubation conditions ($p < 0.01$).

Effect of various allergens on the permeability of LY

The next experiment was performed to determine which concentration or structural property of Pen j 1 affected the PR level of LY. Gly m Bd 30 K (the soybean allergen) and BSA (the beef allergen) were used as reference proteins in this experiment. Figure 3B shows that neither Gly m Bd 30 K (0.5 or 2 mg/mL) nor BSA (5 mg/mL) affected PR of LY, but that Pen j 1 at concentrations of 2 and 5 mg/mL significantly increased it. The effect of Pen j 1 on the permeability of LY was dose-dependent ($1.45 \pm 0.2\%$ to $5.4 \pm 0.4\%$), suggesting that this allergen might be able to disturb the integrity of the tight junctions between epithelial cells due to its unique structure of two-stranded α -helical coiled-coils and without intermolecular disulfide bonds.

Absorption of ^{125} I-Pen j 1 in everted gut sacs from rats

The model system using everted gut sacs from rats has been used as a useful semi-biological method for monitoring the transportation and accumulation of various compounds in the small intestine.^{40–43} We also investigated the digestibility and absorbability of Pen j 1 in the small intestine with the *ex vivo* system. Each everted gut sac, which had been prepared as described in the Materials and Methods section, was bathed in a saline solution containing ^{125} I-labeled Pen j 1, and the radioactivity recovered from the basolateral side of the sac was measured. As shown in Fig. 4A, the radioactivity recovered from the everted sacs from two rats (nos. 1 and 3) of the three tested was about 0.1% of ^{125} I-Pen j 1 added to the apical side after a 60-min incubation. The other rat (no. 2) exhibited higher recovery of the radioactivity (0.6%). More than 95% of the radioactivity in the basolateral solution was recovered in the TCA-soluble fraction from all the rats, suggesting that most of the radioactive compounds in the basolateral solution were peptides with low molecular mass obtained by digestion. The radioactivity recovered in the TCA-soluble fraction of the apical solution was, in contrast, less than 10%, except for the case of rat no. 2, indicating that Pen j 1 predominantly remained intact on the apical side (data not shown). We

studied the molecular details of the radioactivity by using gel-filtration chromatography to clarify the reason for the marked difference among the animals.

The radioactive compounds in the solutions from both sides were fractionated by Sephacryl S-100 column chromatography (Fig. 4B and C). The figures show representative results obtained from rat nos. 2 and 3. The radioactivity on the apical side of the intestine from rat no. 3 predominantly remained in the form of peptide fractions with high molecular mass (Fig. 4B; $\circ$ – $\circ$). In the case of rat no. 2, however, the radioactivity was approximately divided into that from three peptides with molecular masses of 3.5 kDa, 2.0 kDa and less than 1 kDa (Fig. 4B; $\bullet$ – $\bullet$). The chromatograms (Fig. 4C) of the solutions from the basolateral side exhibited similar separation patterns to those from the apical side of rat no. 2. It can be assumed from these results that gastrointestinal digestive enzymes on the apical side surface of the everted sacs from rat no. 2 had been poorly washed out, resulting in the activated degradation of Pen j 1.

Transportation of Pen j 1 into the portal vein of rats after an oral administration

Figure 5 shows the time-course characteristics for the concentration of Pen j 1 or OVA as a reference protein in portal blood after an oral administration. Portal blood samples were collected 15 and 30 min after oral the administration, and the concentrations of their components were measured by sandwich ELISA. Figure 5B shows that a small amount of a substance reactive to the antibody specific for Pen j 1 was detected in the plasma collected after 15 and 30 min, although this was only detected in a limited number of rats (1/6 or 2/6). The concentrations of Pen j 1 in the portal plasma tended to increase in a time-dependent manner. In the case of OVA, however, the immunoreactive substance was detected in the plasma of only one rat collected after 30 min, but not after 15 min. Although the difference in transport rate into the portal plasma was not statistically significant between Pen j 1 and OVA, OVA may have been more slowly absorbed *via* the gastrointestinal tract in comparison with Pen j 1.

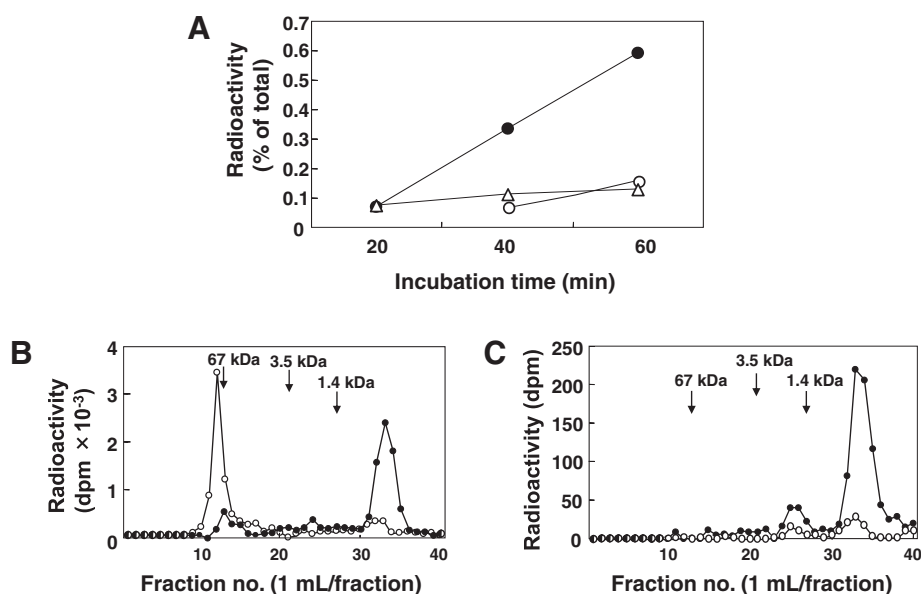


Fig. 4. Radioactive Compounds in the Apical-Side and Basolateral-Side Solutions after Supplementing ^{125}I -Labeled Pen j 1 to the Apical Side of the Everted Gut Sacs.

Rat no. 1 ($\circ$), no. 2 ($\bullet$) and no. 3 ($\triangle$) were used for preparing the everted gut sacs. The transport rate (A) of ^{125}I -Pen j 1 was calculated as the percentage of the radioactivity recovered in the basolateral side solution to the radioactivity ($2\mu\text{Ci}$) administered to the apical side of each everted gut sac. The respective volumes of the apical side and basolateral side solutions collected after incubation in this experiment were about 5 and 0.4 mL. The apical-side (B) and basolateral-side (C) solutions after a 60-min incubation with the everted gut sacs from rat no. 2 ($\bullet$) and no. 3 ($\circ$) were subjected to this analysis. The apical side and the basolateral solutions were collected after incubation as already described, and 10- μL aliquots were applied to a Sephacryl S-100 ($1 \times 10\text{ cm}$) column. Arrows indicate the elution positions of protein size markers (BSA, 67,000 Da; insulin β chain, 3,450 Da; vitamin B_{12} , 1,355 Da).

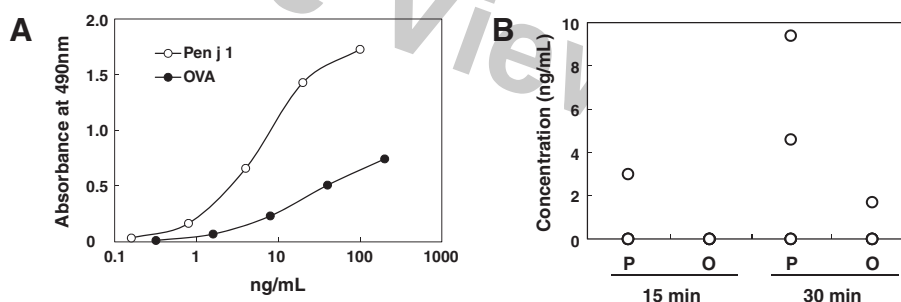


Fig. 5. Transportation of Pen j 1 and OVA after the Oral Administration.

Panel A shows the standard sandwich ELISA curves for Pen j 1 and OVA obtained by the method described in the text. Pen j 1 or OVA (5 mg/rat) was orally administered to the rats through a stomach tube. The portal blood was collected after 15 min and 30 min, and the concentration of immunoreactive substances containing their proteolytic compounds in the plasma was determined by sandwich ELISA (panel B). Each symbol represents an individual rat ($n = 6$). P, Pen j 1; O, OVA.

Digestibility and gastrointestinal absorbability of Pen j 1 after an oral administration

The degradation of Pen j 1 was examined *in vivo* by analyzing intact Pen j 1 and its proteolytic products remaining in the gastrointestinal tract after the oral administration by sandwich ELISA and immunoblotting. Pen j 1 or OVA was orally administered to the rats (5 mg/rat), and the contents of the gastrointestinal tract were taken out from the stomach and 6 sections of the small intestine as described in the Materials and Methods section. The amounts of immunoreactive substances in the contents recovered 15 and 30 min after the oral administration were evaluated by sandwich ELISA (Fig. 6). The amounts of immunoreactive substances remaining in the stomach 15 and 30 min after orally administering Pen j 1 were respectively estimated to be from 70 to 840 μg and from 3 to 145 μg (Fig. 6Aa). Those present in each small intestinal section were

detected at a lower level in only one part (section no. 2) after 15 min. Intact OVA and its proteolytic products present in the stomach 15 min after the oral administration were estimated to be from 9 to 550 μg , but they had disappeared from the stomach 30 min after (Fig. 6Ab). However, intact OVA and its proteolytic products were predominantly present in the small intestine, especially in section nos. 5 and 6 for at least 30 min. The location of these peptides derived from OVA changed from the stomach to the distal part of the small intestine as time elapsed after the administration.

The substances present in the luminal contents were characterized by SDS-PAGE and immunoblotting analyses (Fig. 6B). Some protein bands corresponding to intact Pen j 1 and the proteolytic products with high molecular mass (34–20 kDa) were observed in immunoblots of the contents of the stomach 15 and 30 min after the oral administration, and some in section no. 2 of the

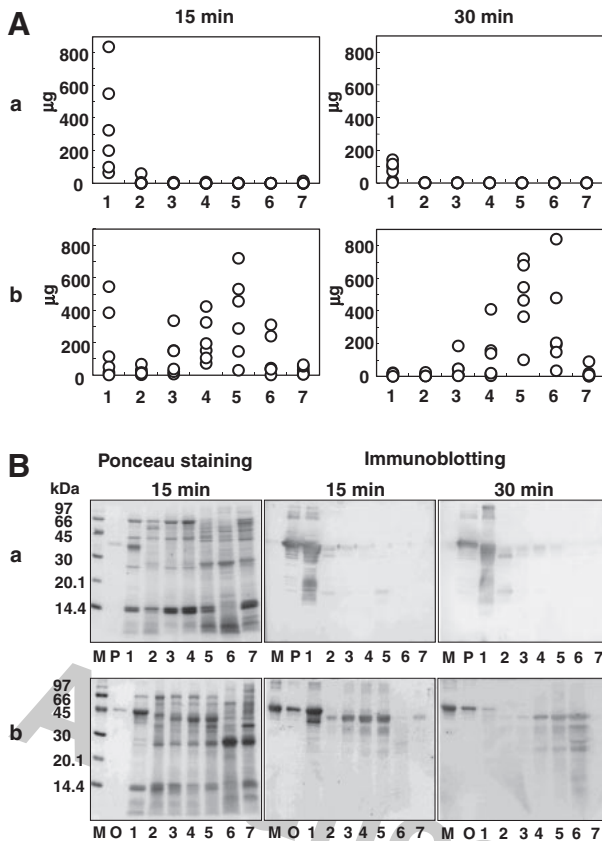


Fig. 6. Distribution of Pen j 1 (a) and OVA (b) in the Gastrointestinal Tract Contents after the Oral Administration.

The administration of the allergens and the preparation of tissue samples were carried out as described in the text. The small intestine was divided into six equal sections, and the contents of each section and the stomach were washed out with 1 mL of saline. The contents were divided into the supernatant and precipitate by centrifugation, and the concentration of the allergen containing its compounds digested in each supernatant was measured by sandwich ELISA (panel A). Each symbol expresses an individual rat ($n = 6$). Each extract from the digested contents obtained as just described was subjected to SDS-PAGE and immunoblotting analyses (panel B). The peptides electrotransferred on to nitrocellulose membranes from each gel after SDS-PAGE (15% gel) were stained with Ponceau S and then immunostained with the anti-Pen j 1 antibody or anti-OVA antibody. Each number shows the stomach (1) and small intestine which was divided into 6 sections numbered from the proximal to distal end (2–7). M, protein size marker; P, Pen j 1; O, OVA.

small intestine (Fig. 6Ba). In the case of OVA (Fig. 6Bb), two proteins (45 and 43 kDa) corresponding to intact OVA and its proteolytic products were predominantly detected only in the contents of the stomach 15 min after the oral administration. The 43-kDa peptide derived from OVA remained predominantly in the contents from the middle parts (section nos. 3–5) of the small intestine 15 min after the oral administration, and various proteolytic peptides with a broad range of molecular mass (43–10 kDa) appeared mainly in the contents of the distal parts (section nos. 5 and 6) of the small intestine after 30 min. These results indicate that Pen j 1 was much more susceptible to proteolytic hydrolysis by intestinal digestive enzymes than OVA and that Pen j 1 tended to remain in the stomach rather than in the small intestine.

We also examined the uptake of their allergens into gut tissues after the oral administration. The immunor-

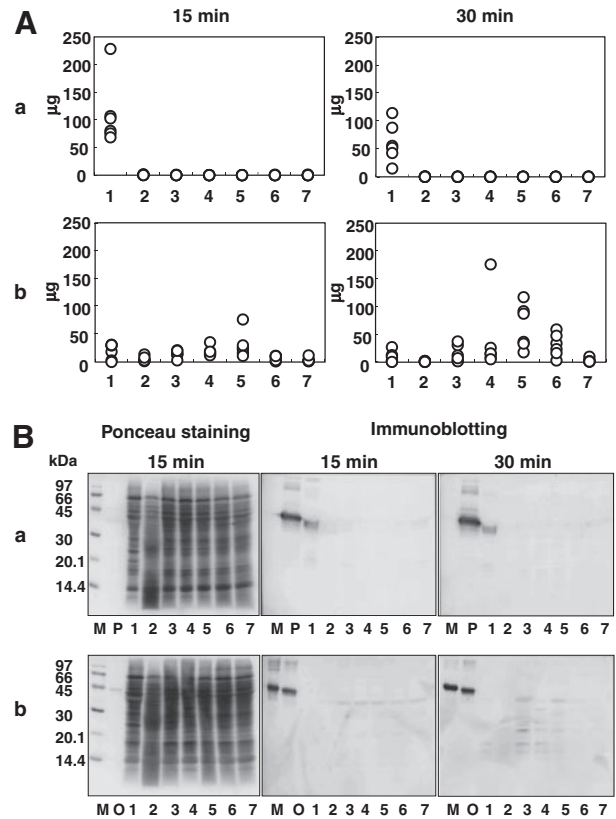


Fig. 7. Distribution of Pen j 1 (a) and OVA (b) in the Gastrointestinal Tract Tissues after the Oral Administration.

The stomach and small intestinal tissues were homogenized in saline containing a protease inhibitor, and then the supernatant and precipitate were obtained by centrifugation of each homogenate as described in the text. The concentration of the immunoreactive substances containing its compounds digested in each supernatant was measured by sandwich ELISA (panel A). Each symbol expresses an individual rat ($n = 6$). The same tissue samples were subjected to SDS-PAGE and immunoblotting analyses (panel B) which are described in the footnote to Fig. 6. Each number shows the stomach (1) and small intestine which was divided into 6 parts numbered from the proximal to distal end (2–7). M, protein size marker; P, Pen j 1; O, OVA.

active substances in the extracts obtained from the stomach and each section of the small intestine were analyzed in the same manner as that used for analyzing the luminal contents. The amounts of immunoreactive substances in the extracts of the stomach taken out 15 and 30 min after administering Pen j 1 were respectively estimated to be from 70 to 230 µg and from 10 to 115 µg (Fig. 7Aa). However, no immunoreactive substances could be detected in the sections of the small intestine taken out after 15 and 30 min. Only the 33-kDa peptide derived from Pen j 1 in the stomach extracts was immunoblotted (Fig. 7Ba). In the case of OVA, the immunoreactive substances in the tissue extracts were detected in only trace amounts in the stomach taken out after 15 and 30 min (Fig. 7Ab). The distribution pattern of the immunoreactive substances from OVA in the tissue extracts of the small intestine was very similar to that in the luminal contents (Fig. 7Ab and Bb).

Trans epithelial transport of food allergens

The initial stage of food allergy is the allergen uptake into the lamina propria mucosa of the digestive tract.

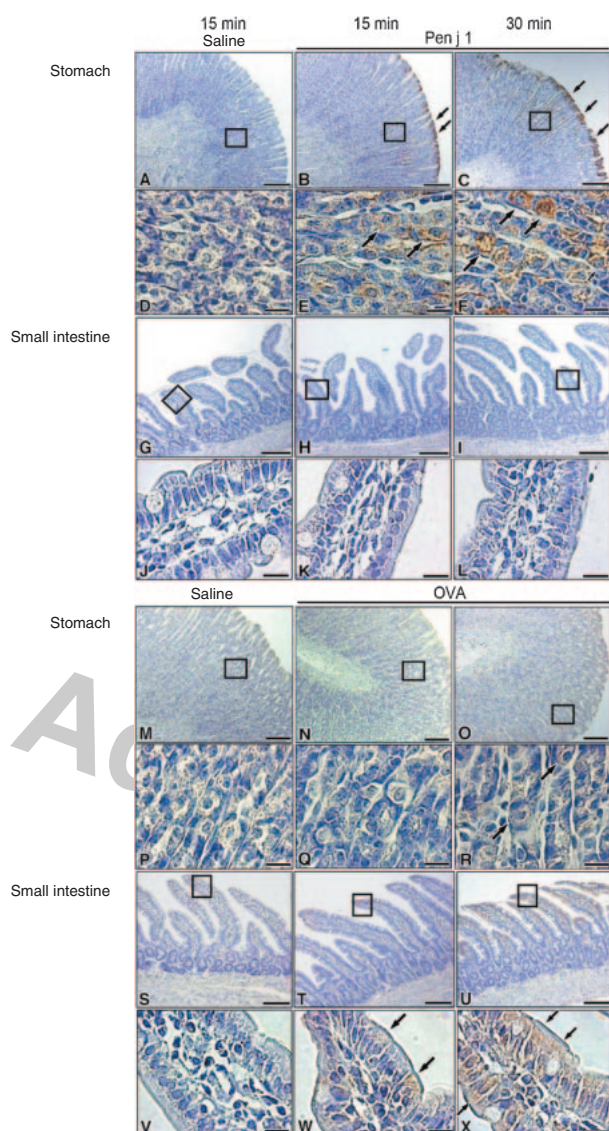


Fig. 8. Immunolocalization of Pen j 1 and OVA in Mucous Tissues from the Gastrointestinal Tract after the Oral Administration.

The rats were treated orally with 5 mg of Pen j 1 (B, C, E, F, H, I, K and L), OVA (N, O, Q, R, T, U, W and X) or physiological saline as a control (A, D, G, J, M, P, S and V). Fifteen minutes and 30 min after the oral administration, the immune-histochemical localization of Pen j 1 and OVA in the rat stomach and small intestine (jejunum) were investigated as described in the text. Immunopositive staining is indicated by a brown color (arrows). Bars in A-C, G-I, M-O and S-U, and in D-F, J-L, P-R and V-X respectively represent a 100- μ m width and a 10- μ m width.

However, it is not clearly understood how allergens pass through the epithelium. We used immunohistochemical staining with anti-Pen j 1 and anti-OVA antibodies in an attempt to reveal the location of the allergen within the gastrointestinal tissues (stomach and jejunum) at different times after the oral administration. The immunoreactive substances from Pen j 1 were intensely apparent in the superficial gastric wall and in the cytoplasm of the gastric parietal cells, and the immunoreactivity of the parietal cells was enhanced in a time-dependent manner, indicating that Pen j 1 moved deeper into the gastric mucosa as time elapsed after the oral administration (Fig. 8E and F). In contrast, the jejunal absorptive cells were not stained at all with anti-Pen j 1 (Fig. 8H, I, K and L). The studies with OVA showed that only weak

immunoreactivity was present in a few parietal cells (Fig. 8R). Interestingly, the OVA-immunopositive reactions were intensely apparent in the jejunal surface absorptive cells (Fig. 8W and X). These results suggest that Pen j 1 may have been absorbed by gastric mucosal surface cells and parietal cells, while OVA was absorbed by jejunal absorptive epithelial cells and then transferred to the circulation.

Discussion

Protein stability is likely to be the most important factor in understanding the allergenicity of food allergens. Food allergens generally tend to be soluble, low-molecular-weight glycoproteins with an acidic isoelectric point. They are usually stable to heat and acidic conditions and relatively resistant to proteolytic digestion.^{44–46} Tropomyosin from kuruma prawn was elucidated in the previous study to be a major allergen and to have no cysteine residue on the polypeptide;²⁴ tropomyosin has been reported to form a coiled coil structure that binds head-to-tail along the helical actin filament.⁴⁷ Tropomyosin is also known to be soluble, and to have an acidic isoelectric point (pH 4.5) and heat stability.⁴⁸ However, the behavior of tropomyosin during digestion in the gastrointestinal tract had not previously been examined. We investigated in this study the behavior of various allergens, including Pen j 1, by incubating with pepsin as SGF and trypsin as SIF (Fig. 1). Pen j 1 was found to be relatively more stable to SGF digestion than BSA, but it was rapidly degraded by incubating with SIF. OVA, a major egg allergen, and BSA were highly resistant to digestion with SIF. Tong-Jen Fu *et al.*⁴⁹ have measured the stability of a group of food allergens and a group of non-allergenic proteins for digestion with SGF and SIF, and compared them between two groups. They concluded from data obtained that food allergens were not necessarily more resistant to digestion than non-allergenic proteins. This finding suggests that it would be difficult to rank the allergenic potential of proteins on the basis of their digestibility by SGF and SIF. The evidence that Pen j 1 was relatively stable to SGF, and that the digested fragments remained in the form of peptides with a molecular mass of more than 20 kDa for at least 24 h suggesting that Pen j 1 had a unique property to trigger an allergic reaction.

The intestinal epithelium is the primary site for the absorption of dietary components and plays an important role in sensitization with food allergens. We examined in this study the molecular mechanism for the intestinal absorption of Pen j 1 by Caco-2 cells as a model of absorptive enterocytes. The transportation of Pen j 1 in Caco-2 cells was enhanced in a dose-dependent manner (Fig. 3). Although the respective percentages of transported Pen j 1 and LY were 0.0008% and 0.11%, after a 3-h incubation of Pen j 1 at 0.5 mg/mL, both substances had significantly increased when Caco-2 cells were incubated with Pen j 1 at more than 2 mg/mL. The previous reports have indicated that the transepithelial electrical resistance (TEER), an evaluating marker for a well-defined tight junction, as well as LY, was affected by food components (*e.g.*, food grade surfactants, capsaicins, and sodium caprate).^{50–53} A decrease in TEER or an increase in PR of LY is a clear indication of

an increase in cell permeability caused by substances enhancing tight-junction permeability. On the basis of the foregoing reports, we speculated that intact Pen j 1 would be absorbed across the Caco-2 monolayer *via* the transcellular pathway. Gly m Bd 30K, a major soybean allergen, or BSA tested at the same concentration as that of Pen j 1 used did not influence the permeability of LY through the Caco-2 monolayer (Fig. 3B). These results suggest that Gly m Bd 30K and BSA would not affect tight junction integrity and support the evidence presented in the previous reports.^{54,55} We have described that Pen j 1 in each basolateral solution was the only intact protein, suggesting that the allergen was not digested by the protease expressed in the brush border membrane of absorptive enterocytes. A high concentration of Pen j 1 might be able to disturb the integrity of the tight junctions between the epithelial cells. A protein that rapidly increased the permeability for LY *via* tight junctions in the Caco-2 cell monolayers has been found in the water-soluble fraction of the mushroom, *Flammulina velutipes*.⁵⁶ Interestingly, the novel 30-kDa protein was non-glycosylated without intermolecular disulfide bonds and exhibited similar structural properties to those of Pen j 1. Under the conditions of the *in vitro* model with Caco-2 monolayers, in which digestive enzymes such as pepsin and trypsin were not expressed, systemic absorption of Pen j 1 may have occurred by self-enhanced paracellular transport.

The experiments with everted gut sacs from rats (Fig. 4A) gave the percentage radioactivity transported in the basolateral solution of 0.1–0.6% of ¹²⁵I-Pen j 1 added to the apical side after a 60-min incubation, and more than 95% radioactivity was located in the TCA-soluble fraction. This result suggests that most of the compounds absorbed across enterocytes *ex vivo* were peptides with low molecular mass or amino acids. Furthermore, the elution patterns obtained by Sephacryl S-100 chromatography showed that the radioactivity in the basolateral solution was widely distributed in the peptides with molecular masses of 1 kDa and 3.5 kDa, in addition to the less than 1-kDa peptides (Fig. 4C). The radioactive compounds with low molecular mass were also present in the apical solution, in which the radioactive peptide corresponding to intact Pen j 1 was found. On the basis of these results, we conclude that Pen j 1 was rapidly degraded by proteolytic enzymes in the intestinal tract or by proteases adsorbed to the surface of the brush border membrane, and that degradation products with low molecular mass were predominantly transported across the enterocytes, while intact Pen j 1 was not.

Pen j 1 orally administered into the rat stomach resulted in a small amount of immunoreactive Pen j 1 and its derivatives being detected in the portal plasma after 15 and 30 min (Fig. 5B). In the case of OVA, these could not be found in the portal blood until 30 min after the administration. Furthermore, intact Pen j 1 could be clearly detected in the contents recovered from stomach and in the stomach tissue even 30 min after the administration, but not in the contents recovered from the small intestine and in the small intestinal tissue (Figs. 6 and 7). In contrast, intact OVA tended to remain in the contents recovered from the stomach and not from the small intestine, although the immunoreactive sub-

stances detected in the contents recovered from the small intestine consisted of proteolytic products with relatively high molecular mass (43–10 kDa) instead of intact OVA. Little of the peptides obtained from OVA could be detected in either the stomach and small intestinal tissues even 15 min after the administration. Moreover, the results of immune-histochemical analyses (Fig. 8) confirmed that Pen j 1 and OVA were predominantly absorbed from the epithelial cells of the stomach and the small intestine, respectively. These observations suggest that intact Pen j 1 could be absorbed through the gastric mucosa more efficiently in comparison with OVA.

Mayer *et al.*⁵⁷ have proposed that the immune response of a food allergen strongly depended on its intestinal absorption and that the nature of the antigen could determine the route of transport and the type of immune response generated. Although the intestinal epithelium theoretically acts as a barrier to restrict the permeation of macromolecules through tight junctions, proteins have been reported to cross the intestinal barrier in an intact form. The gastrointestinal absorption of such intact food allergens as OVA,⁵⁸ proteins from digested peanuts⁵⁹ and Gly m Bd 30K⁵⁴ has been reported by using *in vivo* models. Tsume *et al.*⁵⁸ have demonstrated that both the small intestine and the stomach could absorb OVA, and that OVA absorbed from the stomach was transferred almost exclusively to the blood circulation. This is not surprising, since some studies have demonstrated the ability of gastric mucosa to uptake macromolecules by *in vitro* and *in vivo* experiments.^{60,61} Curtis and Gall⁶⁰ have also reported that macromolecular transport from the stomach occurred *via* an energy-dependent trans-cellular process.

A small amount of ingested food antigens is normally absorbed and transported into the body in an immunologically intact form, and oral tolerance prevails.^{25,62} Dietary antigens transported by enterocytes are likely to induce tolerance.^{63,64} Zimmer *et al.*⁶⁵ have reported that OVA, which easily induces oral tolerance, was rapidly taken up by enterocytes and that it was detected within MHC class II-positive late endosomes. In addition, Dirks *et al.*⁶⁶ have demonstrated that allergenic peanut allergens could be absorbed *via* buccal mucosa, explaining the very acute clinical reactions induced immediately after ingesting offending food by patients with allergy. This evidence, taken together, indicates that antigens have the ability to dictate the route of transport and the type of the ensuing immune response.

We have demonstrated for the first time in the present study that Pen j 1 was rapidly degraded to small peptides and amino acids by intestinal proteases and absorbed from enterocytes, and could be absorbed *via* the gastric mucosa prior to being attacked by digestive enzymes in the intestines. These results strongly suggest that Pen j 1 may have allergic activity in part due to transportation across the gastric mucosa.

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References

- 1) Imai T and Ebisawa M, "Report of Scientific Research by Ministry of Health, Labour and Welfare" (2002).
- 2) Waring NP, Daul CB, Deshaza RD, McCants ML, and Lehrer SB, *J. Allergy Clin. Immunol.*, **76**, 440–445 (1985).
- 3) Daul CB, Morgan JE, Waring NP, McCants ML, Hughes J, and Lehrer SB, *J. Allergy Clin. Immunol.*, **80**, 716–722 (1987).
- 4) "Report of the FDA *ad hoc* Committee on Hypersensitivity to Food Constituents," Food and Drug Administration, Washington, (1996).
- 5) Bush RK and Hefle SL, *Crit. Rev. Food Sci. Nutr.*, **36**, S119–S163 (1996).
- 6) Daul CB, Slattery M, Reese G, and Lether SB, *Int. Arch. Allergy Clin. Immunol.*, **105**, 49–55 (1994).
- 7) Shanti KN, Martin BM, Nagpal S, Metcalfe DD, and Rao PV, *J. Immunol.*, **151**, 5354–5363 (1993).
- 8) Leung PSC, Chu KH, Chow WK, Ansari A, Bandea CI, Kwan HS, Nagy SM, and Gershwin ME, *J. Allergy Clin. Immunol.*, **94**, 882–890 (1994).
- 9) Leung PSC, Chen YC, Mykles DL, Chow WK, Li CP, and Chu KH, *Mol. Mar. Biol. Biotechnol.*, **7**, 12–20 (1998).
- 10) Mykles DL, Cotton JL, Taniguchi H, Sano K, and Maeda Y, *J. Muscle Res. Cell Motil.*, **19**, 105–115 (1998).
- 11) Leung PSC, Chen YC, Gershwin ME, Wong SH, Kwan HS, and Chu KH, *J. Allergy Clin. Immunol.*, **102**, 847–852 (1998).
- 12) Miyazawa H, Fukamachi H, Inagaki Y, Reese G, Daul CB, Lehrer SB, Inouye S, and Sakaguchi M, *J. Allergy Clin. Immunol.*, **98** (Part 1), 948–953 (1996).
- 13) Ishikawa M, Ishida M, Shimakura K, Nagashima Y, and Shiomi K, *J. Food Sci.*, **63**, 44–47 (1998).
- 14) Breiteneder H and Ebner C, *J. Allergy Clin. Immunol.*, **106**, 27–36 (2000).
- 15) Sampson HA, *J. Allergy Clin. Immunol.*, **103**, 717–728 (1999).
- 16) Breiteneder H and Radauer C, *J. Allergy Clin. Immunol.*, **113**, 821–830 (2004).
- 17) Sanchez-Monge R, Gomez L, Barber D, Lopez-Otin C, Armentia A, and Salcedo G, *Biochem. J.*, **281**, 401–405 (1992).
- 18) Batanero E, Villalba M, and Rodriguez R, *Mol. Immunol.*, **31**, 31–37 (1994).
- 19) Hiemori M, Bando N, Ogawa T, Shimada T, Tsuji H, Yamanishi R, and Terao J, *Int. Arch. Allergy Immunol.*, **122**, 238–245 (2000).
- 20) Tsuji H, Hiemori M, Kimoto M, Yamashita H, Kobatake R, Adachi M, Fukuda T, Bando N, Okita M, and Utsumi S, *Biochim. Biophys. Acta*, **1518**, 178–182 (2001).
- 21) Reese G, Ayuso R, and Lehrer SB, *Int. Arch. Allergy Immunol.*, **119**, 247–258 (1999).
- 22) Santos ABR, Chapman MD, Aalberse RC, Vailes LD, Ferriani VPL, Oliver C, Rizzo MC, Naspitz CK, and Arryda LK, *J. Allergy Clin. Immunol.*, **104**, 329–337 (1999).
- 23) Ayuso R, Reese G, Leong-Kee S, Plante M, and Lehrer SB, *Int. Arch. Allergy Immunol.*, **129**, 38–48 (2002).
- 24) Kunimoto A, Shishino T, Sakai K, Matsumoto T, Takahashi K, Yamashita H, Hiemori M, Tsuji H, and Kimoto M, *Biosci. Biotechnol. Biochem.*, **73**, 840–848 (2009).
- 25) Chehade M and Mayer L, *J. Allergy Clin. Immunol.*, **115**, 3–12, quiz 3 (2005).
- 26) Lehrer SB, Horner E, and Reese G, *Crit. Rev. Food Sci. Nutr.*, **36**, 553–564 (1996).
- 27) Fuchs RL and Astwood JD, *Food Technol.*, **50**, 83–88 (1996).
- 28) Aalberse RC, *J. Allergy Clin. Immunol.*, **106**, 228–238 (2000).
- 29) Bredehorst R and David K, *J. Chromatogr. B Biomed. Sci. Appl.*, **756**, 33–40 (2001).
- 30) Moreno FJ, Rubio LA, Olano A, and Clemente A, *J. Agric. Food Chem.*, **54**, 8631–8639 (2006).
- 31) Rytönen J, Valkonen KH, Virtanen V, Foxwell RA, Kyd JM, Cripps AW, and Karttunen TJ, *J. Agric. Food Chem.*, **54**, 1500–1507 (2006).
- 32) Heyman M, *Proc. Nutr. Soc.*, **60**, 419–426 (2001).
- 33) Heyman M, Grasset E, Ducroc R, and Desjeux JF, *Pediatr. Res.*, **24**, 197–202 (1988).
- 34) Clark EC, Patel SD, Chadwick PR, Warhurst G, Curry A, and Carlson GL, *Gut*, **52**, 224–230 (2003).
- 35) Clark EC, Hoare C, Tanianis-Hughes J, Carlson GL, and Warhurst G, *Gastroenterology*, **128**, 1258–1267 (2005).
- 36) Madgula VL, Avula B, Reddy VLN, Khan IA, and Khan SI, *Planta Med.*, **73**, 330–335 (2007).
- 37) Swoboda BE and Massey V, *J. Biol. Chem.*, **240**, 2209–2215 (1965).
- 38) Ogawa T, Bando N, Tsuji H, Okajima K, Nishikawa K, and Sasaoka K, *J. Nutr. Sci. Vitaminol.*, **37**, 555–565 (1991).
- 39) Bando N, Tsuji H, Hiemori M, Yoshizumi K, Yamanishi R, Kimoto M, and Ogawa T, *J. Nutr. Sci. Vitaminol.*, **44**, 655–664 (1998).
- 40) Pato J, Mora M, Naisbett B, Woodly JF, and Duncan R, *Int. J. Pharm.*, **104**, 227–237 (1994).
- 41) Naisbett B and Woodley JF, *Int. J. Pharm.*, **110**, 127–136 (1994).
- 42) Carreno-Gomez B, Woodley JF, and Florence AT, *Int. J. Pharm.*, **183**, 7–11 (1999).
- 43) Barthe L, Bessouet M, Woodley JF, and Houin G, *Int. J. Pharm.*, **173**, 255–258 (1998).
- 44) Hefle SL, *Food Technol.*, **50**, 86–92 (1996).
- 45) Astwood JD, Leach JN, and Fuchs RL, *Nat. Biotechnol.*, **14**, 1269–1273 (1996).
- 46) Deshpande SS and Nielsen J, *J. Food Sci.*, **52**, 1326–1329 (1987).
- 47) Smillie LB, *Trends Biochem. Sci.*, **4**, 151–155 (1979).
- 48) Daul CB, Slattery M, Reese G, and Lehrer SB, *Int. Arch. Allergy Immunol.*, **105**, 49–55 (1994).
- 49) Fu TJ, Abbott UR, and Hatzos C, *J. Agric. Food Chem.*, **50**, 7154–7160 (2002).
- 50) Jensen-Jarolim E, Gajdzik L, Haberi I, Kraft D, Scheiner O, and Graf J, *J. Nutr.*, **128**, 577–581 (1998).
- 51) Soderholm JD, Oman H, Blomsquist L, Veen J, Lindmark T, and Olaison G, *Dig. Dis. Sci.*, **43**, 1547–1552 (1998).
- 52) Hashimoto K, Kawagishi H, Nakayama T, and Shimizu M, *Biochim. Biophys. Acta*, **1323**, 281–290 (1997).
- 53) Mine Y and Zhang JW, *Int. Arch. Allergy Immunol.*, **130**, 135–142 (2003).
- 54) Weangsripanaval T, Murota K, Murakami Y, Kominami M, Kusudo T, Moriyama T, Ogawa T, and Kawada T, *J. Nutr.*, **136**, 2874–2880 (2006).
- 55) Hashimoto K, Takeda K, Nakayama T, and Shimizu M, *Biosci. Biotechnol. Biochem.*, **59**, 1951–1952 (1995).
- 56) Watanabe H, Narai A, and Shimizu M, *Eur. J. Biochem.*, **262**, 850–857 (1999).
- 57) Mayer L, Sperber K, Chan L, and Penninks AH, *Allergy*, **56** (Suppl. 67), 12–15 (2001).
- 58) Tsume Y, Taki Y, Sakane T, Nadai T, Sezaki H, Watabe K, Kohno T, and Yamashita S, *Biol. Pharm. Bull.*, **19**, 1332–1337 (1996).
- 59) Chambers SJ, Wickham MS, Regoli M, Bertelli E, Gunning PA, and Nicoletti C, *Biochem. Biophys. Res. Commun.*, **325**, 1258–1263 (2004).
- 60) Curtis GH and Gall DG, *Am. J. Physiol.*, **25**, G1033–G1040 (1992).
- 61) Hatz RA, Bloch KJ, Harmatz PR, Gonnella PA, Ariniello PD, Walker WA, and Kleinman RE, *Gastroenterology*, **98**, 894–900 (1990).
- 62) Sampson HA, *J. Allergy Clin. Immunol.*, **113**, 805–819 (2004).
- 63) Hershberg RM and Mayer LF, *Immunol. Today*, **21**, 123–128 (2000).
- 64) Brandtzaeg P, *Ann. NY Acad. Sci.*, **964**, 13–45 (2002).
- 65) Zimmer KP, Buning J, Weber P, Kaiserlian D, and Strobel S, *Gastroenterology*, **118**, 128–137 (2000).
- 66) Dirks CG, Pedersen MH, Platzner MH, Bindsløv-Jensen C, Skow PS, and Poulsen LK, *J. Allergy Clin. Immunol.*, **115**, 1321–1323 (2005).