



Enzyme-Modified Cheese Exerts Inhibitory Effects on Allergen Permeation in Rats Suffering from Indomethacin-Induced Intestinal Inflammation

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We have found that an enzyme-modified cheese (EMC) inhibited the permeation of allergens such as ovalbumin (OVA), using Caco-2 cells as an *in vitro* intestinal epithelial model. In addition, NPWDQ (Asn-Pro-Trp-Asp-Gln, aa 107-111 of α s₂-casein) was isolated from EMC and identified as one of the responsible peptides for this inhibitory activity (Tanabe *et al.*, *J. Agric. Food Chem.*, (2007)). In this study, we aimed to clarify the mechanism by which NPWDQ inhibited allergen permeation *in vitro*, and also to evaluate the effects of EMC on allergen permeation *in vivo*. Intestinal permeability for both fluorescein isothiocyanate conjugated dextran and horseradish peroxidase was decreased in Caco-2 cells by the addition of NPWDQ, indicating that NPWDQ might inhibit both paracellular and transcellular transports. Next, intestinal inflammation was induced by subcutaneous injections of indomethacin to rats. When OVA was injected into the jejunal and ileac loops of indomethacin-administered rats with and without NPWDQ, it was found that the addition of NPWDQ effectively diminished OVA permeation from both loops. Although the plasma OVA concentration of indomethacin-administered rats after oral OVA challenge was markedly elevated over that of normal rats, supplemental administration of EMC to the rats effectively suppressed OVA permeation. These results suggest that EMC is useful for the prevention of food allergy by inhibiting allergen permeation probably by enforcing the intestinal barrier.

Key words: enzyme-modified cheese; casein peptide; allergen permeation; indomethacin; intestinal inflammation

Food allergy/hypersensitivity is an important health problem, and countermeasures are socially needed. It is estimated that up to 8% of children under the age of 3 years and 2% of adults are affected by food allergy.¹⁾ Very recently, it has been reported that, by the age of 3

years, 5–6% of children suffer from food hypersensitivity based on food challenges and good clinical histories.²⁾ Hen's egg, cow's milk, wheat, and peanuts are generally known allergens in food-allergic patients.³⁾

Food allergy, triggered by an aberrant immune response elicited by orally ingested food allergens, is generated through a complicated mechanism. Of several steps, allergen permeation at the intestine is the first. The intestinal epithelium theoretically acts as a barrier restricting the permeation of macromolecules, but a small portion (10^{-3} to 10^{-4}) of dietary proteins can cross the epithelium to access and activate effector cells,⁴⁾ and this becomes a risk factor for food allergy. In addition, it has been reported that antigen permeability through the intestinal tract was enhanced in allergic patients.^{5,6)} Therefore, it is conceivable that enforcing the barrier function of the intestinal epithelium would help to prevent food allergy.

Enzyme-modified cheeses (EMCs) are generally produced by the hydrolysis of cheese with commercial proteases. They are used in food industry 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.⁷⁾ In addition, the enzymatic treatment of cheese has the potential to produce bioactive peptides that can provide nutritional, medical, and health benefits.⁸⁾ For example, Haileselassie *et al.*⁹⁾ isolated antihypertensive peptides, such as YPFPGPI, which inhibits an angiotensin I-converting enzyme, from an EMC. Recently, we found that EMC inhibited the permeation of allergens such as ovalbumin (OVA), using Caco-2 cells as an *in vitro* intestinal epithelial model.¹⁰⁾ In addition, NPWDQ (Asn-Pro-Trp-Asp-Gln, aa 107-111 of α s₂-casein) peptide has been isolated from EMC and identified as one of the responsible peptides for this inhibitory activity.¹⁰⁾ We have also isolated, from Edam cheese, a peptide with the same inhibitory activity on allergen permeation as NPWDQ, and we identified its amino acid sequence

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as DKHPF (Asp-Lys-Ile-His-Pro-Phe, aa 47-52 of β -casein).¹¹⁾

In this study, we examined to determine whether NPWDQ would inhibit the permeation of fluorescein isothiocyanate conjugated dextran (FITC-dextran) and horseradish peroxidase (HRP) in an *in vitro* Caco-2 cell model, and we examined its inhibitory activity on allergen permeation in an *ex vivo* intestinal loop. With a view to the application of casein peptides, such physiological functional peptides as NPWDQ, on the industrial level, *in vivo* evaluation of EMC was also performed. In this paper, we show that supplemental administration of EMC effectively suppressed OVA permeation in rats suffering from indomethacin-induced intestinal inflammation.

Materials and Methods

Caco-2 cells. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). In this study, cells were used between 45–50 passages. The growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal bovine serum (FBS), 1% nonessential amino acids, and antibiotics (100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 50 μ g/ml of gentamycin). DMEM, non-essential amino acids, penicillin, streptomycin, and gentamycin were all obtained from Invitrogen (Carlsbad, CA). FBS was from ICN Biomedicals (Osaka, Japan). Cells were cultured at 37°C under a humidified 5% CO₂ atmosphere. They were normally grown in 75 cm² tissue culture flasks to approximately 80% confluence, and seeded into a 12-well Transwell cell culture chamber (0.4 μ m in pore size and 12 mm in diameter) (Corning Coster, Cambridge, MA) at a density of 5×10^5 cells/cm². Each well was placed in a cluster plate with an outside medium (basolateral side, 1.5 ml) and an inside medium (apical side, 0.5 ml). The cell monolayers were fed fresh medium every 24 h. After 16 d of culture, transepithelial resistance (TER) was measured using a Millicell-ERS instrument with Ag/AgCl electrodes (Millipore, Bedford, MA). Caco-2 cell monolayers were used when their TER values were > 300 ohm cm².

In vitro permeation study. Synthetic peptide NPWDQ was obtained from Takara Bio (Osaka, Japan). One day before permeability measurements, the monolayers were cultured in the absence (control) or presence of NPWDQ (10^{-6} M in the apical side). FITC-dextran (0.5 mg, Sigma-Aldrich, St. Louis, MO) or HRP (0.5 mg, Sigma-Aldrich) was added to the apical side, and the monolayers were incubated for 1 h, after which the outside medium was collected. The concentration of FITC-dextran in the basolateral side was determined by measuring the fluorescence intensity with a spectrofluorometer (FP-750, Nihon Bunko, Tokyo) at excitation and emission wavelengths of 495 nm and

520 nm respectively. The concentration of HRP in the basolateral side was determined as its enzyme activity using *o*-phenylenediamine (Wako Pure Chemical Industries, Osaka, Japan) as a substrate, based on the absorption at 490 nm. Inhibitory activity (%) was calculated according to $(P_c - P_s)/P_c \times 100$, where P_c and P_s are permeated FITC-dextran or HRP in the absence and the presence of NPWDQ respectively. The assay was repeated at least 2 times independently, and each assay was performed in triplicate.

Rats. Six-week-old Sprague Dawley rats were purchased from Charles River Japan (Kanagawa, Japan), and housed in an air-conditioned room under a 12 h light/dark cycle. They were allowed *ad libitum* access to tap water and a standard diet (MF, Oriental Yeast, Tokyo), unless otherwise stated. The experimental protocols were approved by the Animal Care Committee of the Graduate School of Biosphere Science of Hiroshima University. The rats were randomly assigned to five groups. One was a normal group ($n = 4$). The rats in the other groups ($n = 3$ each) were given two or four subcutaneous injections of indomethacin (7.5 mg/kg/d, Wako Pure Chemical Industries) daily at 24 h intervals, according to the method of Yamada *et al.*¹²⁾ To the normal group, a vehicle (5% NaHCO₃) was injected in the same manner. Indomethacin-administered (twice injected) rats were further randomly assigned to three groups including control (CON), 0.1 g of EMC administration (EMC 0.1), and 0.5 g of EMC administration (EMC 0.5) groups. Separately, for the *ex vivo* loop experiments as described below, the jejunum and ileum were excised from normal and indomethacin-administered (twice injected) rats.

Ex vivo loop experiments. *Ex vivo* closed loop experiments were performed, of which a schematic illustration is depicted in Fig. 1. Following anesthetization of normal and indomethacin-administered rats

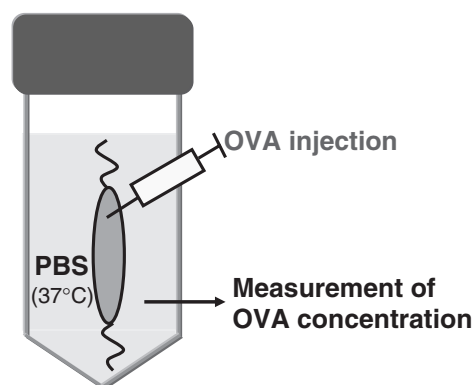


Fig. 1. Schematic Illustration of the *ex Vivo* Loop Experiment.

OVA was injected into the closed loop from normal and indomethacin-administered rats with and without the presence of NPWDQ, and then the loop was placed into a tube that contained PBS. The tube was incubated at 37°C for 2 h, and the outside solution was collected to measure the OVA concentration.

($n = 4$ or 5), 10-cm upper (jejunum) and lower (ileum) segments of the small intestine were excised and rinsed with PBS. PBS (0.1 ml) containing OVA (0.2 mg/ml) was injected into the closed loop with and without NPWDQ (5 μ g), and then the loop was placed into a 15-ml tube that contained 5 ml of PBS warmed to 37°C. The tube was incubated at 37°C for 2 h, and the outside solution was collected to measure the OVA concentration by sandwich ELISA by the method of Watanabe *et al.*¹³⁾ Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-OVA IgG (Millipore/Chemicon) at 4°C overnight and then blocked with Block Ace (Funakoshi, Tokyo) at 37°C for 2 h. Diluted test samples were incubated in the well at 37°C for 2 h. After that, horseradish peroxidase-conjugated rabbit anti-OVA IgG (Rockland, PA) was added and incubated at 37°C for 2 h. Between the steps, the wells were washed with PBS containing 0.02% Tween-20. Finally, *o*-phenylenediamine solution was added, followed by incubation at room temperature. After color developed, the plate was read at 450 nm with a microplate reader.

Preparation of EMC. Denmark skim cheese was hydrolyzed with Protease S, Newlase A, and Umamizyme (Amano Enzyme, Nagoya, Japan), as reported previously.¹⁰⁾ Briefly, Denmark skim cheese was suspended in water (50% w/v), and then the proteases were added to a final concentration of 0.15–0.3% (w/v). EMC was obtained after Protease S treatment at 34°C for 48 h, followed by Newlase A/Umamizyme treatment at pH 4.1 at 34°C for 120 h. The resulting EMC was heat treated at 110°C for 10 min to inactivate the enzymes used in the preparation, and freeze-dried. The composition of EMC was examined by the usual method.¹⁴⁾ Briefly, water and protein contents were analyzed by the air oven method and Kjeldahl's method respectively. Fat content was analyzed by the acid hydrolysis method. Ash content was analyzed by ignition at 550°C. Carbohydrate content was calculated as follows: Carbohydrate (%) = 100 – (water (%) + protein (%) + fat (%) + ash (%)). For analysis of amino acid composition, EMC was hydrolyzed with 6N HCl at 110°C for 24 h. The hydrolysate was analyzed with an amino acid analyzer, except for tryptophan, which was analyzed with HPLC. For half-cystine measurement, performic acid oxidation was done before hydrolysis with 6N HCl.

Time-course of changes in plasma OVA concentration after OVA challenge. Twenty-four hours after the second injection of indomethacin, rats from the CON, EMC 0.1, and EMC 0.5 groups were used in this experiment (Fig. 2). To rats from the EMC groups, EMC (0.1 g or 0.5 g/head) was given orally twice at a 24-h-interval at the same time as subcutaneous injections of indomethacin.

After withholding of food overnight, the rats were orally administered 200 mg of OVA. Blood samples

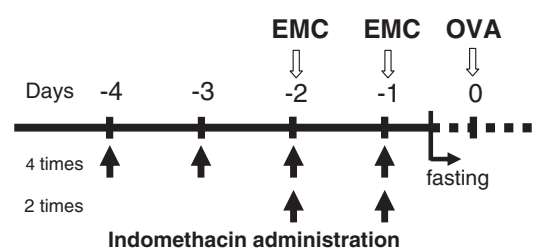


Fig. 2. Experimental Protocol for the Animal Study.

Closed and open arrows indicate subcutaneous injections of indomethacin and oral administrations of EMC and of OVA respectively. Blood samples were collected from the portal vein at 0, 0.5, 1, 2, 4, 8, and 24 h after OVA challenge to measure the plasma OVA concentration.

were collected from the portal vein at 0, 0.5, 1, 2, 4, 8, and 24 h after OVA challenge, and the plasma OVA concentration was measured by sandwich ELISA by the method described above. In addition, AUC (the area under the OVA concentration-time curve) between 0 and 24 h for each rat was calculated.

Statistical analysis. Statistical analysis of differences between the control and test groups was performed by Student's *t*-test.

Results and Discussion

We prepared an EMC by hydrolysis of Denmark skim cheese with Protease S, Newlase A, and Umamizyme. The composition of EMC was determined by the usual method, and is listed in Table 1. EMC was composed mainly of protein (61.6%), and the most abundant amino

Table 1. The Composition of EMC (wt %)

Water	2.3
Protein	61.6
Fat	6.9
Ash	11.7
Carbohydrate	17.5
Total amino acids	
Arg	1.78
Lys	4.89
His	1.86
Phe	3.28
Tyr	3.51
Leu	5.82
Ile	2.96
Met	1.79
Val	3.88
Ala	1.76
Gly	1.14
Pro	6.62
Glx ¹	13.6
Ser	3.50
Thr	2.33
Asx ²	4.31
Trp	0.76
Cys	0.27

¹ Glu+Gln

² Asp+Asn

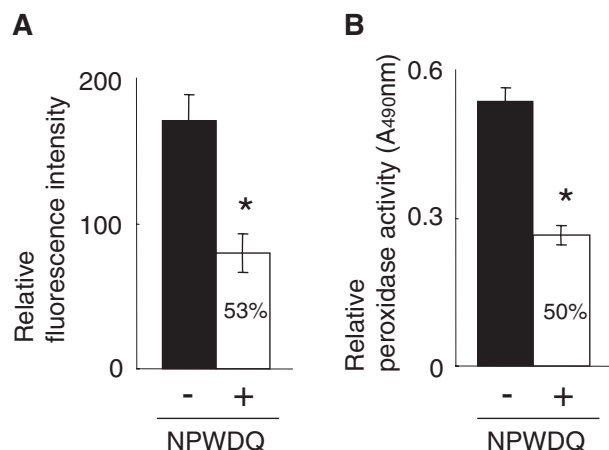


Fig. 3. Effects of NPWDQ on the Permeation of Fluorescein Isothiocyanate Conjugated Dextran (FITC-dextran) (A) and Horseradish Peroxidase (HRP) (B) in Caco-2 Cells.

NPWDQ was added to the apical side of the Caco-2 cell monolayers. After a 24-h incubation, FITC-dextran or HRP was added to the apical side. The monolayers were incubated for 1 h, and the concentration of FITC-dextran or of HRP in the basolateral side was determined. Results are expressed as means $\pm$ SE, $n = 3$. *, $p < 0.05$ between the absence and the presence of NPWDQ.

acids were Glx (Gln + Glu) (13.6%). Since it is well-known that Gln is the preferred fuel source for enterocytes and that it plays a vital role in intestinal function,^{15–17}) it was expected that EMC would show protective activity against intestinal inflammation.

We have reported that NPWDQ (aa 107–111 of α_{s2} -casein), which occurred in the EMC, inhibited OVA permeation in a Caco-2 cell model.¹⁰) In this study, first we examined to determine whether NPWDQ would inhibit the permeation of FITC-dextran (a marker of paracellular transport¹⁸) and HRP (that of transcellular transport¹⁸) *in vitro*. As shown in Fig. 3, NPWDQ significantly decreased the permeation of FITC-dextran (47% inhibition, Fig. 3A). Hence, it was assumed that enforcing a tight junction (TJ) barrier was at least in part involved in the inhibitory activity of NPWDQ on allergen permeation. Permeation of HRP was also decreased by NPWDQ (50% inhibition, Fig. 3B). Therefore, it is highly probable that NPWDQ inhibited both paracellular and transcellular transport in Caco-2 cells. Further study has been done to examine whether NPWDQ affects protein and mRNA expression of TJ-related molecules such as occludin, claudins, and ZO-1.

Caco-2 monolayers with high TER values ($> 300 \text{ ohm cm}^2$) are frequently used as a normal intestinal model. On the while, it has been reported that allergen permeability through the intestinal tract is enhanced in allergic patients.^{5,6}) To mimic this condition of allergic patients, intestinal injury was induced by two or four subcutaneous injections of indomethacin to rats. We examined to determine whether NPWDQ would inhibit allergen permeation in the rat model. The major pathogenesis is basically explained by the fact that indo-

methacin inhibits cyclooxygenase, depletes endogenous and protective prostaglandins in the mucosa, and subsequently impairs mucosal barrier function.¹⁹)

Using the jejunoileal tract from normal and indomethacin-administered (twice injected) rats, we performed an *ex vivo* experiment to clarify the inhibitory activity of NPWDQ on OVA permeation in the intestine. A schematic illustration of the *ex vivo* evaluation of permeability is given in Fig. 1. Two rabbit anti-OVA antibodies were used for sandwich ELISA. The reason we used NPWDQ instead of EMC in this *ex vivo* experiment was that NPWDQ appeared to be relatively resistant to degradation by digestive enzymes due to the presence of the second N-terminal proline residue,²⁰) while orally-administered EMC might be further digested in the stomach and the intestine.

As shown in Fig. 4A, administration of indomethacin almost doubled OVA permeation in the jejunal loop, but the addition of NPWDQ into the loop restored it to the normal level. As for the ileac loop (Fig. 4B), OVA permeation in the loop from normal rats was extremely low, while that from indomethacin-administered rats was remarkable. This increase in ileac OVA permeation by indomethacin was more prominent than that in the jejunal case, suggesting that the ileum was more damaged than the jejunum. Nevertheless, NPWDQ in

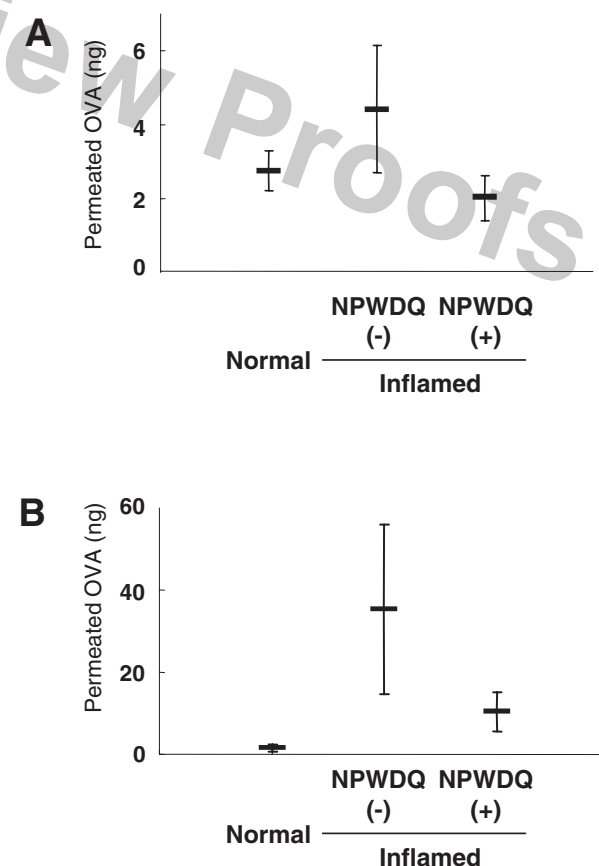


Fig. 4. Effects of NPWDQ on OVA Permeation in the Inflamed Jejunal (A) and Ileac (B) Loops.

See Fig. 1. Results are expressed as means $\pm$ SE, $n = 4$ –5.

Table 2. Time Course of Blood OVA Concentrations and AUCs in Normal and Indomethacin-Administered Rats

Time (h)	Normal	Indomethacin-administered	
		2 times	4 times
	(ng/ml)		
0	0.17 ± 0.01	0.11 ± 0.01	0.11 ± 0.00
0.5	0.23 ± 0.06	2.8 ± 2.6	11 ± 11
1	0.15 ± 0.01	2.5 ± 2.3	6.0 ± 5.9
2	0.14 ± 0.01	0.37 ± 0.26	7.0 ± 6.5
4	0.13 ± 0.01	0.56 ± 0.43	12 ± 6
8	0.13 ± 0.01	1.2 ± 1.1	24 ± 20
24	0.12 ± 0.00	0.22 ± 0.16	0.44 ± 0.05
	(ng·h/ml)		
AUC ¹	3.1 ± 0.1	19 ± 17	297 ± 235

Results are expressed as mean ± SE, n = 3–4.

¹Area under the OVA concentration-time curve.

the ileac loop effectively inhibited OVA permeation.

If peptides with inhibitory effects on allergen permeation are used as health-promoting functional foods, it is likely that raw materials, instead of purified or synthetic peptides, are more acceptable to the industry. Hence, we next performed an *in vivo* experiment to determine whether EMC containing various peptides such as NPWDQ would inhibit allergen permeation in indomethacin-administered rats. Prior to the *in vivo* experiment, the blood OVA concentration was measured in normal rats after oral administration. Table 2 shows the time course of the blood concentration of OVA after oral administration to the rats. Although OVA was detected at low levels 0.5 h after OVA challenge in normal rats, no remarkable transfer of OVA into the peripheral blood was observed after 1 h. Thus, it is possible that OVA is rarely absorbed at the intestine of the normal rat as an intact form.

Contrary to the normal rats, undigested OVA was detected at high levels in the peripheral blood in the indomethacin-administered rats (Table 2). While the AUC for normal rats was 3.14 ± 0.13 , those for twice- and four-time-administered rats were 19 ± 17 and 297 ± 235 (ng·h/ml) respectively. Since severe acute inflammation, such as blistered stomach, discolored liver, and adhesive intestine, was found in the rats administered indomethacin 4 times, this model was judged not to be suitable for this study. Hence, twice-administered rats were used in the subsequent experiment. Since 1 g of EMC contained approximately 2 moles of α s₂-casein, it was calculated to contain 1.3 mg of NPWDQ in the sequence of α s₂-casein if α s₂-casein was not hydrolyzed inside the NPWDQ sequence during the preparation of EMC. Based on this calculation, we decided to administer 0.1 or 0.5 g of EMC daily in the *in vivo* experiment.

Table 3 shows the effects of EMC on the blood concentration of OVA after oral OVA challenge to indomethacin-administered rats. EMC effectively lowered the blood concentration of OVA in a dose-dependent manner; the AUC values for the CON, EMC 0.1, and

Table 3. Effects of Oral EMC Administration on Blood OVA Concentrations and AUCs in Indomethacin-Administered Rats

Time (h)	Control	EMC	
		0.1 g/d	0.5 g/d
	(ng/ml)		
0	0.11 ± 0.01	0.31 ± 0.00	0.04 ± 0.00
0.5	2.8 ± 2.6	0.37 ± 0.00	0.04 ± 0.00
1	2.5 ± 2.3	0.54 ± 0.02	0.07 ± 0.03
2	0.37 ± 0.26	0.10 ± 0.07	0.04 ± 0.00
4	0.56 ± 0.43	0.06 ± 0.03	0.05 ± 0.01
8	1.2 ± 1.1	0.04 ± 0.01	0.04 ± 0.00
24	0.22 ± 0.16	0.03 ± 0.00	0.03 ± 0.01
	(ng·h/ml)		
AUC	19 ± 15	1.0 ± 0.3	0.9 ± 0.1

Results are expressed as mean ± SE, n = 3.

EMC 0.5 groups were 19 ± 15 , 1.0 ± 0.3 , and 0.9 ± 0.1 (ng·h/ml) respectively. Although there were no significant differences between the AUC for the CON group and those for the EMC groups, it was obvious that EMC inhibited OVA permeation *in vivo*. The inhibitory activity of EMC on OVA permeation was also confirmed by the result that the T_{max} (time for maximum concentration) in the EMC groups was delayed (the T_{max} values for the CON and EMC groups were 0.5 and 1 h respectively). However, it remains unclear whether there existed other peptides than NPWDQ with inhibitory activity on OVA permeation. Further study is needed to confirm that NPWDQ itself inhibits OVA permeation *in vivo*.

It is now accepted that enzymatic hydrolysis of casein releases peptides that exhibit various biological activities. For example, the most important casein-derived peptides appear to be phosphopeptides, which have the ability to sequester calcium and possibly other minerals, thus acting as biocarriers.²¹⁾ However, information on milk-derived peptides with inhibitory activities on allergen permeation is relatively poor. Here, we present novel *in vivo* data that indicate an inhibitory effect of EMC on allergen permeation. Increased permeability was involved in pathogenesis not only in food allergy but also in inflammatory bowel disease (IBD).^{22–24)} There exists the possibility that EMC and/or its peptides possessing the NPWDQ sequence can be applied practically in the prevention of these diseases.

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