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To cite this article: Mioko KUSABA-NAKAYAMA, Masami KI, Emiko KAWADA, Masao SATO, Ikuo IKEDA, Toshihiro MOCHIZUKI & Katsumi IMAIZUMI (2001) Intestinal Absorbability of Wheat Allergens, Subunits of a Wheat α -Amylase Inhibitor, Expressed by Bacteria, Bioscience, Biotechnology, and Biochemistry, 65:11, 2448-2455, DOI: [10.1271/bbb.65.2448](https://doi.org/10.1271/bbb.65.2448)

To link to this article: <https://doi.org/10.1271/bbb.65.2448>



Published online: 22 May 2014.



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Intestinal Absorbability of Wheat Allergens, Subunits of a Wheat α -Amylase Inhibitor, Expressed by Bacteria

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Received May 22, 2001; Accepted July 13, 2001

Wheat CM2, CM3 and CM16 proteins are known as subunits of the tetrameric α -amylase inhibitor as well as major allergens to baker's asthma. The purpose of this study is to produce these CM proteins by bacteria in a quantity adequate for studying the penetration characteristics of the CM proteins through intestinal mucosa in rats and Caco-2 cells. cDNAs encoding the mature proteins were expressed in *Escherichia coli* and purified by an Ni^{2+} -chelating column. The recombinant proteins were radioiodinated and administered orally to rats or applied to the apical site of the Caco-2 cell monolayer. The radioactivity in the trichloroacetic acid-insoluble fraction, which was mainly composed of peptides with molecular mass less than that of the intact CM proteins, in the serum and the basolateral medium was highest in recombinant CM3. Accordingly, the intestinal absorption of these three proteins in the form present in wheat should be evaluated.

Key words: allergen absorption; baker's asthma; Caco-2 cell; recombinant protein; wheat α -amylase inhibitor

A variety of α -amylase inhibitors of endogenous and exogenous origins have been found in wheat endosperm.¹⁾ The endogenous α -amylase inhibitors inhibit α -amylases from plants and microorganisms, while the exogenous α -amylase inhibitors inhibit mammalian and insect α -amylases and are grouped into three families with molecular masses of 60, 24 and 12 kDa.²⁾ The 60 kDa family is a tetramer, and the subunits have been identified as CM proteins. In tetraploid wheat (*Triticum durum*), three subunits (CM2, CM3 and CM16) have been reported, with two more subunits (CM1 and CM17) in hexaploid wheat (*Triticum aestivum*).³⁾ Protein inhibitors of α -amylase such as CM proteins have received considerable attention concerning their possible role in plant protection, human nutrition and quality improve-

ment of cooked wheat products.^{4,5)} CM proteins have also been identified as major allergens associated with baker's asthma, a common disease among workers with occupational exposure to cereal flour.^{6–8)} We have reported that CM3 might be an allergen commonly concerned with two types of allergy, baker's asthma and atopic dermatitis, respectively caused by the inhalation and ingestion of wheat products.⁹⁾ In atopic dermatitis patients, the sensitization to allergens is thought to occur predominantly via the gastrointestinal tract. However, there have been few reports that dealt with allergens associated with the ingested wheat allergy.¹⁰⁾ So a better understanding of allergen penetration at the mucosal level might be important to deal with food allergy. Food proteins have been reported to cross the intestinal epithelium in an intact form.¹¹⁾ The absorption of such native proteins as β -lactoglobulin (β -LG), horseradish peroxidase, bovine serum albumin and ovalbumin has been studied in both animals and Ussing chambers *in vitro*.^{12–15)} In contrast to these proteins, it is difficult to obtain a sufficient amount of CM proteins of high purity to study their absorption and transport.

cDNA clones encoding CM proteins have been isolated and characterized.^{16–19)} Lullien-Pellerin *et al.*²⁰⁾ have cloned cDNA encoding CM16 protein in an *Escherichia coli* (*E. coli*) expression vector and obtained the corresponding recombinant protein. The synthesized gene for the subunit of a dimeric α -amylase inhibitor was recently overexpressed in *E. coli*,²¹⁾ and the recombinant subunit was reported to possess similar inhibiting activity to that of the native protein against insect α -amylase. However, there is no report on the simultaneous production of all of the recombinant subunits (CM2, CM3 and CM16) of the tetrameric α -amylase inhibitor. The availability of these recombinant proteins in a reasonable quantity may also help in understanding the susceptibility to the al-

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Abbreviations: β -LG, β -lactoglobulin; IPTG, isopropyl- β -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TCA, trichloroacetic acid

lergy against ingested wheat.

In the present study, cDNAs coding for three CM proteins (CM2, CM3 and CM16) were expressed in *E. coli* and purified. We confirmed their ability to bind to IgG from rabbit anti-CM protein antisera. Subsequently, the transport susceptibility of the recombinant CM proteins through intestinal mucosa was estimated in rats and in the Caco-2 human colon carcinoma cell line.

Materials and Methods

Biological material. In our preliminary study, the steady-state mRNA levels of CM proteins in developing endosperm of *Triticum aestivum* (chikugo-izumi) collected at Kyushu University farm (Fukuoka) were determined by the Northern blotting technique, using inserts encoding CM2, CM3 and CM16 as probes. According to the previous reports,^{16,22} mRNA of CM proteins began to accumulate in developing endosperms as early as 4–7 d after flowering and increased to a maximum 18–21 d after flowering. Compatible results were obtained; mRNA levels increased to a maximum 20–25 d after flowering and then gradually decreased. Hence, endosperms at approximately 20 d after flowering were used for the expression study. Whole ears were immediately frozen in liquid nitrogen and stored at -80°C until being used.

Expression of CM proteins. The endosperm tissues were ground to fine powder under liquid nitrogen from which total RNA was prepared by the SDS-phenol method.²³ Poly(A)⁺ RNA was then purified by oligo(dT)-cellulose (Boehringer Mannheim, Mannheim, Germany). Double-stranded cDNA was synthesized by using the 'You-Prime First-Strand Beads' kit (Amersham Pharmacia Biotech, NJ, USA), starting with 2 μg of poly(A)⁺ RNA. Fragments encoding CM2, CM3 and CM16 in full length were PCR amplified from synthesized cDNA as a template by using the following three oligonucleotide pairs; CM2-1, 5'-GGATACAC CATATG ACAGGCCCGTATTG-3' (first 14 nucleotides from the N-terminus of the mature protein downstream of the underlined *NdeI* site) and CM2-2, 5'-CCGGAATTCCCATCTTTATATGTCCAAACC-3' (nucleotide numbers 424–444 from the start of translation containing the underlined *EcoRI* site); CM3-1, 5'-GGAATTCCATATGTCCGGCAGCTGCGTCCC-3' (first 17 nucleotides from the N-terminus of the mature protein downstream of the underlined *NdeI* site) and CM3-2, 5'-CCG GAATT CATCTCTAGATCCACAGAG-3' (nucleotide numbers 487–504 from the start of translation containing the underlined *EcoRI* site); and CM16-1, 5'-GGGTGCACCATATGATCGGCAATGAAGATTG-3' (first 17 nucleotides from the N-terminus of the mature protein downstream of the underlined *NdeI* site) and CM16-2, 5'-CCGGAATTC

TTGTCTCTAGCTCCACTGAGAC-3' (nucleotide numbers 417–438 from the start of translation containing the underlined *EcoRI* site). PCR amplification was done according to the standard protocols.²⁴ and cloning of the PCR products was done by the pGEM-T vector system (Promega Corporation, WI, USA). The amplified fragments were subjected to agarose gel electrophoresis and their sizes were checked. Three fragments of the expected sizes, containing the entire coding region for the mature CM proteins, were obtained. The fragments encoding CM2, CM3 and CM16 were then ligated into the *NdeI-EcoRI* sites of pET28a (Novagen, WI, USA) which was treated with the same restriction enzymes. The constructions were confirmed by an ALF-Express DNA sequencer (Pharmacia Japan, Tokyo, Japan), indicating no mutations during the amplification or cloning procedure. Expression plasmid vector pET28a was transformed into *E. coli* BL21 (DE3) (Novagen, WI, USA). The transformant was cultured in LB-broth (1% Difco tryptone, 0.5% Difco yeast extract and 1% NaCl) containing 30 $\mu\text{g}/\text{ml}$ of kanamycin at 37°C . Expression was induced by adding 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After 16 h of induction at 25°C , aliquots of the culture were collected and centrifuged. The pellets were suspended in an SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min before being subjected to SDS-PAGE.

Purification of the recombinant CM proteins. Cultured cells were harvested by centrifugation at 2,500 g for 15 min. The resulting pellet was suspended in a 5 mM imidazole solution containing 500 mM NaCl and 20 mM Tris (pH 7.9) and then sonicated. The insoluble fraction was collected by centrifugation at 17,500 g for 20 min and washed three times with 1% Triton X-100. The product was dissolved in an 8 M urea solution containing 5 mM imidazole, 500 mM NaCl and 20 mM Tris (pH 7.9), and then diluted to a 6 M urea concentration. After centrifugation, the supernatant was collected and purified by an Ni^{2+} -chelating column (Novagen, WI, USA), utilizing the His-Tag sequence of six histidine residues designed to express at the N-terminal end of the target protein. The column was washed with 10 volumes of a 6 M urea solution containing 5 mM imidazole, 500 mM NaCl and 20 mM Tris (pH 7.9), and then by 6 volumes of a 6 M urea solution containing 60 mM imidazole, 500 mM NaCl and 20 mM Tris (pH 7.9). The recombinant proteins were eluted with a 6 M urea solution containing 1 M imidazole, 500 mM NaCl, and 20 mM Tris (pH 7.9). To prepare for an absorption study, a 10% SDS solution was added to the purified proteins (to a final concentration of 1%), and the mixture dialyzed against phosphate buffered saline for 48 h to remove SDS from the sample.

Preparation of antisera for the α -amylase inhibitor subunits. Nihon white rabbits (female, 2 kg; Seac Yoshitomi, Fukuoka, Japan) were used to obtain antisera. Each wheat protein (500 μ g each of CM2, CM3 and CM16), which had been purified according to the method of Sanchez-Monge *et al.*⁷⁾ with our modification,⁹⁾ was subcutaneously injected into the rabbit dorsum with Freund's complete adjuvant (Difco Laboratories, MI, USA). After 3 and 5 weeks, subsequent injections of the purified wheat proteins (150 μ g) were administered with Freund's incomplete adjuvant (Difco Laboratories, MI, USA). A week after the last injection, the rabbits were killed by withdrawing blood from the aorta, and then antisera were obtained.

This experiment was done under the control of the Guidelines for Animal Experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

SDS-PAGE and immunoblotting. The recombinant proteins dissolved in a 6 M urea solution were subjected to SDS-PAGE (12.5% gel) under reducing conditions.²⁵⁾ Western blotting was performed with semi-dry blotting apparatus. Nonspecific binding was eliminated by incubating the nitrocellulose membrane (Shleicher & Schuell Co., Dassel, Germany) for 1 h at room temperature in a 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.05% Tween 20, and 5% skim milk. IgG-immunodetection, using the antisera prepared from rabbits, was carried out by treating the membrane with an antiserum (1:1,000) and peroxidase-conjugated anti-rabbit IgG (1:2,000). The immune complexes were visualized by applying 0.5 mg/ml of a diaminobenzidine solution containing 0.013% H₂O₂ and 50 mM Tris (pH 7.5).

¹²⁵I-labelling of the recombinant CM proteins. The terminal amino groups (lysine residues) of the recombinant proteins and β -LG (20 μ g) were radioiodinated with the ¹²⁵I-Bolton-Hunter reagent (ICN Pharmaceuticals, CA, USA) according to the manufacturer's recommendations. The reaction mixture was passed through a Sephadex G-25 column to remove free ¹²⁵I. The number of lysine residues per molecule was 3, 3, 2 and 16 for CM2, CM3, CM16 and β -LG, respectively. The his-tag sequence had no lysine residue.

Intestinal absorption of the recombinant CM proteins in rats. Male Sprague Dawley rats (Seac Yoshitomi, Fukuoka, Japan) weighing 120–150 g were given *ad libitum* a commercial non-purified diet (type NMF; Oriental Yeast Co., Tokyo, Japan) and drinking water. After overnight fasting, the rats were orally given 1.5 ml of a test solution containing 5 mg

of one of the recombinant proteins, CM2 (rCM2), CM3 (rCM3) or CM16 (rCM16), 0.75 ml of phospholipid-stabilized 10% soybean oil, (Intralipid; Pharmacia, Stockholm, Sweden) and the ¹²⁵I-recombinant protein (1000–1200 cpm/ng) as a tracer (9–13.5 μ Ci). This lipid emulsion was used to facilitate the protein absorption.^{15,26,27)} Serum samples were taken from the caudal vein at appropriate intervals for 6 h and immediately mixed with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml of leupeptin and 2 μ g/ml of aprotinin). An aliquot of each serum sample (20 μ l) was measured for its radioactivity. The sera were analyzed for trichloroacetic acid (TCA)-insoluble and -soluble radioactivity, after adding 10% TCA (to a final concentration of 5%) and centrifuging. The total volume of serum per rat was estimated as 4.5% of the body weight. The transport rate of the labeled material was calculated as the percentage of radioactivity in the total serum against the total radioactivity administered to each rat. The sera were also subjected to SDS-PAGE (17.5% gel) under reducing condition.²⁵⁾ Each gel was dried and subjected to autoradiography, and the radioactivity of the gel was analyzed with a bio-imaging analyzer (BAS-1000, Fuji Photo Film Co., Tokyo, Japan).

Cell culture. The human colon adenocarcinoma cell line, Caco-2 (passage 37, obtained from American Type Culture Collection, Rockville, MD, USA), was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technologies, UK) supplemented with 10% fetal bovine serum (Gibco-BRL, Life Technologies, UK), 3.7 g/l of sodium bicarbonate, 1% non-essential amino acids (Dainippon Pharmaceutical Co., Tokyo, Japan), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml) and insulin (10 μ g/ml) in 10-mm Petri dishes. These were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and the medium was changed every other day. After one week, the cells were passaged by trypsinizing with 0.25% trypsin (Difco Laboratories, MI, USA) in a Ca²⁺-, Mg²⁺-free phosphate-buffered saline (PBS) solution containing 0.01% EDTA. The cells were then grown in a Biocoat cell culture insert with a 3- μ m pore size and 23.1-mm diameter membrane coated with type I collagen (Becton Dickinson Labware, MA, USA). The monolayer became confluent 7 d after seeding, and cells that had been cultured for 21 d were used for the subsequent experiments.

Transport of the recombinant CM proteins across the Caco-2 monolayers. The recombinant CM proteins were added to the apical side of the well at a final concentration of 0.4 mg/ml, and then incubated at 37°C. The ¹²⁵I-recombinant CM proteins (2.2–3.3 μ Ci/ μ g of protein), which had been labeled as

already described, were also added as a tracer (0.1–2.6 μ Ci). In addition, β -LG was used in this study to compare its permeability through the Caco-2 monolayers with that of the recombinant CM proteins. β -LG was chosen, since it has a similar molecular weight to that of the CM proteins and is mostly responsible for cow's allergy.¹²⁾ The media on both sides of the well were collected at appropriate intervals for 3 h. A 20% TCA solution was added (final concentration of 5%) to an appropriate aliquot of the collected medium (1 ml), which was then centrifuged and the radioactivity of the TCA precipitate determined. The collected medium was also subjected to Sephadex G-25 gel filtration column chromatography, using 2% bovine serum albumin in PBS as the elution buffer. The eluate was fractionated and collected in 1-ml fractions, and the radioactivity of each fraction was measured.

Statistics. Data were analyzed by one-way ANOVA followed by a *post-hoc* test (Fisher's protected least significant difference test). The statistical analyses were carried out with the Excel 98 Macintosh Editor (Microsoft, Tokyo, Japan).

Results

Expression of the recombinant CM proteins in E. coli

Transformed *E. coli* cells were cultured in LB-broth and induced with IPTG. The harvested cells were then subjected to SDS-PAGE. A histidine tag sequence with 21 amino acids, including six histidine residues, was designed to express at the N-terminal end of the target protein. Coomassie-stained bands with expected molecular sizes (2.5 kDa larger than those of the native proteins) were detected after IPTG induction (Fig. 1). The expression levels of the recombinant proteins approached 30% of the cellular protein, as estimated by Coomassie blue staining. The insoluble fractions of the harvested cells were subjected to SDS-PAGE and then electroblotted to a nitrocellulose filter. The filter was then probed with the rabbit anti-CM2, -CM3 and -CM16 sera. All recombinant proteins reacted with the corresponding rabbit antisera (data not shown). Approximately 3–5 mg of purified recombinant proteins were routinely recovered from a 100-ml bacterial culture after Ni^{2+} -chelating column purification (Fig. 2). Co-elution of higher-molecular-weight proteins from the column was also detected. Since then molecular weights of the contaminating proteins were similar to the protein bands that cross-reacted with the rabbit antisera, these proteins may have been aggregated or reassociated forms of the recombinant proteins. Most of these recombinant proteins were accumulated in an insoluble form, possibly as an inclusion body. It has previously been reported that certain

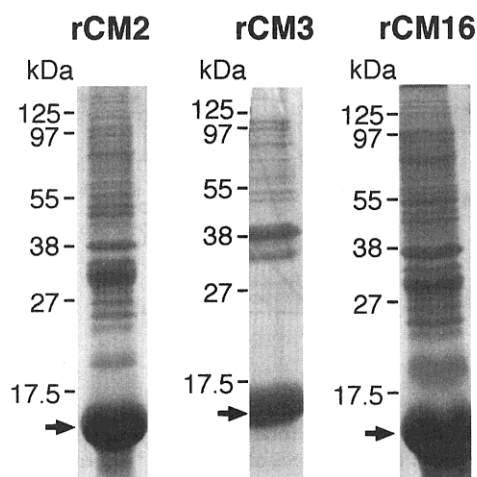


Fig. 1. SDS-PAGE Analysis of the Total Bacterial Proteins. Arrows indicate the recombinant CM proteins. Molecular markers are indicated on the left.

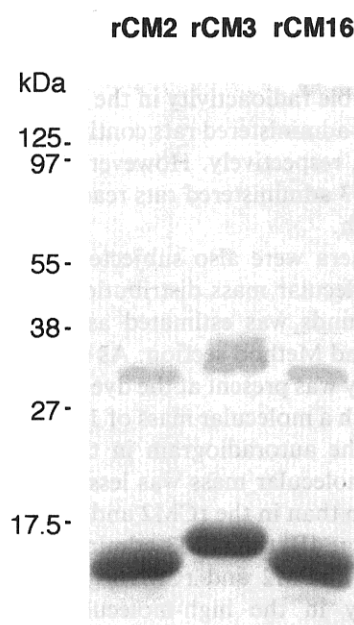


Fig. 2. SDS-PAGE Analysis of the Recombinant CM Proteins after Purification in an Ni^{2+} -Chelating Column. Molecular markers are indicated on the left.

cultivation conditions for *E. coli* such as the temperature and level of induction could prevent the formation of inclusion bodies.^{28,29)} We examined the effects of a lower temperature for cultivation and the concentration of IPTG on the solubility of the recombinant proteins; however, in accordance with the results of Lullien-Pellerin *et al.*,²⁰⁾ no soluble proteins were produced.

Intestinal absorption of the recombinant CM proteins in rats

The total radioactivity in rat serum administered rCM3 was significantly higher throughout the period

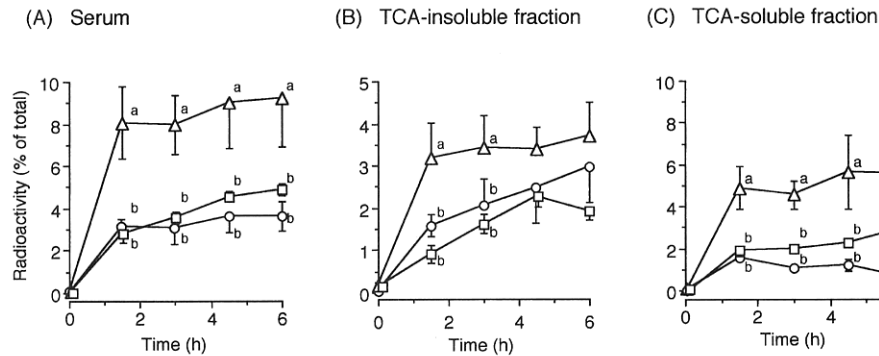


Fig. 3. Recovery of Radioactivity in Serum after the Oral Administration of ^{125}I -Labeled rCM2 (○), rCM3 (△) and rCM16 (□) to Rats. (A) Recovery in the total serum. (B) Recovery in the TCA-insoluble fraction. (C) Recovery in the TCA-soluble fraction. Each point represents the mean $\pm$ SE of four rats. ^{ab}Different letters show significant difference at $p < 0.05$.

than that in the other two recombinant proteins (Fig. 3). The radioactivity of TCA-insoluble (intact protein and relatively large peptides) and -soluble (free iodine, small peptides and amino acids) materials in the sera tended to be higher in the rCM3-administered rats than in the other two groups. The TCA-insoluble radioactivity in the sera of the rCM2- and rCM16-administered rats continued increasing to 4 h and 6 h, respectively. However, the radioactivity in the rCM-3 administered rats reached its maximum level at 1.5 h.

The rat sera were also subjected to SDS-PAGE, and the molecular mass distribution of the radioactive compounds was estimated as described in the Materials and Method section. As shown in Fig. 4A, radioactivity was present at the dye front and in those peptides with a molecular mass of 10–12 kDa. The intensity of the autoradiogram in the peptide region with high molecular mass was less prominent in the rCM3 group than in the rCM2 and rCM16 groups. As shown in Fig. 4B, the rCM3-administered rats, compared to the rCM2 and rCM16 groups, had lower radioactivity in the high-molecular-mass peptides and the radioactivity was predominant at the dye front, where small peptides had comigrated.

Transport of the recombinant CM proteins across the Caco-2 monolayers

The radioactivity recovered from the basolateral side of the Caco-2 monolayer was less than 3% after ^{125}I -rCM proteins or β -LG had been added to the apical side for 3 h. The radioactivity in the medium and in the TCA-insoluble fraction tended to be higher in the CM3 group than in the other groups (Fig. 5). In particular, the radioactivity at 1 h and 3 h of the rCM3 group was higher than that in the other groups. The increase in radioactivity of the β -LG group was less prominent than that of the other groups.

The molecular mass distribution of the ^{125}I -rCM3-containing medium was examined by Sepadex G25 column chromatography (Fig. 6). On the apical side,

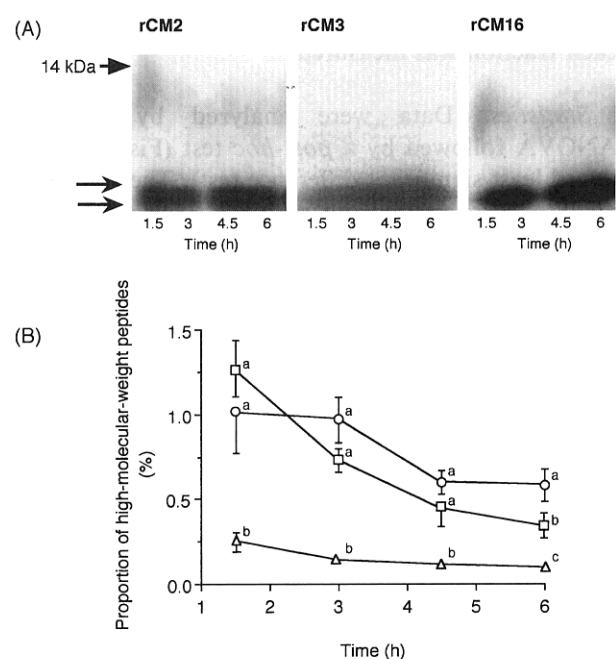


Fig. 4. Autoradiogram (A) and Radioactivity Distribution in the High-Molecular-Mass Fraction (B) of Serum Separated by SDS-PAGE after the Oral Administration of ^{125}I -labeled rCM2 (○), rCM3 (△) and rCM16 (□) to rats.

Double arrows indicate the dye front. The radioactivity of the high-molecular-weight peptides (10–12 kDa) and dye front were determined by a Bio-Imaging analyzer, as described in the text. The proportion in the high-molecular-weight peptides was then calculated.

the radioactivity predominantly remained in the region with a molecular mass greater than 5 kDa throughout the incubation period. On the basolateral side, the radioactivity was approximately distributed in two regions, that between 5 kDa and 1 kDa and that less than 1 kDa. The radioactivity in these regions during incubation was greater for the CM3 group than for the CM2, CM16 or β -LG group, excepting the radioactivity of rCM 2 at 3 hr. The radioactivity in the high-molecular-weight and low-molecular-weight regions in the β -LG group was very

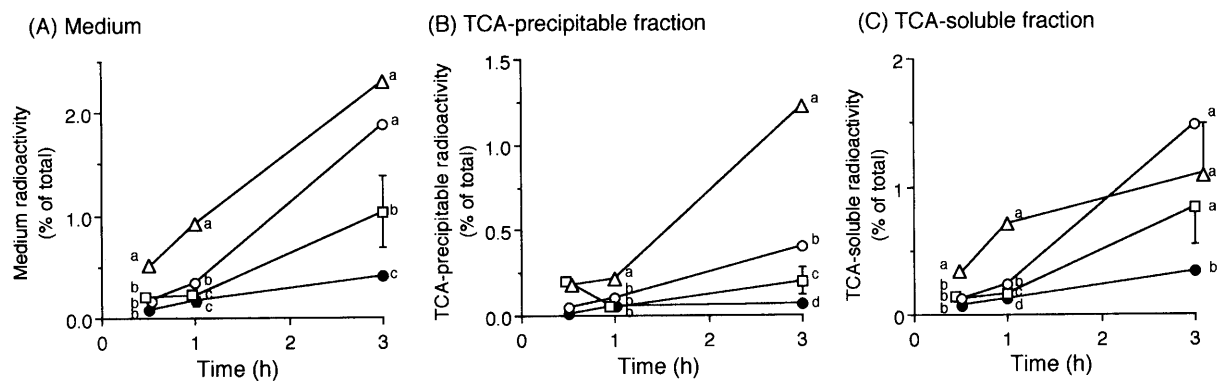


Fig. 5. Recovery of Radioactivity in Basolateral Side Medium after Supplementation of ^{125}I -Labeled rCM2 ($\circ$), rCM3 (Δ), rCM16 ($\square$) and β -LG ($\bullet$) to the Apical Side of the Caco-2 cell Monolayer.

(A) Recovery in the medium. (B) Recovery in the TCA-insoluble fraction. (C) Recovery in the TCA-soluble fraction. Each point represents the mean $\pm$ SE of three wells. ^{abc}Different letters show significant difference at $p < 0.05$.

small in comparison with that in the CM groups.

Discussion

We succeeded in producing recombinant CM proteins (rCM2, rCM3 and rCM16) in an amount that allowed us to evaluate their intestinal absorption and transport in rats. As revealed by Western blotting, these recombinant proteins maintained immunological reactivity to the antibodies raised against the CM proteins that had been prepared from wheat. This immunological reactivity does not necessarily mean that the rCM proteins maintained all aspects of the structure of the corresponding native CM proteins for the following reasons. First, the recombinant proteins contained the His tag portion at the N-terminal of the rCM proteins. Secondly, since there were 10 cystein residues in each CM protein,^{2,3)} the bacterium might have produced rCM proteins that had the wrong intramolecular disulfide bonds. Thirdly, the recombinant proteins might not have been free of SDS, although we tried to remove as much SDS as possible by washing exhaustively by dialysis. Accordingly, the digestibility and intestinal transport of the rCM proteins might have differed from those of the corresponding native proteins. These points should be kept in mind, when extrapolating the present results concerning the absorption of the rCM proteins to the case of the native CM proteins. We therefore tried to focus on comparing the absorption characteristics among the rCM proteins.

When the ^{125}I labeled rCM proteins were administered orally to the rats or supplemented to the apical-side medium of the Caco-2 cells, the radioactive proteins were recovered as TCA-insoluble materials at the level of 1–3% in the blood serum and 0.2–1.2% on the basolateral side. The majority of the radioactivity in the TCA-insoluble fractions seems to have been derived from peptides smaller than the intact rCM proteins, since the autoradiogram in Fig. 4

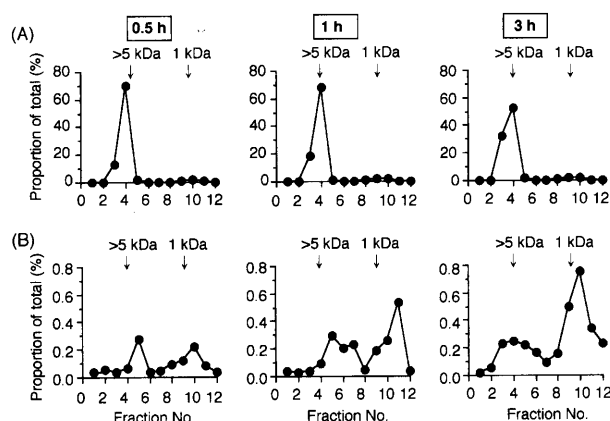


Fig. 6. Fractionation by Sephadex G25 Column Chromatography of the Apical Side (A) and Basolateral Side (B) Media after Supplementation of ^{125}I -Labeled rCM3 to the Apical Side of the Caco-2 cell Monolayer.

Each point represents the mean of three wells.

reveals that the most of the radioactivity in our SDS-PAGE system was present in the dye front where free amino acids and peptides of less than 10 kDa had comigrated. Furthermore, the elution patterns by Sephadex G25 (Fig. 6) of the medium derived from the basolateral side show that the radioactivity was distributed between molecular masses of 1 kDa and 5 kDa, in addition to that greater than 5 kDa. The latter results are in agreement with those of Heyman *et al.*,³⁰⁾ who reported that ^3H -labeled horse radish peroxidase transport across the monolayer of Caco-2 cells in Ussing chambers occurred along the degradative pathway, possibly through the lysosomal system, allowing the transport of peptides and amino acids, but the amount transmitted *via* a direct pathway, allowing transport of intact protein, was very small. It has been suggested that β -LG is the main factor responsible for milk protein immunoreactivity and intolerance, since β -LG was efficiently absorbed by the intestinal mucosa of adult animals, partly in an

intact antigenic form.¹²⁾ In the present study, the transport of β -LG across the Caco-2 cell monolayer was less than that of the rCM proteins. It remains to be determined if the native CM proteins from wheat would also result in an increased absorption compared to β -LG, when tested in the Caco-2 cells.

The increased transport of intact protein through the intestinal barrier may influence immunological sensitization to food allergens.¹⁰⁾ In terms of the comparative immunogenicity, there appear to be sequences that were predominantly IgE- and/or IgG-reactive, since β -LG-derived peptides prepared chemically or by trypsin and bacterial protease with a molecular mass of less than 3,500 were selective for IgG and/or IgE antibody recognition.^{31,32)} In our previous study, CM3 reacted to the sera of allergy patients most strongly among the three subunits.⁹⁾ In this context, rCM3 relative to rCM2 or rCM16 may be more important immunologically, since the recovery as TCA-insoluble materials of ¹²⁵I labeled rCM3 was the greatest in the rat serum and on the basolateral side of the Caco-2 cells.

The recovery of rCM protein in the TCA-insoluble fraction of rat serum was much higher than that in the corresponding basolateral medium fraction, suggesting that the rCM proteins were absorbed efficiently in the rat intestine and/or through a route other than the intestine. In fact, food proteins that were orally injected into rats were absorbed through the stomach³³⁾ and intestine *via* blood circulation and the lymphatic system.¹⁶⁾

In summary, the present study has shown the efficient absorption of rCM3 in comparison with that of rCM2 or rCM16 and suggests that the high absorption rate of rCM3 might be involved in the high titer of IgE specific to the corresponding native protein in bakers' asthma. To confirm this hypothesis, a more precise evaluation of the transport susceptibility of the native CM proteins through the mucosa should be carried out.

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