

Comparative Study of *in Vitro* Digestibility of Food Proteins and Effect of Preheating on the Digestion

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Information on the comparative digestibility of food allergens and non-allergenic proteins is crucial when stability to digestion is to be used as a criterion to assess the allergenic potential of novel proteins. Preheating effect on *in vitro* digestibility has not been fully examined. In this study we investigated the preheating effect of *in vitro* digestibility of several proteins and their proteolytic fragments in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Five major food allergens, ovalbumin (OVA), ovomucoid (OVM), β -lactoglobulin (BLG), bovine serum albumin (BSA), soybean trypsin inhibitor (STI), four proteins of unproven allergenicity, horseradish peroxidase (HRP), ribulose-1,5-bisphosphate carboxylase/oxidase (RBC), phosphinothricin acetyltransferase (PAT) and zein from corn, and plant lectin, concanavalin A (Con A) were preheated (at 100 °C for 5 min) or not preheated, and then digested in SGF or SIF. Food allergens were relatively stable in both SGF and SIF. Among the allergens, digestibility of OVA in both SGF and SIF was markedly decreased, and BLG and STI were relatively stable after preheating. Digestibility of ConA in SGF and SIF was markedly decreased by preheating. Digestibility of non-allergenic proteins in SGF was higher than the allergenic proteins. From these results, because of the marked increase of the digestibility in several proteins by preheating, systematic information concerning the effect of food treatment on protein digestion is necessary to assess the relationship between allergenic potential and the digestibility of food protein.

Key words food allergen; digestion; preheating; simulated gastric fluid; simulated intestinal fluid

Food allergy is mediated by IgE antibodies to food components, usually proteins, and the digestion resistance of each protein is considered to be one of important parameters for its allergenic potential.^{1–4)} The safety of foods derived from genetically modified plants must be carefully assessed, especially in regard to food allergy.^{5–12)} The International Food Biotechnology Council and the International Life Sciences Institute jointly developed a step-wise approach to assess the safety of newly expressed proteins.¹²⁾ Their recommendations are based on similarities to known protein allergens and resistance to digestion of the proteins.

However, the digestibility of a protein measured by the *in vitro* assay in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) is greatly influenced by the assay conditions.^{13–15)} The ratio of pepsin (or pancreatin) to food proteins during the digestion process significantly affects the rate of digestion. Thus, evaluation of relative digestion resistance of various allergenic and non-allergenic proteins under standardized conditions is very useful to obtain the relationship between allergenic potential and digestibility.

In addition, the allergenic potential of food is altered by various manufacturing processes,¹⁶⁾ and also the digestibility of food proteins can be affected by food processing, for example, heating, chemical-treatment, exposure to high-pressure, fermentation, etc.

Since little is known about changes in the digestibility as a result of food processing, we also investigated the reduction in digestion resistance by preheating, the most popular processing method.

We tested five food allergens, ovalbumin (OVA), ovomucoid (OVM), β -lactoglobulin (BLG), bovine serum albumin (BSA), soybean trypsin inhibitor (STI). We also studied the digestibility of four food proteins, horseradish peroxidase (HRP), ribulose-1,5-bisphosphate carboxylase/oxidase (RBC), phosphinothricin acetyltransferase (PAT) and corn

storage protein, zein, whose allergenicity have not been reported. A plant lectin, concanavalin A (Con A) was investigated for reference.

MATERIALS AND METHODS

Materials Pepsin (catalog number P6887), pancreatin (catalog number P8096), and the test proteins were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) in the purest form available. The concentrations of all test proteins except PAT, RBC and zein were 5 mg/ml water for SGF and 2 mg/ml water for SIF. PAT and RBC were dissolved in 50 mM Tris-HCl (pH 9.5) and zein was dissolved in 10% dimethylsulfoxide (final concentration in the test solution was 0.5%). Gels and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Invitrogen (Carlsbad, CA, U.S.A.).

Preparation of SGF and SIF Pepsin (3.8 mg; approximately 13148 units of activity) was dissolved into the 5 ml gastric control solution (G-con; 2 mg/ml NaCl, pH adjusted to 2.0 with dilute HCl) and the activity of each newly prepared SGF solution was defined as production of a ΔA_{280} of 0.001 per min at pH 2.0 at 37 °C measured as trichloro-acetic acid (TCA) -soluble products with hemoglobin as substrate. SIF was prepared as described in the United States Pharmacopoeia, 24th edition, p2236, and 10 mg/ml pancreatin was dissolved in intestinal control solution (I-con; 0.05 M KH_2SO_4 , pH 6.8). Both solutions were used within the same day.

Incubation in SGF or SIF SGF (1520 μl) was incubated at 37 °C for 2 min before addition of 80 μl of test protein solution (5 mg/ml) at time zero. At each scheduled time point (0.5, 2, 5, 10, 20, 30, 60 min), 200 μl of the reaction mixture was removed and added to a separate sampling tube containing 70 μl 5 $\times$ Laemmli buffer (40% glycerol, 5% 2-

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mercapto-ethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8) and 70 μ l 200 mM Na_2CO_3 . In the zero-point samples, test protein solution (10 μ l) was added to neutralize SGF (190 μ l SGF, 70 μ l 5 $\times$ Laemmli buffer, and 70 μ l 200 mM Na_2CO_3). All neutralized samples were boiled at 100°C for 3 min and subjected to SDS-PAGE gel. For the digestion study in SIF, SIF (1710 μ l) was incubated at 37°C for 2 min before addition of the 90 μ l of test protein solution (2 mg/ml) at time zero. At the scheduled time points (2, 5, 10, 20, 30, 60, 90, 120 min), 200 μ l of the reaction mixture was removed, and immediately after addition to a separate sampling tube containing 50 μ l 5 $\times$ Laemmli buffer, the tube was boiled at 100°C for 5 min, and the contents were subjected to SDS-PAGE gel. For the 0 min samples, test protein solution (10 μ l) was added to 190 μ l of SIF heat-inactivated at 100°C for 5 min and 50 μ l 5 $\times$ Laemmli buffer, and the solution was boiled at 100°C for 5 min.

Table 1. Digestibility in SGF

Allergenic proteins

Protein		Appearance (min) ^{a)}	Max. intensity (min) ^{b)}	End point (min) ^{c)}	Score ^{d)}
OVA	O (45.9 kDa) ^{e)}	NA ^{f)}	NA	60	E
	F1 (42.1 kDa) ^{g)}	2	60	60	
	F2 (4.1 kDa)	5	unclear	30	
OVM	O (35–46 kDa)	NA	NA	0–0.5	B
	F (7.0 kDa)	0.5	2	30	
BLG	O (17.7 kDa)	NA	NA	60	E
BSA	O (63.1 kDa)	NA	NA	0–0.5	B
	F1 (7.6 kDa)	0.5	0.5	10	
	F2 (5.9 kDa)	0.5	0.5	20	
	F3 (3.9 kDa)	0.5	0.5	20–60	
STI	O (20.0 kDa)	NA	NA	60	E

Non allergenic proteins

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
HRP	O (48.8 kDa)	NA	NA	0.5	A
RBC	O (52.0 kDa)	NA	NA	0	A
PAT	O (22.3 kDa)	NA	NA	0	A
zein	O (19.5 kDa)	NA	NA	0	A

Plant mitogen

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
Con A	O1 (27.9 kDa)	NA	NA	60	E
	O2 (14.7 kDa)	NA	NA	20	
	O3 (10.2 kDa)	NA	NA	20	
	F1 (22.3 kDa)	0.5	20	60	
	F2 (3.9 kDa)	5	30	60	

a) The first time point when the fragment band was detected. b) Time point when the band intensity was maximum. c) The last time point when the band was observed. d) Digestion Score: A. Rapidly digested (less than 2 min), B. Rapid with fragment, C. Intermediately labile, D. Intermediately labile with fragment, E. Stable. e) O: Originally detected protein. f) NA: Not applicable. g) F: Fragment. OVA: ovalbumin, OVM: ovomucoid, BLG: β -lactoglobulin, BSA: bovine serum albumin, STI: soybean trypsin inhibitor, HRP: horseradish peroxidase, RBC: ribulose-1,5-bisphosphate carboxylase/oxidase, PAT: phosphothricin acetyltransferase, Con A: concanavalin A.

SDS-PAGE Analysis and Staining Procedure Samples were loaded on 10–20% polyacrylamide Tris/Tricine gel at 15 μ l/lane and electrically separated. The gels were fixed for 5 min in 5% TCA, washed for 2 h with SDS Wash (45.5% methanol, 9% acetic acid), stained for 10 min with CBB staining solution (0.1% Coomassie Brilliant blue R, 15% methanol, 10% acetic acid), and destained with 25% methanol and 7.5% acetic acid. The stained gel images were analyzed by using Image Gauge V3.1 (Fuji Film, Tokyo, Japan) and the density of each band was quantified. Western blotting was performed as described previously¹⁵⁾ with laboratory-prepared mouse anti-OVA antiserum.

RESULTS

Digestion Patterns in SGF and Effect of Preheating

As the minimal molecular mass to elicit immunogenicity and allergenicity appeared to be 3 kDa,¹⁷⁾ we monitored the time-course digestibility of whole protein and the fragments which molecular weight were more than 3 kDa.

Table 1 summarizes the digestibility in SGF of five proteins that are known allergens, three of non-allergenic proteins and one plant lectin. Table 2 shows the effect of preheating on the digestibility of these proteins. Fig. 1A shows the results of SDS-PAGE analysis of the OVA degradation products in SGF as a typical digestion pattern, and Fig. 1B shows the time course of digestion determined by densitometry of the stained bands. Original OVA (45.9 kDa) was gradually decreased with formation of digested fragments, but both the original protein and the main fragment (40.5 kDa) persisted until the end of the study (60 min). Preheating, however, significantly accelerated OVA digestion (Fig. 1C), with the original band rapidly disappearing within 0.5 min and the fragments disappearing within 2 min. A similar effect

Table 2. Digestibility of Heat-Treated Proteins in SGF

Allergenic proteins

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
OVA	O (45.9 kDa)	NA	NA	0	B
	F1 (6.3 kDa)	NA	NA	0.5	
	F2 (3.8 kDa)	NA	NA	0.5	
OVM	O (35–46 kDa)	NA	NA	0–0.5	A
BLG	O (17.7 kDa)	NA	NA	60	E
BSA	O (63.1 kDa)	NA	NA	0–0.5	B
	F1 (7.6 kDa)	0.5	0.5	0.5	
	F2 (3.9 kDa)	0.5	0.5	2	
STI	O (20.0 kDa)	NA	NA	60	E

Plant lectin

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
Con A	O1 (27.9 kDa)	NA	NA	0	B
	O2 (14.7 kDa)	NA	NA	0.5	
	O3 (10.2 kDa)	NA	NA	0.5	
	F1 (6.8 kDa)	0.5	0.5	5	
	F2 (5.1 kDa)	0.5	0.5	2	
	F3 (3.9 kDa)	0.5	0.5	5–10	

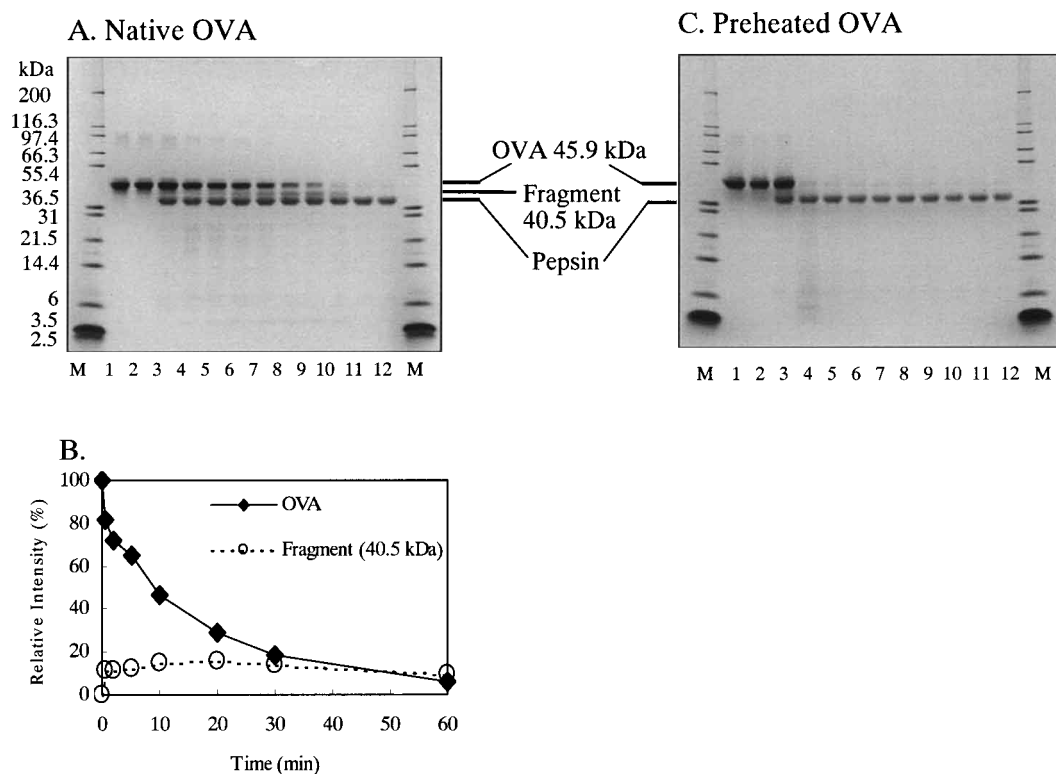


Fig. 1. SGF-Digestion Pattern of OVA

SDS-PAGE analysis of the SGF digestion of native (A) and preheated (C) OVA, and quantification of the density of bands in panel A (B). Lanes M: molecular weight markers; Lanes 1 and 2: OVA controls at $t=0$ and 60 min; Lanes 3—10: SGF-digestion pattern of OVA at $t=0, 0.5, 2, 5, 10, 20, 30$ and 60 min; Lanes 11 and 12: SGF controls at $t=0$ and 60. "Relative intensity" of each band means the ratio of the density of the band to the density of the OVA band at $t=0$ (Lane 3). Values are the mean of the duplicate analysis. Similar results were observed in another experiments.

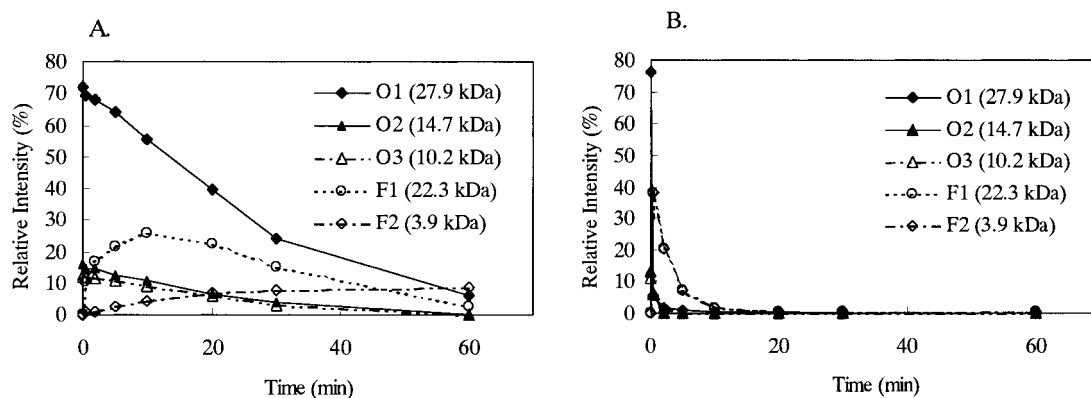


Fig. 2. Quantification of the SGF-Digestion Pattern of Native (A) and Preheated (B) Con A

"Relative intensity" of each band means the ratio of the density of the band to the total density of the originally detected bands at $t=0$. O: original band. F: fragment band. Values are the mean of the duplicate analysis. Similar results were observed in another experiments.

of preheating was observed in regard to Con A digestion (Figs. 2A, B). As a result of SDS-PAGE, Con A sample used was separated into three bands, which seemed to correspond to the primary gene product (MW 27.9 kDa) and two naturally occurring fragments (MW 14.7 kDa and 10.2 kDa)^{18,19}. They were gradually digested in SGF and formed several fragments during the digestion test (Fig. 2A). However, all of the original bands and fragments of preheated Con A almost completely disappeared within 10 min in SGF (Fig. 2B).

BLG and STI decreased very slowly, and no fragments were observed. After heating, the proteins decreased during the initial 0.5 min of the incubation period, but some of the proteins were persisted until final time point (60 min).

Since the pepsin band (approx. 39.0 kDa) interfered with a broad band corresponding to highly glycosylated OVM (35—46 kDa) under the SDS-PAGE conditions, quantification of OVM and its fragment was difficult (Fig. 3).

BSA was also digested very rapidly, and some fragments were produced (Table 1). The most stable fragment (3.9 kDa) was observed until the final time point in the experiment (60 min). After preheating, all fragments digested more rapidly and the 3.9 kDa-fragment was disappeared within 5 min (Table 2).

The allergenicity of HRP, RBC, PAT and zein has not been reported, and they were all rapidly digested without fragments (Table 1).

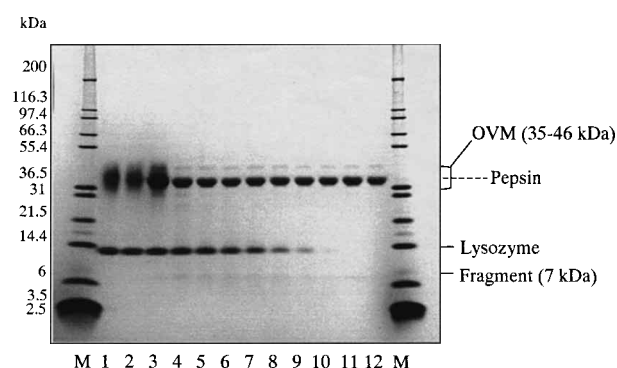


Fig. 3. SDS-PAGE Analysis of the SGF Digestion of Native OVM

Lanes M: molecular weight markers; Lanes 1 and 2: OVA controls at $t=0$ and 60 min; Lanes 3—10: SGF-digestion pattern of OVA at $t=0$, 0.5, 2, 5, 10, 20, 30 and 60 min; Lanes 11 and 12: SGF controls at $t=0$ and 60. Lysozyme (14 kDa) was contaminated in the used OVM standard. Two experiments were performed and similar results were observed in another experiments.

Table 3. Digestibility in SIF

Allergenic proteins

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
OVA	O (45.9 kDa)	NA	NA	120	E
	F (40.5 kDa)	2	2	120	
OVM	O (35—46 kDa)	NA	NA	60	C
	O (17.7 kDa)	NA	NA	0—2	
BLG	F (10.2 kDa)	2	2	2	B
	O (63.1 kDa)	NA	NA	120	
BSA	F1 (53.0 kDa)	2	90—120	120	E
	F2 (45.0 kDa)	2	2	120	
	F3 (16.8 kDa)	2	2	120	
	F4 (6.0 kDa)	2	60—120	120	
STI	O (20.0 kDa)	NA	NA	120	E

Non allergenic proteins

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
PAT	O (22.3 kDa)	NA	NA	0	A
	O (19.5 kDa)	NA	NA	0	

Plant lectin

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
Con A	O1 (27.9 kDa)	NA	NA	120	E
	O2 (14.7 kDa)	NA	NA	5	
	O3 (10.2 kDa)	NA	NA	5	
	F (11.0 kDa)	2	2	5	

Digestion Patterns in SIF and Effect of Preheating

The results of digestion of five allergenic proteins and plant lectin in SIF are shown in Table 3, and the results for preheated proteins are shown in Table 4. Figure 3A shows the results of SDS-PAGE analysis of the OVA digestion products in SIF. Pancreatin consists of many enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. As a result, many bands corresponding to these enzymes were observed

Table 4. Digestibility of Heat-Treated Proteins in SIF

Allergenic proteins

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
OVA	O (45.9 kDa)	NA	NA	0	A
OVM	O (35—46 kDa)	NA	NA	30	C
BSA	O (63.1 kDa)	NA	NA	120	E
	F1 (53.0 kDa)	2	10—20	120	
	F2 (45.0 kDa)	2	2	60	
STI	O (20.0 kDa)	NA	NA	120	E

Plant lectin

Protein		Appearance (min)	Max. intensity (min)	End point (min)	Score
Con A	O1 (27.9 kDa)	NA	NA	0	B
	O2 (14.7 kDa)	NA	NA	0	
	O3 (10.2 kDa)	NA	NA	0	
	F (3.9 kDa)	2	2	5—10	

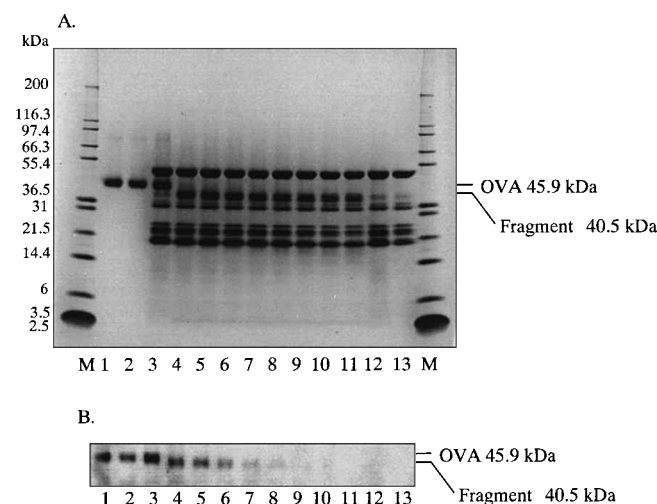


Fig. 4. SDS-PAGE Analysis of the SIF Digestion of Native OVA (A) and Western Blotting with Mouse Anti-OVA Antiserum (B)

Lanes M: molecular weight markers; Lanes 1 and 2: OVA controls at $t=0$ and 120 min; Lanes 3—11: SIF-digestion pattern of OVA at $t=0$, 2, 5, 10, 20, 30, 60, 90 and 120 min; Lanes 12 and 13: SIF controls at $t=0$ and 120. Two experiments were performed and similar results were observed in another experiments.

in the SDS-PAGE gel loaded with SIF-digested proteins, making it difficult to determine the digestibility of some proteins.

The original band of OVA rapidly decreased, but a small amount of the original band and a fragment band (40.5 kDa) persisted until the final time point (120 min). Since the OVA-derived fragment coeluted with a weak band of pancreatin components, we confirmed the OVA digestion pattern by Western blotting with a mouse anti-OVA antiserum (Fig. 3B). Further, as shown in Table 4, preheating markedly increased digestibility of OVA, and the preheated OVA disappeared within 2 min without any fragments.

BLG was stable in SGF, but was easily digested in SIF. By contrast, BSA and OVM, relatively labile in SGF, resisted SIF-digestion and preheating had no effect on BSA stability.

STI, stable in SGF was also very stable in SIF and preheating had no effect on the digestion of STI in SIF.

The digestion pattern of Con A in SIF was similar to the pattern in SGF. It was digested gradually and detected until the final time point, and it yielded a fragment, that rapidly disappeared. Preheating greatly accelerated Con A digestion in SIF.

DISCUSSION

Besler *et al.* reviewed the stability of the allergenicity of processed foods, whose allergenic activity was altered by several processing procedures.¹⁶⁾ Since food containing allergenic proteins is usually edible after treatment, the digestibility of the proteins after treatment should be assessed, otherwise the relationship between protein digestibility and the contribution of the protein to food hypersensitivity may be unclear.

We have previously described that newly expressed proteins Cry 1Ab in GM-corns and 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) in GM-soybeans were easily digested in SGF but resistant to SIF-digestion, and preheating dramatically increased the SIF-digestibility of them.¹⁵⁾ Since people usually eat corns and soybeans after heating, our findings about the increase of their digestibility were very important to assessment their allergenic potential.

The preheating condition, at 100°C for 5 min, is usually used for pretreatment of canned corn and other treated foods. And a remarkable reduction in allergenicity of milk can be observed after boiling for 10 min, while boiling for 2 and 5 min induced no significant change in skin prick test and dot immunoblotting experiments.¹⁶⁾ Therefore, we used the preheating condition, at 100°C for 5 min, which does not cause the complete disappearance of allergic potential of major allergens.

In our present experiments, preheating increased the digestibility of some proteins in SGF and SIF, and the digestion pattern, including fragment formation, significantly changed. Preheating markedly accelerated the *in vitro* digestibility of OVA and Con A both in SGF and in SIF (Figs. 1, 2), however, did not affect their mobility in SDS-PAGE which suggested that the conformation of the OVA and Con A molecules was changed by preheating that resulted in easy digestion by pepsin, trypsin, or other proteases. However, the digestibility of some proteins was unaffected by preheating.

Food allergens are considered to be stable following various digestive treatments,⁴⁾ and some major allergens have been reported to be highly resistant to pepsin digestion. And it has been reported that when the ratio is changed, the digestion patterns and fragment formation of some proteins dramatically altered.^{13,14)} The pepsin/protein ratio we used was 10 unit/ μ g protein. Under the condition, OVM disappeared within 0–0.5 min (Table 1), which was delayed to 5–10 min when the ratio was changed to 1 unit/ μ g protein, and at 0.1 unit/ μ g protein, OVM persisted until the end of the study (60 min, data not shown).

And some proteolytic fragments retain the allergenic po-

tential the same as the original proteins.^{20,21)} Thus, even if proteins were rapidly digested, their proteolytic fragments could cause allergy. For example, Matsuda *et al.* reported that peptic digestion did not completely destroy OVM antigenic sites, and that antigenic fragments were produced.²⁰⁾ A weak fragment around 7 kDa was observed under the SGF-digestion condition, but it was too weak to determine quantitatively (Fig. 3), and the 7 kDa-fragment was not observed in SGF-digestion of preheated OVM.

We investigated OVM-specific IgE in egg white allergy patients' sera and detected it in 16 of 18 serum samples, and 13% of OVM-positive sera reacted with OVM-derived 7 kDa-fragment by Western blotting (unpublished data).

Further study of digestibility with consideration for the ratios of the enzymes to the test proteins, fragment formation, and the allergenicity of the fragments may elucidate the relationship between protein digestibility and allergenicity.

Systematic information concerning the effect of food treatment on protein digestion is necessary to assess the relationship between allergenic potential and the digestibility of food protein.

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