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Identification of a Peptide in Enzymatic Hydrolyzate of Cheese That Inhibits Ovalbumin Permeation in Caco-2 Cells

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Because the first step in the triggering of food allergy is the permeation of the allergen through the intestine, enhancement of the intestinal barrier function is thought to be effective for preventing food allergy. In this study, a peptide that inhibits ovalbumin (OVA) permeation in an *in vitro* Caco-2 cell model was isolated from enzymatic hydrolyzate of cheese (EHC). Amino acid sequence analysis identified the active peptide as GPIVLNPWDQ, a sequence identical to amino acids 102−111 of αs_2-casein. The decapeptide significantly inhibited OVA permeation at a concentration of 10^{-6} M. In addition, it was found that a pentapeptide half, NPWDQ, is essential for the inhibitory activity because NPWDQ but not GPIVL had nearly the same inhibitory activity as GPIVLNPWDQ. The possibility exists that EHC and/or peptides possessing the NPWDQ sequence can be practically applied to the prevention of food allergy.

KEYWORDS: Enzymatic hydrolyzate of cheese; allergen; permeation; Caco-2; αs_2-casein; ovalbumin

INTRODUCTION

Allergic diseases are the most common of all immunologically mediated conditions, and they are increasing in prevalence in most developed countries. Food allergy has been thought to involve an excessive immune reaction to allergens permeating from the intestinal tract (*1*). After a specific food allergen is ingested, a sensitized individual may experience gastrointestinal symptoms such as diarrhea and nausea and extraintestinal symptoms can occur in the skin and airways (*2*). The intestinal epithelium theoretically acts as a barrier restricting the permeation of macromolecules. However, a small proportion (from 10^{-3} to 10^{-4}) of dietary proteins can cross the epithelium to access and activate effector cells, resulting in food allergy (*3*). Moreover, it has been reported that antigen permeability through the intestinal tract is enhanced in allergic patients (*4*, *5*) and patients with Crohn's disease (*6*). In the light of these facts, it is conceivable that enhancing the barrier function of the intestinal epithelium would help to prevent food allergy.

There are various physiologically functional (bioactive) peptides derived from milk proteins, and these have been the subject of intensive studies (*7*−*9*). For example, the most important casein-derived peptides are casomorphins, which possess opioid-like activity; immunopeptides, which have an immunostimulating activity; peptides with antihypertensive activities; and phosphopeptides with the ability to sequester calcium and possibly other minerals, thus acting as biocarriers. Therefore, we aimed to search for milk-protein-derived peptides that inhibit allergen permeation at the intestine. For this purpose, we chose enzymatic hydrolyzate of cheese (EHC) as a starting material.

EHCs are generally produced by the hydrolysis of cheese with commercial proteases and are used in the food in several ways, such as the sole source of cheese flavor in a product, to intensify an existing cheesy taste, or to give a specific cheese character to a more bland-tasting cheese product (*10*). In addition, the enzymatic treatment of cheese has the potential to produce bioactive peptides that may provide nutritional, medical, and/or health benefits. For example, Haileselassie et al. (*11*) isolated antihypertensive peptides, such as YPFPGPI, which inhibits the angiotensin I-converting enzyme (ACE), from an EHC.

Human adenocarcinoma cell lines Caco-2 cells, derived from a colon carcinoma, have been used as an *in vitro* model to estimate the bioavailability of dietary components involving calcium ions (*12*−*15*), amino acids and peptides (*16*, *17*), and polyphenols (*18*−*22*). Caco-2 cells differentiate under standard culture conditions to form confluent monolayers and acquire many features of absorptive intestinal cells during culture (*23*). Caco-2 cells spontaneously exhibit various enterocytic characteristics including the expression of brush-border enzymes, nutrient transporters, and the formation of intercellular tight junctions (TJ) (*24*, *25*). Recently, our group has established an

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in vitro system to evaluate allergen permeation using differentiated Caco-2 cells grown on a permeable filter (*26*, *27*).

In the present study, we investigated the ability of three types of EHCs obtained by a series of enzymatic treatments to inhibit allergen permeation using Caco-2 cells as an intestinal model and ovalbumin (OVA) as a representative food allergen. Furthermore, an active peptide was isolated from one EHC and characterized.

MATERIALS AND METHODS

Materials. Denmark skim milk cheese was used as the starting material, and the proteases used for EHC preparation included proteases N, protease S, umamizyme, newlase A (Amano Enzyme, Nagoya, Japan), flavourzyme (Novozymes Japan, Chiba, Japan), and sumizyme FP (Shin Nihon Chemical, Anjyo, Japan). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, penicillin, streptomycin, gentamycin, and Hanks' balanced salt solution (HBSS) were all from Invitrogen Corp. (Carlsbad, CA). Fetal calf serum (FCS) was obtained from ICN Biomedicals, Inc. (Osaka, Japan). Transwell cell-culture chambers (0.4 μm in pore size and 12 mm in diameter) and a Millicell-ERS instrument with Ag/AgCl electrodes were purchased from Corning Coster (Cambridge, MA) and Nihon Millipore (Tokyo, Japan), respectively. The synthetic peptides (GPIVLNPWDQ, GPIVL, and NPWDQ) were obtained from Takara Bio (Osaka, Japan). Fluorescein isothiocyanate (FITC) and OVA were from Sigma-Aldrich (Tokyo, Japan), and trifluoroacetic acid (TFA) was from Wako Pure Industries (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of EHC. Denmark skim milk cheese was hydrolyzed by a mixture of two or three enzymes: protease N (*Bacillus subtilis*), umamizyme (*Aspergillus oryzae*), and flavourzyme (*Aspergillus oryzae*) for the preparation of EHC-1; protease S (*Bacillus stearothermophilus*), newlase A (*Aspergillus niger*), and umamizyme for the preparation of EHC-2; and protease N and sumizyme FP (*Aspergillus oryzae*) for the preparation of EHC-3.

Denmark skim milk cheese (500 g, 6 months ripening) was grated and suspended in 500 mL of deionized water. The suspension was adjusted to pH 5.0 with citric acid solution. A total of 3 g of protease 1 (EHC-1 and EHC-3, protease N; EHC-2, protease S) was added to the suspension and held for 48 h at 34 °C with stirring. Then, the suspention was readjusted to pH 4.1, and protease 2 and 3 (EHC-1, 1.5 g of umamizyme and 1.5 g of flavorzyme; EHC-2, 1.5 g of umamizyme and 1.5 g of newlase A; EHC-3, 3 g of sumizyme FP) were added and held for 120 h at 34 °C. The resulting suspension was adjusted to pH 5.0 with sodium hydroxide solution and heated at 110 °C for 10 min to inactivate the enzymes used.

The resulting EHCs were freeze-dried. Each EHC was suspended in water (20 wt %/vol) and centrifuged at 3000*g* for 10 min. The water-soluble fractions (final concentration of 0.01 mg/mL) were used for the first assay using Caco-2 cells described below.

FITC Labeling of OVA. OVA was conjugated with FITC according to the method of Maeda et al. (*28*). The reaction mixture was dialyzed overnight against running water, and the nondiffusible fraction was concentrated and passed through a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column to remove free FITC.

Cell Culture. All Caco-2 cells in this study were used between 70 and 110 passages. The growth medium consisted of DMEM with 20% FCS, 1% nonessential amino acids, and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamycin). Cells were cultured at 37 °C under a humidified 5% CO_2 atmosphere. Cells were normally grown in 75 cm^2 tissue culture flasks to approximately 80% confluence and seeded into a Transwell cell-culture chamber at a density of 2×10^5 cells/cm^2.

Inhibitory Activity against OVA Permeation. For the evaluation of OVA permeability, Caco-2 cells were grown in a Transwell cell-culture chamber. After 10−20 days of culture, transepithelial electrical resistance (TEER) was measured using a Milicell-ERS. Monolayers of Caco-2 cells were used when their TEERs were >600 Ω cm^2. Each well was placed in a cluster plate with outside medium (basolateral side of 1.5 mL) and inside medium (apical side of 0.5 mL). The cell monolayers were fed fresh medium every 24 h. One day before permeability measurements, the monolayer was cultured in the absence (control) or presence of a test sample. After washing twice with HBSS, 0.5 mL of HBSS containing the test sample and FITC−OVA (0.5 mg) was added to the apical side and 1.5 mL of HBSS containing the test sample was added to the basolateral side. A similar procedure was carried out in the absence of the test sample as a control. The monolayers were incubated for 2 h, after which the outside medium was collected. The concentration of FITC−OVA in the basolateral side was determined by measuring the fluorescence intensity with a spectrophotofluorometer (Nihon Bunko, FP-750) at excitation and emission wavelengths of 495 and 520 nm, respectively. Inhibitory activity (%) was calculated according to $(P_c - P_s)/P_c \times 100$, where P_c and P_s are permeated OVA in the absence and presence of the sample, respectively. The assay was repeated at least 2 times independently, and each assay was performed in triplicate unless otherwise stated. A schematic illustration of the inhibitory activity measurement assay using Caco-2 cells is depicted in **Figure 1**.

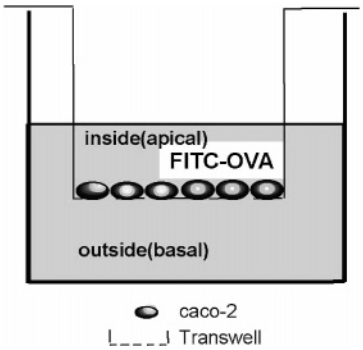

Figure 1. Schematic illustration of the evaluation of OVA permeability using Caco-2 cells seeded onto Transwell. Each well was placed in a well of a 12-well cluster plate with outside medium (basolateral side, 1.5 mL) and inside medium (apical side, 0.5 mL). For the permeability assay, 0.5 mL of HBSS containing the test sample and FITC–OVA (0.5 mg) was added to the apical side and 1.5 mL of HBSS containing the test sample was added to the basolateral side. The monolayers were incubated for 2 h, after which the outside medium was collected. The concentration of FITC–OVA in the basolateral side was determined.

Purification of an Active Peptide from EHC. EHC-2 (1 mg) was dissolved with 0.1% TFA (buffer A) and loaded onto C18 Sep-pak cartridges (Waters, Milford, MA). Elution was achieved using a three-step procedure involving a mixture (8 mL) of buffer A and 80% acetonitrile in 0.085% TFA (buffer B), with the buffer A/B mixture ratios (v/v) being 75:25, 50:50, and 25:75. The eluates in the buffer A/B mixture ratio of 75:25, 50:50, and 25:75 were designated as fractions 1, 2, and 3, respectively. The collected fractions were dried in vacuo and subjected to Caco-2 assay. Fractions with the inhibitory activity against OVA permeation were further purified by high-performance liquid chromatography (HPLC) using a Capcell pak C18 (MG II) column (4.6 × 250 mm, Shiseido, Tokyo, Japan) at a 1 mL/min flow rate using a linear gradient of 0−100% buffer B over 60 min. Elution was monitored at 220 nm. The collected peaks were dried in vacuo and subjected to the Caco-2 assay. The amino acid sequence of the isolate was analyzed with a protein sequencer (G1005A, Hewlett-Packard Co., San Jose, CA).

RESULTS AND DISCUSSION

Although EHCs are widely used in the food industry as cheese flavor enhancers, their physiological functions have not been fully evaluated. In this study, we used Caco-2 cells to investigate the ability to inhibit allergen permeation of three types of EHCs obtained by a series of enzymatic treatments. Caco-2 is a human colon carcinoma cell line that exhibits various enterocytic characteristics such as intracellular TJ and the presence of

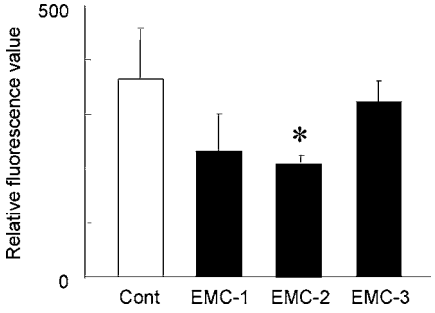

Figure 2. Effects of EHC-1, EHC-2, and EHC-3 on OVA permeability through Caco-2 monolayers. The water extracts of each EHC (final concentration, 0.01 mg/mL) were used for the assay. Data represent means ± standard deviation (SD) ($n = 3$). (*) Significantly different ($p < 0.05$) from the control sample.

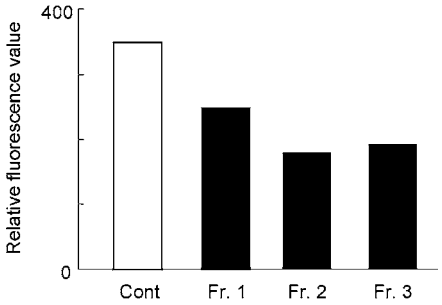

Figure 3. Effects of fractions 1, 2, and 3 of EHC-2 on OVA permeability through Caco-2 monolayers. Data represent the average ($n = 2$). Fraction 2 was submitted to reversed-phase HPLC (**Figure 4**) to isolate the active peptide(s).

nutrient transporters and brush-border enzymes. We used Caco-2 cell monolayers as a model of the small intestinal tract, and OVA was used as a typical allergen.

In the first assay of allergen permeation, the three EHCs were assayed for their inhibitory activity toward OVA permeation at a concentration of 0.01 mg/mL. The water extract of EHC-2, which was produced by protease S, newlase A, and umamizyme, showed the most potent activity among the three EHCs (**Figure 2**). The results also indicated that the inhibitory activity of the hydrolyzates depended largely upon the enzymes used.

The water extract of EHC-2 was fractionated on a C18 Sep-pak cartridge. The fractions adsorbed by the Sep-pak and eluted by 20, 40, and 60% acetonitrile were designated as fractions 1, 2, and 3, respectively. Although all three fractions inhibited OVA permeation, the activities of fractions 2 and 3 were higher than that of fraction 1 (**Figure 3**). Fraction 2 was then submitted to reversed-phase HPLC to isolate the active peptide(s). As shown in **Figure 4**, more than 10 peaks were detected. The inhibitory activities toward OVA permeation of these peaks were preliminarily measured by the same method, and the peaks numbered P1, P2, and P3 were found to be active when assayed using a single well (instead of the usual three wells) for each peak (data not shown). For further analysis, although none of the three peaks possessed significant inhibitory activities, maybe because of the low amounts of sample, only P1 exerted inhibitory activity reproducibly (**Figure 5**).

Therefore, P1 was submitted to an amino acid sequence determination, using a protein sequencer, with the determined sequence being Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln (GPIVLNPWDQ). This amino acid sequence agrees with the sequence of amino acids 102−111 of αs_2-casein (NCBI accession number NP 776953). To confirm these data, the decapeptide GPIVLNPWDQ was synthesized and its activity−concentration relationship was measured (**Figure 6**). As a result, the peptide was found to produce concentration-dependent inhibition of OVA permeation. We next examined what portions of the decapeptide are essential for the inhibitory activity. For this purpose, the two pentapeptide halves, GPIVL (amino acids 102−106) and NPWDQ (amino acids 107−111), were also synthesized and their activities were evaluated. Interestingly, it was found that the inhibitory activity of NPWDQ but not GPIVL was almost the same as that of GPIVLNPWDQ (**Figure 7**). Thus, it was concluded that only the C-terminal-half peptide is essential for the activity.

We have previously analyzed the molecular-size distribution in the basolateral-side medium by SDS−PAGE with fluorescence detection and demonstrated that OVA was transported

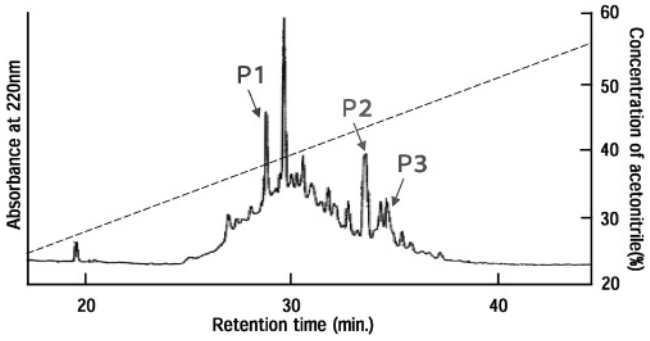

Figure 4. Reversed-phase HPLC chromatogram of fraction 2 from EHC-2. In a preliminary assay, the numbered peaks, P1, P2, and P3, were found to be active.

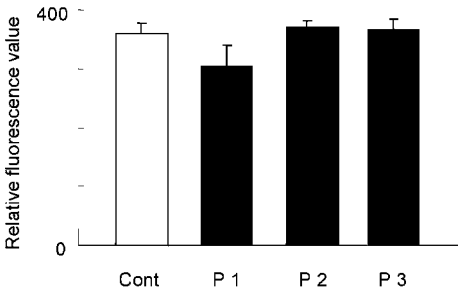

Figure 5. Effects of peaks 1, 2, and 3 on OVA permeability through Caco-2 monolayers. Data represent means ± SD ($n = 3$). Among the three peaks, only P1 exerted inhibitory activity. Amino acid sequence analysis identified P1 as GPIVLNPWDQ.

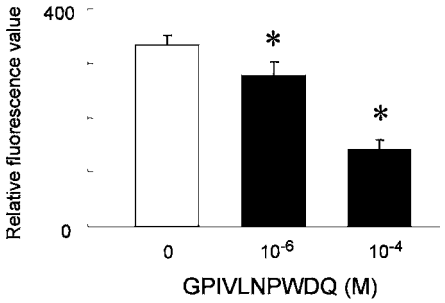

Figure 6. Effects of the decapeptide on OVA permeability through Caco-2 monolayers. Data represent means ± SD ($n = 3$). (*) Significantly different ($p < 0.05$) from the control sample.

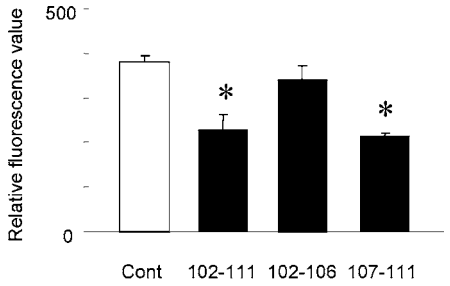

Figure 7. Effects of the decapeptide (102–111) and its two pentapeptide fragments, GPIVL (102–106) and NPWDQ (107–111), on OVA permeability through Caco-2 monolayers. Peptides were added at 10^{-4} M. Data represent means ± SD ($n = 3$). (*) Significantly different ($p < 0.05$) from the control sample.

through a Caco-2 cell monolayer as almost an intact form in this assay using Caco-2 cells and the Transwell system (*27*). Thus, it is assumed that GPIVLNPWDQ and NPWDQ directly inhibited the uptake of OVA.

Recently, Watanabe et al. (*29*) reported that tryptophan is favorable for the inhibition of OVA permeation. In addition, we have found that DKIHPF [β-casein f (47−52)], which is present in Edam cheese, has the same activity (*27*). Therefore, it is highly probable that the P, W, and D residues in NPWDQ [αs_2-casein f (107−111)] would be, at least in part, important for the inhibition of OVA permeation. Investigations are needed into whether the existence of these P, W, and D residues is sufficient or whether the specific amino acid sequence is indispensable for the inhibition of OVA permeation.

There exist at least two possible mechanisms by which GPIVLNPWDQ and NPWDQ could inhibit OVA permeation in Caco-2 cells: inhibition of the endocytosis of OVA and/or the enforcement of TJ functions. However, because the TEER value, which reflects TJ conditions, did not change appreciably following the addition of GPIVLNPWDQ or NPWDQ (data not shown), the former mechanism appears to be more likely.

The results obtained in this study present a novel point concerning the significance of lactation in preventing protein (allergen) permeation in the small intestine, because this kind of peptide would be produced from αs_2-casein by proteases in the infant digestive tract and its production could prevent allergen permeation. Further studies on the stability of the active peptides during gastrointestinal digestion are needed to clarify this hypothesis, although proline-containing peptides are generally resistant to degradation by digestive enzymes (*30*). In addition, unlike other bioactive peptides, such as ACE inhibitory peptides, which have to reach the target system (organ or tissue) in an active form to exert the effect, the peptides in this study act in the small intestine. Therefore, the bioavailability of the active peptides is expected to be rather high.

In conclusion, the peptide GPIVLNPWDQ derived from αs_2-casein, which is present in EHC, inhibits OVA permeation in a Caco-2 cell model. A means to apply EHC, which has been widely used in the food industry, to the prevention of food allergy is now being planned using an animal model.

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