

An Active Compound against Allergen Absorption in Hypoallergenic Wheat Flour Produced by Enzymatic Modification

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Hypoallergenic wheat flour produced by modification with cellulase and actinase showed inhibitory activity against ovalbumin permeation in an *in vitro* model by using the Caco-2 cell monolayer. The activity was found in the cellulase preparation used for producing the flour. An active compound was isolated by HPLC and identified as Trp-Ser-Asn-Ser-Gly-Asn-Phe-Val-Gly-Gly-Lys by ¹H-NMR data and Edman degradation. The undecapeptide, some oligopeptides with the N-terminal sequences and Trp ethyl ester showed activity at 10⁻⁷ M, acetyl Trp being active at 10⁻² M. These data suggest that the Trp residue without a free carboxyl group would be required for the inhibitory activity of ovalbumin absorption through the intestinal tract.

Key words: Caco-2; hypoallergenic wheat flour; endo-1,4- β -xylanase; allergen absorption

Allergic diseases are the most common of all immunologically mediated conditions and are increasing in prevalence in most countries of the developed world. Food allergy has been thought to involve an excessive immune reaction against allergens absorbed from the intestinal tract.¹⁾ After ingesting a specific food antigen, 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 part (10⁻³ to 10⁻⁴) of dietary proteins can cross the epithelium to access and activate effector cells, resulting in food allergy.³⁾

Yu *et al.*⁴⁾ have reported that sensitization to an antigen enhanced CD23 expression in intestinal epithelial cells, with a resulting increase in the transport

kinetics of the antigen. Moreover, it has been reported that antigen permeability through the intestinal tract was enhanced in allergic patients^{5,6)} and Crohn's disease patients.⁷⁾ In the light of these facts, it is conceivable that enhancing the barrier function of the intestinal epithelium would help to prevent food allergy.

Our group has been undertaking studies on wheat allergens and has proposed a procedure for producing hypoallergenic wheat flour by enzymatic modification.⁸⁾ This procedure involves the digestion of wheat flour with cellulase and actinase and successfully reduced the allergenicity.⁸⁾ Since hypoallergenic flour has poor functionality for food processing and needs to be processed into easy-to-eat foods, we have proposed methods to prepare some food items, including noodles and bread.^{8,9)} The majority of wheat-allergic patients did not show allergic symptoms after a hypoallergenic flour intake in our clinical test (unpublished data). Moreover, we have recently reported that the hypoallergenic flour actively suppressed the allergic reactions in an animal model.¹⁰⁾

We hypothesized that some components in hypoallergenic wheat flour showed inhibitory activity against allergen absorption, and that the inhibitor(s) contributed to the allergy-suppressive effect of the hypoallergenic wheat flour. To isolate the allergen absorption inhibitor(s), we selected a monolayer of Caco-2, the human colon carcinoma cell line, as an intestinal model. This monolayer exhibits microvilli and polarized morphology when grown on a permeable filter.¹¹⁾ Ovalbumin (OVA), the major allergen of egg white, was used as an allergen model. This study demonstrates that an undecapeptide in the hypoallergenic wheat flour strongly inhibited OVA permeation through the Caco-2 monolayer, and that the Trp-

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Abbreviations: BSA, bovine serum albumin; Dig, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; ODS, octadecylsilyl; OVA, ovalbumin; PBS, 10 mM phosphate-buffered saline; TEER, transepithelial electrical resistance; TFA, trifluoroacetic acid

residue with the bound carboxyl group of the peptide was important for its inhibitory activity.

Materials and Methods

Materials. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, penicillin, streptomycin, gentamycin and HBSS were all from Gibco Life Technologies (Grand Island, MD, USA). Fetal calf serum (FCS) was obtained from ICN Biomedicals (Osaka, Japan), and the type-I collagen solution was from Nitta Gelatin (Osaka, Japan). Transwell cell culture chambers of 0.4 μ m in pore size and 12 mm in diameter were purchased from Coster (Cambridge, MA, USA). OVA was obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Cellulase TDF from *Trichoderma viride*, (86.8 units/mg of solid) and actinase (250 Tyr units/mg of solid) were purchased from Amano Enzyme Inc. (Tokyo, Japan) and Kaken Pharma Co. (Tokyo, Japan), respectively. Trp-Ser-Asn-Ser-Gly-Asn-Phe-Val-Gly-Gly-Lys, Trp-Ser-Asn-Ser-Gly and Trp-Gly were obtained from Peptide Institute (Osaka, Japan). Trp-Ser-Asn and Trp-Ser were synthesized by the solid-phase method with a peptide synthesizer (Shimadzu PSSM-8) and then purified by HPLC, the conditions being the same as those in the first HPLC run described later, except for the linear gradient of 10–50% acetonitrile in 0.1% trifluoroacetic acid (TFA). The retention times of Trp-Ser-Asn and Trp-Ser were 10.8 and 5.9 min, respectively. Other reagents used were all of normal reagent grade.

Preparation of the hypoallergenic wheat flour. Soft flour was successively treated with cellulase at 50°C for 1 h and then with actinase at 40°C for 1 h at pH 7 by our reported method.⁸⁾

Preparation of aqueous methanol-soluble fractions from the hypoallergenic flour and its materials. Each sample (1 g) of hypoallergenic flour, intact flour, cellulase and actinase was extracted with 50% methanol (10 ml), and the extract was dried under vacuum. The residue was submitted to activity measurements.

Extraction and pretreatment of an active fraction. Cellulase (50 g) was mixed with 50% methanol (500 ml) and then centrifuged at 5000 $\times$ g for 20 min to obtain a supernatant. The supernatant was evaporated under vacuum. The residue was dissolved in 0.1% TFA (500 ml), and an aliquot (25 ml) of the solution was treated in an octadecylsilyl (ODS) column (Waters, Sep-Pak C18). The column was washed with the same solvent (10 ml) and then eluted with 80% methanol in 0.1% TFA.

Isolation of the active compound. The fraction adsorbed by ODS and eluted by 80% methanol was separated by the first HPLC run in a reversed phase column (Shodex RSpak RP18-415, 4.6 $\times$ 150 mm). Linear gradient elution was used starting with 40% methanol in 0.1% TFA and ending with 60% methanol in 0.1% TFA at a flow rate of 1 ml/min at 20°C. Detection was at the wavelength of 220 nm. The eluates collected near and at each peak were dried and then submitted to activity measurements. The active peak obtained by the first HPLC run was separated by the second HPLC run in the reversed-phase column (Shodex RSpak ODP-50-4D, 4.6 $\times$ 150 mm) with elution by 17.5% acetonitrile in 0.1% TFA (isocratic), the other conditions being the same as those used in the first run. The active peak obtained by the second HPLC run was separated by a third run in the reversed-phase column (Shodex RSpak RP18-415, 4.6 $\times$ 150 mm) by linear gradient elution with 40–50% methanol in 10 mM ammonium acetate, the other conditions being the same as those used in the first run. The active peak obtained by the third HPLC run was desalted in the same manner in the second HPLC run.

Identification of the active compound. ¹H-NMR and 2D HH COSY spectra were recorded by a Bruker AMX500 spectrometer operated at 500 MHz and 24°C. Chemical shifts were found relative to the residual methyl proton signal (δ 3.30 ppm) of methanol-*d*₄. The active fraction was separately determined for its N-terminal amino acid sequence by auto-Edman degradation with an amino acid sequencing system (Hewlett Packard G1005A). The amino acid sequence similarity of the active compound and other proteins was checked by a computer program (Fast Homology Search CDS, GenBank).

Cell culture. The Caco-2 cells used in this study were between 30 and 50 passages. The growth medium contained DMEM with 20% FCS, 1% nonessential amino acids and antibiotics (100 IU/ml of penicillin, 100 mg/ml of streptomycin and 50 mg/ml of gentamycin). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C and were normally grown to confluence in 75-cm² tissue-culture flasks. The cells were then seeded into transwell cell culture chambers coated with type-I collagen at a density of 2 $\times$ 10⁵ cells/cm². Each well was placed in a cluster plate with the external medium (basolateral side, 1.5 ml) and the internal medium (apical side, 0.5 ml). The cells were fed with fresh media every 24 h. The transepithelial electrical resistance (TEER) of the cell monolayer reached more than 300 Ω /cm² after 14–21 days of culture, and this layer was used for the activity measurements. TEER was measured with a Millicell ERS instrument with Ag/AgCl electrodes (Nihon Milli-

pore, Tokyo, Japan).

Measurement of inhibitory activity against OVA permeation. One day before the activity measurements, the cells were cultured in DMEM with or without (control) the test sample. The cells were then washed twice with Hanks' balanced salt solution (HBSS) and incubated for 30 min in the external medium (1.5 ml of HBSS containing a test sample) and internal medium (0.5 ml of HBSS containing both OVA (0.5 mg) and the test sample). In the case of the undecapeptide, the peptide was dissolved in the internal medium alone, or in both the internal and external media. At the end of the incubation period, the external medium was taken, and permeated OVA was determined by competitive ELISA. Microtiter plates were coated with the rabbit anti-OVA antiserum (100 µl, 1:5,000 dilution, Nordic Immunology, The Netherlands) and left overnight at 4°C. Unoccupied sites in the wells were saturated by incubating with 2% bovine serum albumin (BSA, 200 µl) at 37°C for 1 h. A mixture (100 µl) of a given concentration of digoxigenin (DIG)-labeled OVA and the external medium from the Caco-2 culture was incubated in the well at 37°C for 1.5 h. This was followed by the addition of peroxidase-conjugated anti-DIG (Boehringer Mannheim Co., Germany) and then *o*-phenylenediamine (Wako Pure Chemical Ind., Japan). The wells were washed between each addition with 0.05% Tween-20 in PBS. After the reaction, the absorbance at 490 nm was read by a microplate reader (Bio-Rad model 450). The inhibitory activity for OVA permeation is expressed as $(P_c - P_s)/P_c \times 100$ (%), where P_c and P_s are permeated OVA in the absence and presence of the sample, respectively.

Results and Discussion

The hypoallergenic wheat flour did not develop any allergic symptoms, but consumption of the flour sometimes suppressed the allergic reactions in clinical and animal tests.¹⁰ We thus hypothesized that this effect might have been, at least in part, a result of the hypoallergenic flour possessing some inhibitory functions for allergen absorption. Caco-2 is a human colon carcinoma cell line which exhibits various enterocytic characteristics such as the intercellular tight junction, nutrient transporters and brush-border enzymes. We used the monolayer of Caco-2 cells as a model of the small intestinal tract, and OVA was used as a typical allergen.

The 50% methanol-soluble fraction from the hypoallergenic flour was assayed for its inhibitory activity toward OVA permeation at a concentration of 1 mg/ml, being judged to be active. The fraction mainly consisted of oligosaccharides and peptides derived from the enzymatic process used in the

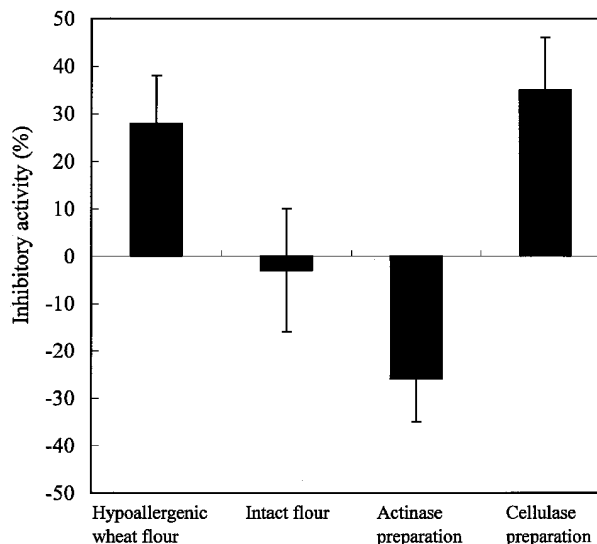


Fig. 1. Inhibitory Activities against OVA Permeability through Caco-2 Monolayers in the Presence of 50% Methanol Extracts from Hypoallergenic Wheat Flour, Intact Flour and Enzyme Preparations of Cellulase and Actinase.

The concentration of each extract was set at 1 mg/ml. Data bars represent the mean $\pm$ s.e. The s.e. of the control (without any of the extracts) was ± 8 . Inhibitory activity is expressed as $(P_c - P_s)/P_c \times 100$ (%), where P_c and P_s are permeated OVA in the absence and presence of the sample, respectively.

hypoallergenic flour production.⁸⁾ The hypoallergenic flour also contained enzyme preparations of cellulase and actinase as minor components. To isolate the active compound(s) we first assayed the activity of the 50% methanol extracts from intact flour and from the two enzyme preparations. Figure 1 shows that the cellulase preparation was active.

The extract from the cellulase preparation was pretreated with ODS (Sep-Pak) and then chromatographed by the first HPLC run. One peak (Fig. 2-A, asterisk) was active. The active peak was separated by the second HPLC run to obtain an active peak (asterisked in Fig. 2-B). This active peak was submitted to the third HPLC run, eluting with a methanol-ammonium acetate system. The active peak (Fig. 2-C, asterisk) was desalted to obtain a white powder (about 0.1 mg from 50 g of the cellulase preparation).

The ¹H-NMR spectrum of the active compound indicated its peptide nature. Unfortunately, spectral complication and the small sample amount made it difficult to unambiguously assign each amino acid residue. 2D HH COSY data partly clarified the proton networks. Four coupled aromatic protons at δ 7.67–7.06–7.14–7.38 and a multi-proton broad singlet at δ 7.26 indicate Trp and Phe, respectively. Sequentially coupled side-chain protons at δ 4.44–1.77 / 1.93–1.46–1.66–2.91 correspond to α to ϵ protons of Lys. An α proton whose coupling extends to high-field signals, δ 4.09–2.16–0.95, shows Val. Typical ABX patterns, δ 4.32–3.85 / 3.89 and 4.49–3.80 / 3.86,

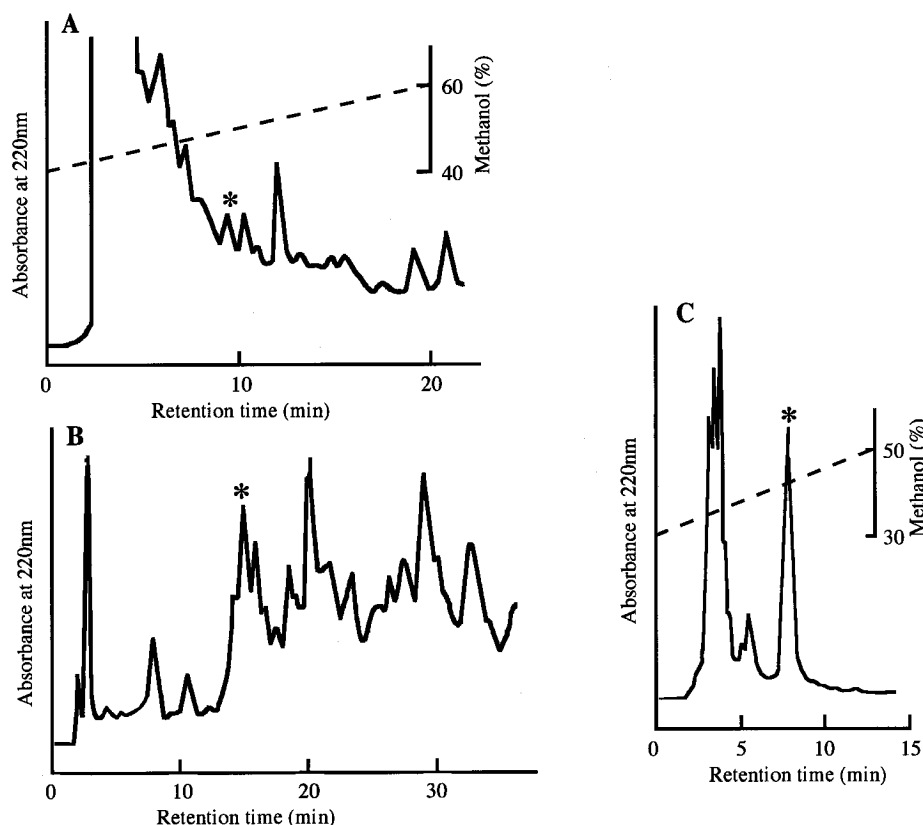


Fig. 2. Reversed-phase HPLC Patterns of an Extract from the Cellulase Preparation.

A, ODS-adsorbed-80% methanol-eluted fraction of the cellulase preparation. B, Active peak of the first HPLC run (asterisked in Fig. 2-A). C, Active peak of the second HPLC run (asterisked in Fig. 2-B). In each chromatogram, an asterisked peak shows the inhibitory activity against OVA permeation, the vertical axis being drawn to an arbitrary scale.

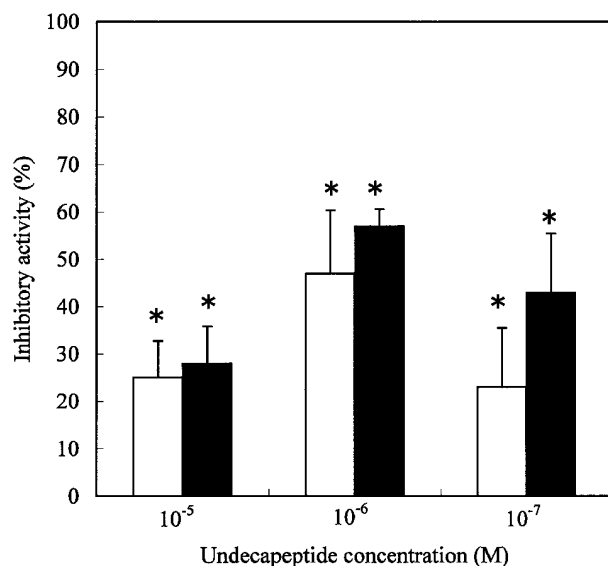


Fig. 3. Effect of the Undecapeptide on the Permeability of OVA through the Caco-2 Monolayers.

Filled column: The undecapeptide was added to both the external and internal media. Unfilled column: The undecapeptide was added only to the internal medium. In both cases, no activity was found at 10^{-8} M. Data bars represent the mean $\pm$ s.e. The s.e. of the control (without the undecapeptide) was ± 12 . *Significantly different ($P < 0.05$) from the control sample.

suggest two residues of Ser as well as δ 4.62–2.62/2.71 for Asx.

The compound was then submitted to an amino acid sequence determination. The determined structure was Trp-Ser-Asn-Ser-Gly-Asn-Phe-Val-Gly-Gly. The amino acid sequence similarity shows that the active compound agreed with the 39–48th amino acid sequence of *Tricoderma* endo-1,4- β -xylanase (EC 3.2.1.8). The C-terminal amino acid residue of the compound should be Lys according to the NMR data. Summarizing the analytical data, we identified the compound as the undecapeptide of Trp-Ser-Asn-Ser-Gly-Asn-Phe-Val-Gly-Gly-Lys.

The activity-concentration relationship was measured by using a synthetic peptide. Figure 3 shows that the undecapeptide was active at concentrations between 10^{-5} and 10^{-7} M, when it existed only in the internal medium or both internally and externally. The data suggest that the ingested peptide would be effective if it were transported to the small intestine. We next examined which amino acid residue(s) of the undecapeptide were essential for the activity. A half-sized peptide of Trp-Ser-Asn-Ser-Gly and smaller peptides of Trp-Ser-Asn and Trp-Ser were all active at 10^{-7} M (Fig. 4). Trp-Gly was also active at the same molar concentration (Fig. 4), suggesting that the Trp

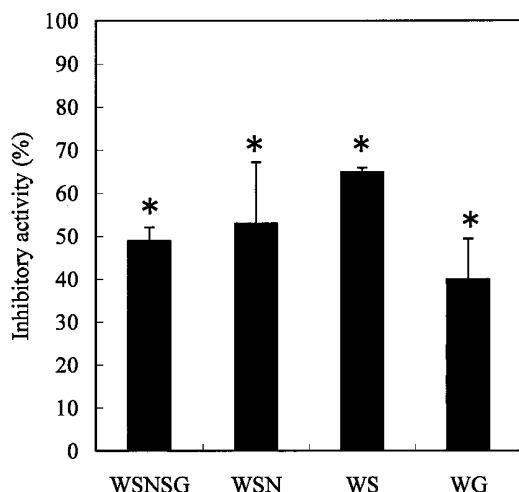


Fig. 4. Effect of the Peptide Fragments on the Permeability of OVA through the Caco-2 Monolayers.

The concentration of each of the fragments was 10^{-7} M. No activity was found at 10^{-8} M in all the cases. Data bars represent the mean $\pm$ s.e. The s.e. of the control (without any of the fragments) was ± 8 . *Significantly different ($P < 0.05$) from the control sample.

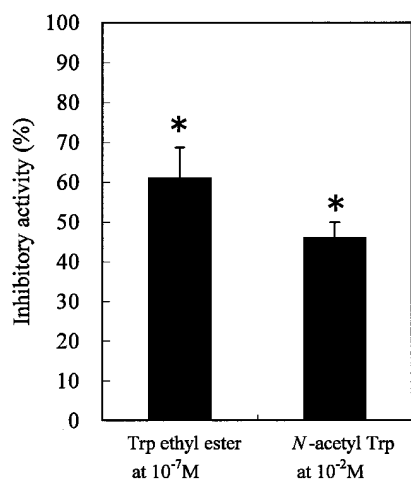


Fig. 5. Effect of the Trp Ethyl Ester and *N*-acetyl Trp on the Permeability of OVA through the Caco-2 Monolayers.

No activity was found at 10^{-8} M of the Trp ethyl ester and at 10^{-3} M of *N*-acetyl Trp. Data bars represent the mean $\pm$ s.e. The s.e. of the control (without the Trp ethyl ester and *N*-acetyl Trp) was ± 8 . *Significantly different ($P < 0.05$) from the control sample.

residue would have contributed to the activity development.

Figure 5 shows that the Trp ethyl ester and acetyl Trp were active at molar concentrations of 10^{-7} and 10^{-2} , respectively. The data also suggest that the Trp residue without a free carboxyl group would be favorable for the inhibitory activity. Summarizing the data obtained in this work, we conclude that the Trp residue with the bound carboxyl group is required for the activity inhibiting OVA absorption. The hypoallergenic flour probably contains a

sufficient concentration of the active undecapeptide and its fragments, and the peptides would function as an inhibitors of OVA permeation.

The active undecapeptide and its fragments possibly contribute to the allergy-suppressive effects of the hypoallergenic flour. We also expect that the hypoallergenic flour would function as a preventive foodstuff against allergies induced by other allergens including egg and milk.

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