研究成果の刊行に関する一覧表

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Fragmentation of DNAs of Processed Foods Made from Genetically Modified Soybeans

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Abstract

We studied the degradation of DNA molecules of soybean during various processes of food production. The validity of quantification of a recombinant DNA in "tofu" was confirmed using the processed food made from a mixture of non-genetically modified (GM) and pesticide-resistant (PR) soybeans. The quantitative PCR method is useful for determining the amount of PR soybeans in the foods processed by treatments at about 100℃. On the other hand, severe DNA degradation was occurred in the some kind of foods processed such as deep roast and fermentation, in which the length of DNA fragment was shorter than almost 95 bp. Therefore, in order to estimate PR soybean content in these processed foods, new probes and primers for PCR amplifying shorter than 100 bp of the DNA fragment for the target DNA will be needed.

Key words: genetically modified soybean, processed food, fragmentation, quantitative PCR, Tofu

I. Introduction

Recently, many genetically modified (GM) crops have been developed, the safety of which has been assessed by government and national organizations. Regulations on GM crops have been discussed and legislated in various countries, and in Japan the safety assessment for GM crops has become obligatory and a new labeling system for processed foods containing GM crops has been in force since April 2000. Labeling such foods as "genetically modified" or "not segregated from GM product" has been mandatory in the cases of GM soybeans and corns when they make up more than 5% of the total weight of a food product and is one of the top three components by weight. Therefore, GM food guidelines for a quantitative detection method were announced by the Japanese Ministry of Health, Labor and Welfare (MHLW) and the Japanese Ministry of Agricultural Forestry and Fishers (MAFF) in March 2001. It is important that recombinant DNA extracted from food products made using GM crops should be not only qualitatively but also quantitatively detected using reliable methods to verify the labeling. However, only GM crop grains can be quantitatively determined using the detection methods under the present circumstances. Polymerase chain reaction (PCR) methods used to detect recombinant DNA in foods containing GM crops have been reported worldwide to be reliable, and have been tested on crops such as soybean [1)-7)], maize [8)-14)], potato [15)-17)] and papaya [18)]. There have been some reports about the purification of DNA and a method for detecting recombinant DNA in processed soyfoods [3)-7)]. However, the way in which DNA molecules of soybeans are qualitatively decayed during various kinds of food processing has not been investigated. There are many kinds of foods made from soybeans in Japan and they are processed using various manufacturing methods. In this paper, we examined the effect of the process of food production on the degradation of DNA in soybean. The validity of quantification of the recombinant DNA in "tofu", which is an extremely conventional and food familiar to the Japanese public, was confirmed in the processing of "tofu" using various mix ratios of non-GM and PR soybeans.

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II. Materials and Methods

1. Soybeans

Seeds of Roundup Ready Soybean, which is a pesticide-resistant (PR) soybean, imported from the USA were provided by the Ministry of Health, Labor and Welfare (MHLW) in Japan, who also provided the non-GM soybeans.

2. Processed soybean foods on the market

The soy-foods tested here were purchased from markets. They were "tofu", "okara", "tohnyu", "yuba" (the solid ingredient formed on the surface of boiling "tohnyu"), canned whole soybean, packed "tofu", "koori-tofu" (dried "tofu"), "edamame" (boiled soybean with salts), "abura-age" ("tofu" mildly fried in vegetable oil), "iridaizu" (roasted soybean), "kinako" (roasted soybean subjected to a powdering process), "konbu-mame" (boiled and sterilized soybean with some seasonings and seaweed), "miso", "natto", and soy sauce (fermented and processed soybean). Both processed and fermented foods were used in this study.

3. Preparation of handmade tofu from soybeans

PR soybean and non-GM soybean seeds were mixed to be contents of 1%, 5%, 25%, 50%, 75% and 100% (w/w) of PR soybean seeds. The seeds (20 g) were well washed in tap water and soaked in sterilized distilled water (60 ml) overnight. The water was discarded and the soybeans pulverized using a blender in 40 ml of sterilized distilled water and then filtered through cotton gauze. In Japan, the filtrate and the solid ingredient left on the gauze are called "tohnyu" and "okara", respectively. The "tohnyu" was heated to just below boiling, cooled to about 80℃ and then "nigari", which is composed of some salts such as magnesium sulfate, was poured into molds. Following this, the "tohnyu" was cooled down in water and solidified to "tofu".

4. Extraction of DNA from soybeans and foods made from soybeans

To extract the DNAs from samples, we used the official method in the guidelines determined by the MHLW in Japan. DNAs were extracted from soybean seeds and hand-made processed foods as "tofu" using a DNeasy Plant Mini Kit (QIAGEN). G-tip (QIAGEN) were used in the other processed foods. The concentration of the DNA in the extracts was measured using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, INC.).

5. PCR amplification of DNAs using primers

In order to design the primer sequences to give several lengths of DNA fragments amplified from the introduced gene, a DNA fragment was amplified by PCR with the primers 5'-CCTTCGCAAGACCCTTCCTGTATA-3' and 5'-ATCCTGGCGCCATGGCCTGCATG-3', published by Yamaguchi et al.[5], using the DNA prepared from PR soybeans as a template. The reaction mixture of 50 μL consisted of 5.0 μL of 10 x reaction buffer, 2.0 μL (50 ng) of the template DNA, 0.25 nmol/L of dNTPs, 0.25 μ mol/L of the primers and one unit of *Taq* DNA polymerase (TaKaRa Biochemicals, Japan). The PCR proceeded using the GeneAmp PCR System 9600 (Applied Biosystems, Japan) as follows: 94℃ for 2 minutes, 40 cycles of denaturing at 94℃ for 30 seconds, annealing at 58℃ for 1 minute and extension at 72℃ for 1 minute. These conditions were also used for qualitative PCR. The amplified DNA fragment was cloned into Bluescript SK+ and the nucleotide sequence of the double strands determined using a LICOR-4000 L DNA sequencer (LI-COR, Inc.). The determined nucleotide sequence of the introduced gene (designated "*epsps*" below) consisted of the cauliflower mosaic virus 35S promoter, the chlorophyll transit peptide sequence from *Petunia hybrida*, and the glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. We designed the primer pairs expected to amplify the DNA fragments of various lengths derived from the introduced gene of the PR soybean as shown in Table 1. The amplified DNA fragment using the primer sets 5'-GGCTGATAACACACTCTATTATTGT-3' and 5'-TGATGGATCTGATAGAATTGACGTT-3', derived from the *lectin 1* gene published by Yamaguchi et al.[5], were subcloned, and the nucleotide sequence determined as above. Then, we also designed the primer sets required to give the amplified DNA fragments of various lengths as shown in Table 2.

6. Quantification of PR soybean contents in the various samples

To quantify the amount of PR soybean in the samples, we used the official method in the guidelines determined by the MHLW and the MAFF in Japan. The number of gene copies was measured by this quantitative PCR method based on the principle of Taqman chemistry and using the control plasmid. This plasmid contained both sequences of genetically modified gene and the endogenous DNA of soybean and used as reference materials for this method[19]. The total volume of the reaction mixture was 25 μL, in which the 2×TaqMan Universal PCR Master Mix was 12.5 μL, the template DNA was 2.0 μL (50 ng), the primer was

Table 1. PCR primer list for the *epsps* gene

Primer name	Sequence	Length of amplified DNA fragment (bp)
101F	5'-GAAGTTCATTTCATTTGGAGAGGAC-3'	101
101R	5'-AGGGTTTGTATCCCTTGTGCC-3'	
201F	5'-TATAAGGAAGTTCATTTCATTTGGAGAG-3'	201
201R	5'-AGAATTTGCTGAATTTTTCAGTTTTTTA-3'	
402F	5'-TCATTTCATTTGGAGAGGACACG-3'	402
402R	5'-TCAAAGAACCTCTGGGTCCAAG-3'	
513F	5'-CCTTCGCAAGACCCTTCCTCTATA-3'	513
513R	5'-ATCCTGGCGCCCATGGCCTGCATG-3'	

Table 2. PCR primer list for the *lectin 1* gene

Primer name	Sequence	Length of amplified DNA fragment (bp)
95F	5'-CCTCTCCCGATGTGGTCGATT-3'	95
95R	5'-TGCGATTCCCCAGGTATGTC-3'	
195F	5'-TGACTCCCCATGCATCACAG-3'	195
195R	5'-GATCATGTTTGGTTGCTTCGG-3'	
295F	5'-GCTATTGTGACCTCCTCGGG-3'	295
295R	5'-GCGACGACTTGATCACCAGAC-3'	
395F	5'-ACCAGCAAGGCAAACTCAGC-3'	395
395R	5'-GTCAAACTCAACAGCGACGACT-3'	
495F	5'-CCGCTTCCTTCAACTTCACC-3'	495
495R	5'-CTAGCGTGTGGCAAATTGGA-3'	
595F	5'-AAGGCAAACTCAGCGGAAC-3'	595
595R	5'-TCGACCACATCGGAGAGGAT-3'	

0.5 μ mol/L and the probe was 0.2 μ mol/L. The PCR proceeded as follows: 50℃ for 2 minutes, 95℃ for 10 minutes, 40 cycles of denaturation at 95℃ for 30 seconds, and annealing and extension at 59℃ for 1 minute, using a 7700 Sequence Detector (Applied Biosystems, Japan).

III. Results and Discussion

1. Quantification of PR soybean contents in processed foods using TaqMan PCR

As a model experiment to determine the content of PR soybeans in the processed soybean foods using the quantitative PCR method, the PR soybean seeds were mixed in proportions of 1%, 5%, 50% and 100% with non-GM soybean seeds, and then "tofu", "okara" and "tohnyu" were prepared. Because the PR soybean was developed from a soybean cultivar that produced soybean oil and was not suitable for producing "tofu", the "tofu" produced here using 100% PR soybean seeds did not solidify and was likely to produce "kumi-dofu" (soft "tofu"). Fragmentation of genomic DNA has not obviously occurred at the level of affecting the results of qualitative PCR we used (Fig. 1).

Before performing quantitation of PR soybean in processed food, we quantified processed ingredient powder mixed at a weight ratio specifying PR and non-GM soybean. As shown in Fig. 2, good correlation was found by comparing the weight ratio between obtained quantitative value and mixed one. The PR soybean seeds using this method for the standard curve were contents of 1%, 5%, 25%, 50%, 75% and 100%. Quantitative PCR was conducted on various processed foods and each quantitative value was obtained. When the quantitative value in "tofu", "okara" and "tohnyu" was compared with the weight ratio, 20% of bias was obtained (Table 3). These results indicated that the quantitative PCR method is adequate in determining the content of PR soybeans in processed foods. Recently, Kakihara et al[7]. reported the detection of recombinant DNA from commercial tofu and they estimated their PR soybean contents by the same quantita-

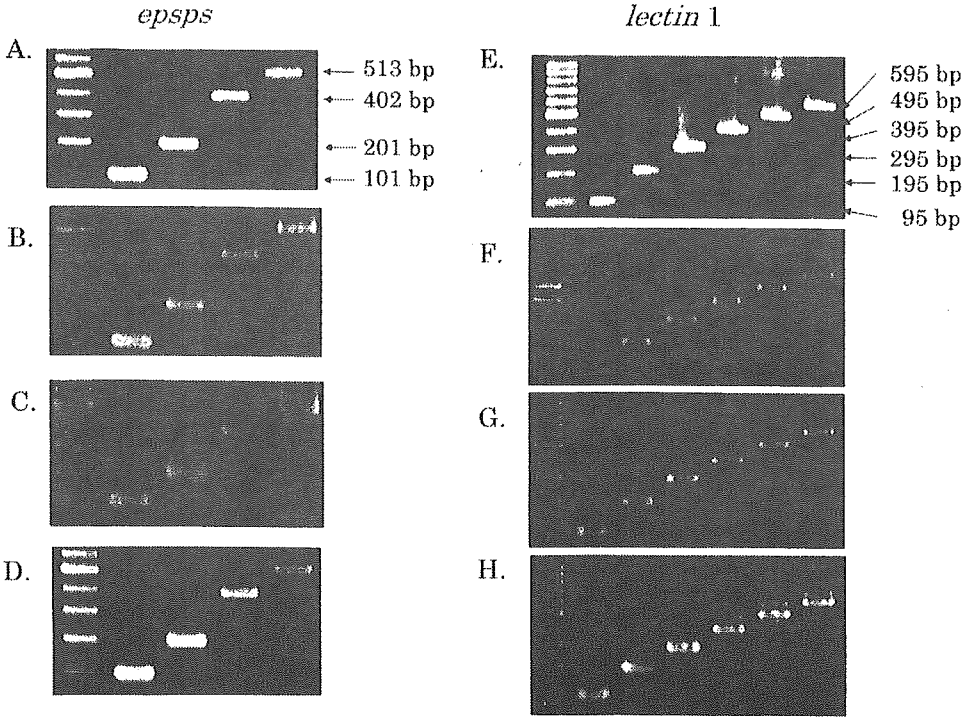

Fig. 1. Agarose gel electrophoresis of the amplified products by PCR using DNAs extracted from soybeans (non-treated) (A and E), hand made "tofu" (B and F), hand made "okara" (C and J) and hand made "tohnyu" (D and H), with *epsps* (A to D) and *lectin1* gene (E to H) as the target genes.

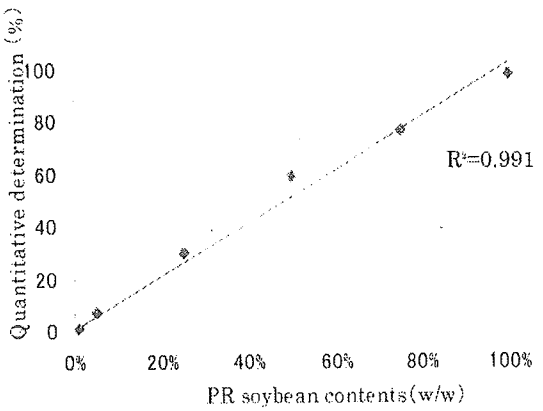

Fig. 2. Analytical curve of quantification of PR soybean contents in processed foods by quantitative PCR.

Table 3. Quantification of PR soybean contents in processed foods using quantitative PCR method

PR soybean contents (w/w) / Items	1%	5%	50%	100%
soybean (non-treated)	0.80	6.6	58	98
tofu	0.84	3.8	50	112
okara	0.66	4.6	50	111
tohnyu	0.60	4.2	49	105

tive PCR method as that using in this report. But, they did not mention whether the method is adequate for the estimation of GM contents in processed foods.

2. Fragmentation of DNA from processed soybean foods on the market

Representative PCR profiles of the DNAs prepared from the processed foods made from the soybeans are shown in Fig. 3. Amplified bands up to 595 bp were observed in "tofu", while canned soybeans and "kinako" only gave bands up to 195 bp and 95 bp, respectively. In foods prepared by fermentation, only "miso" gave the band of 95 bp. All results are summarized in Table 4. The soybean genomic DNAs prepared from the 7 foods (okara, tohnyu, yuba, tofu, packed tofu, koori-dofu, and edamame) processed at just below boiling (about 100℃) were amplified, even at lengths of 595 bp. This suggested that boiling process did not cause serious damage to the DNA for PCR amplification.

"Iridaizu" was roasted soybeans and "kinako" was made by pulverizing roasted soybeans. The amplicon from the sample of "iridaizu" was detected even when the primer pair of 595F and 595R were used. But a DNA fragment up to only 195 bp from "kinako" was amplified. In addition, the intensity of the DNA fragment of 195 bp from "kinako" were very weak. This discrepancy might be explained as follows. Soybean seeds for "kinako" were pulverized to be fine powders and roasted more deeply than "iridaizu" in order that heat penetrate into the center of the seeds. Therefore, severe fragmentation of DNAs was occurred by deep roast, high temperature treatment with long time, in soybean seeds for "kinako". Furthermore, no amplicon were observed at lengths longer than 195 bp in "konbu-mame" and canned soybean. These were cooked by autoclave at temperatures of about 121℃, which were lower temperature than that for roasting. As a control experiment, soybean seeds were autoclaved, and the genomic DNA prepared from them gave amplified DNA fragments shorter than 195 bp. These results indicated that both high temperature and high pressure promote the degradation and fragmentation of soybean genomic DNA.

In the case of "miso", DNA fragments longer than 195 bp were not amplified. Furthermore, DNA fragments from "natto" and

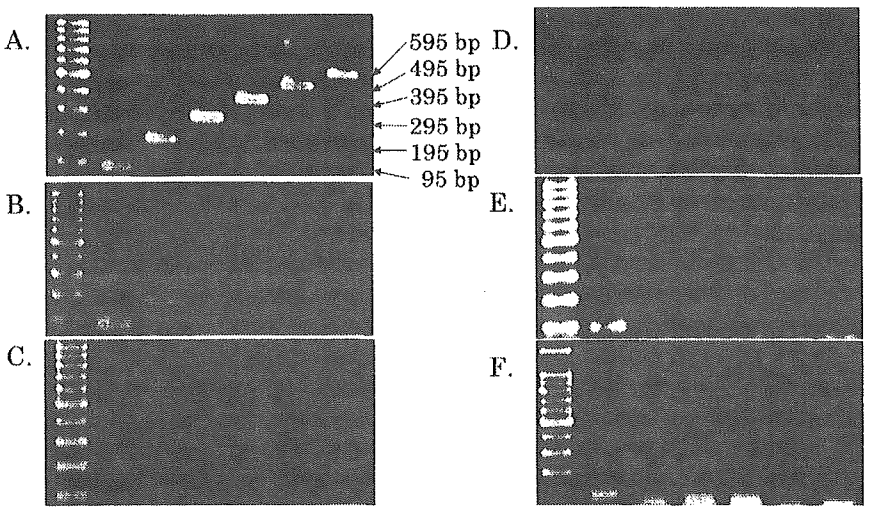

Fig. 3. Agarose gel electrophoresis of the PCR products using *lectin 1* primers derived from DNAs extracted from soybeans and major foods made from soybeans on the market. A, soybean (untreated); B, canned soybean; C, soy sauce; D, "tofu"; E, "kinako"; F, "miso".

Table 4. Fragmentation of the DNA extracted from various processed foods on the market made from soybeans

Treatment	Items	Fragment length of DNA amplified by PCR (bp)					
		95	195	295	395	495	595
control	soybean (non-treated)	A	A	A	A	A	A
	soybean (121℃, 15min)	A	A	N	N	N	N
boiled	okara	A	A	A	A	A	A
	tohnyu	A	A	A	A	A	A
	yuba	A	A	A	A	A	A
	tofu	A	A	A	A	A	A
	packed tofu	A	A	A	A	A	A
	koori-tofu	A	A	A	A	A	A
	edamame	A	A	A	A	A	A
fried	abura-age	A	A	A	A	A	A
roasted	iridaizu	A	A	A	A	A	A
	kinako	A	A	N	N	N	N
autoclaved	konbu-mame	A	A	N	N	N	N
	canned soybean	A	A	N	N	N	N
fermented	miso	A	N	N	N	N	N
	natto	N	N	N	N	N	N
	soy sauce	N	N	N	N	N	N

A: amplified
N: non-amplified

soy sauce were not amplified even when the primer pairs of 95F and 95R were used. During the fermentation process, the soybean DNAs might have been digested by DNase derived from microorganisms.

Our results indicate that the quantitative PCR method is reliable and can be used to determine the content of PR soybeans in foods processed by heating at about 100℃. However, deep roast, autoclaving and fermentation processes cause to severe degradation or fragmentation of the DNA in foods. The official quantitative method using the TaqMan method noticed by the MHLW in Japan is to use primer pairs producing 118bp amplicons of *Lectin 1* control sequence and 121bp amplicons for the specific sequence of PR soybean. Amplification of DNA in some processed food was not found in our study using primer set which amplifies the shortest length of 95 bp. In conclusion, it was suggested that DNA did not remain for obtaining precise quantitative value. Therefore, considering our results, the official method might not be applicable for these foods made with deep roasting, autoclaving and/or fermentation processes unless new primers amplifying shorter than 95 bp of the DNA fragment for the target DNA will be designed.

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論　　文

遺伝子組換え大豆から製造された加工食品におけるDNA断片化

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概　要

さまざまな大豆加工食品における組換え遺伝子の含量とDNA断片化の程度を調べることにより、厚生労働省により通知された公定法記載の定量PCR法の加工食品への適用可能性について検証した。豆腐のように100℃程度で加熱加工された場合は、適用できることがわかった。しかし、市販されている加工食品のように加熱され、物理的力が更にかかるオートクレーブ処理などが行われた場合、あるいは発酵食品の場合、100bp程度までDNAの厳しい断片化が引き起こされることが明らかとなった。従って、これらの加工食品中の遺伝子組換え大豆の定量を行うには95bpより短いプライマー、プローブを開発する必要があると考えられた。

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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 ΔA280 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× 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 μl 200 mM Na_2CO_3. In the zero-point samples, test protein solution (10 μl) was added to neutralize SGF (190 μl SGF, 70 μl 5× Laemmli buffer, and 70 μl 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 μl) was incubated at 37°C for 2 min before addition of the 90 μl 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 μl of the reaction mixture was removed, and immediately after addition to a separate sampling tube containing 50 μl 5× 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 μl) was added to 190 μl of SIF heat-inactivated at 100°C for 5 min and 50 μl 5× 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	
	F1 (42.1 kDa)[g]	2	60	60	E
	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 kD)	NA	NA	60	E
BSA	O (63.1 kDa)	NA	NA	0—0.5	
	F1 (7.6 kDa)	0.5	0.5	10	B
	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	
	O2 (14.7 kDa)	NA	NA	20	
	O3 (10.2 kDa)	NA	NA	20	E
	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: phosphinothricin acetyltransferase, Con A: concanavalin A.

SDS-PAGE Analysis and Staining Procedure Samples were loaded on 10—20% polyacrylamide Tris/Tricine gel at 15 μl/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 previously[15] 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	
	F1 (6.3 kDa)	NA	NA	0.5	B
	F2 (3.8 kDa)	NA	NA	0.5	
OVM	O (35—46 kDa)	NA	NA	0—0.5	A
BLG	O (17.7 kD)	NA	NA	60	E
BSA	O (63.1 kDa)	NA	NA	0—0.5	
	F1 (7.6 kDa)	0.5	0.5	0.5	B
	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	
	O2 (14.7 kDa)	NA	NA	0.5	
	O3 (10.2 kDa)	NA	NA	0.5	B
	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	

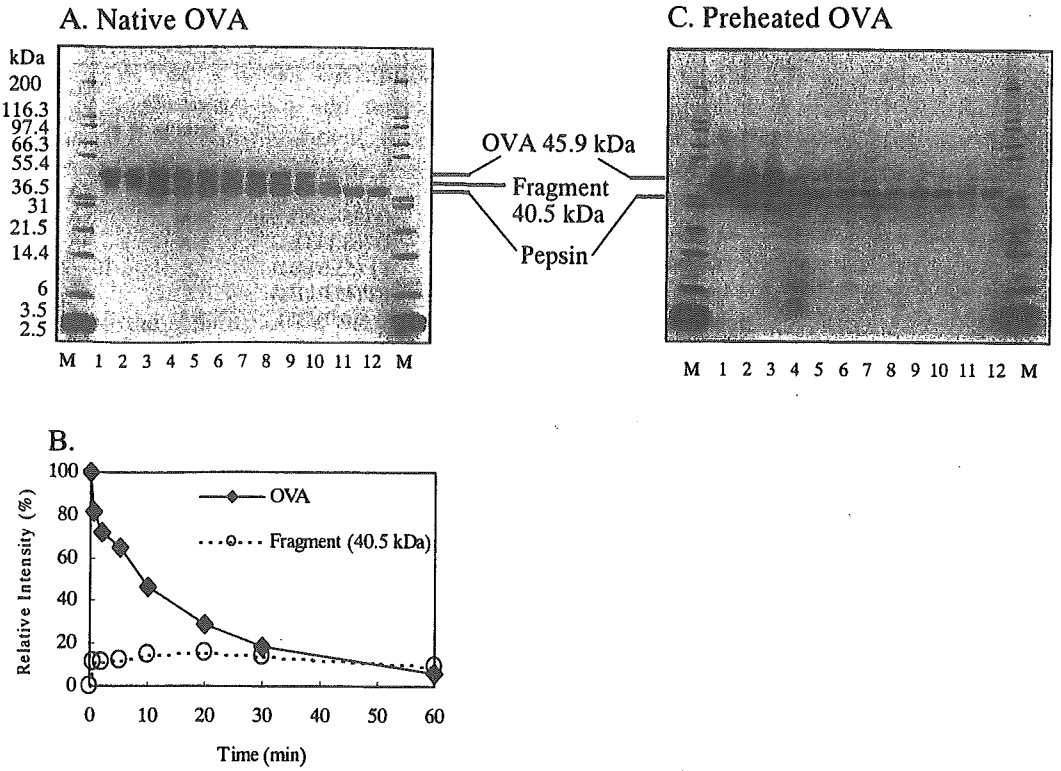

Fig. 1. SGF-Digestion Pattern of OVA

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.

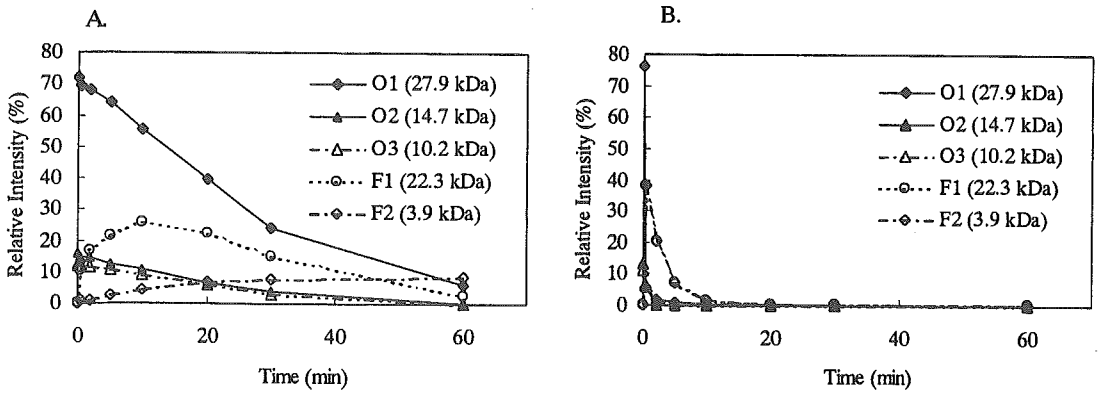

Fig. 2. Quantification of the SGF-Digestion Pattern of Native (A) and Preheated (B) Con A

"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).

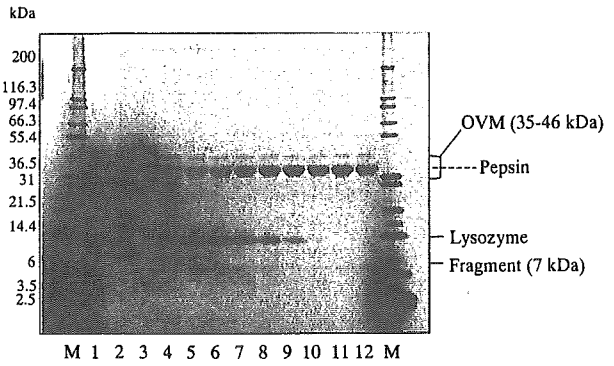

Fig. 3. SDS-PAGE Analysis of the SGF Digestion of Native OVM

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
BLG	O (17.7 kD)	NA	NA	0—2	B
	F (10.2 kDa)	2	2	2	
BSA	O (63.1 kDa)	NA	NA	120	
	F1 (53.0 kDa)	2	90—120	120	
	F2 (45.0 kDa)	2	2	120	E
	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
zein	O (19.5 kDa)	NA	NA	0	A

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

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	

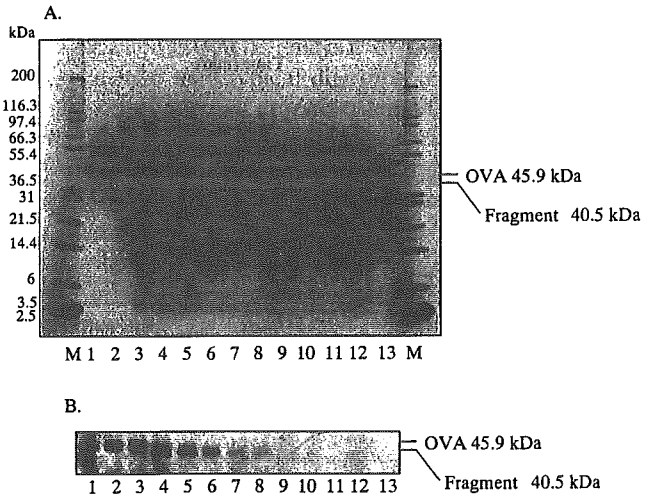

Fig. 4. SDS-PAGE Analysis of the SIF Digestion of Native OVA (A) and Western Blotting with Mouse Anti-OVA Antiserum (B)

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.

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 descried 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/μg 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/μg protein, and at 0.1 unit/μg protein, OVM persisted until the end of the study (60 min, data not shown).

And some proteolytic fragments retain the allergenic potential 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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Application of Human FcεRI α-Chain-Transfected RBL-2H3 Cells for Estimation of Active Serum IgE

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We transfected the α-chain of human FcεRI into rat basophilic leukemia cell line RBL-2H3, established several stable transfected cells, and screened them by β-hexosaminidase release induced by sensitization with human IgE and stimulation with anti-human IgE antibody. A cloned cell line RBL-hEIa-2B12 was the strongest responder among the transfected cell clones. The concentrations of cytosolic free Ca^{2+} concentration in the human IgE-sensitized cells increased after stimulation with anti-human IgE antibody. Thus, it is suggested that the α-chain of human FcεRI is associated with the β-chain and/or γ-chain of rat FcεRI, and that they form functional high affinity IgE receptor complexes. The total IgE concentrations of the sera from allergic patients were determined by using the β-hexosaminidase release assay, where the transfected cells were sensitized with diluted and heat-inactivated (at 56 °C for 30 min) serum and stimulated with anti-human IgE antibody. The IgE concentration obtained correlated with those measured by an enzyme immunoassay method. β-Hexosaminidase release induced by stimulation with 5 times diluted serum was sometimes less than the release induced by the same serum; diluted 25 times or 125 times, suggesting that these serum contained factors that blocked IgE binding to FcεRI or cross-linking by anti-human IgE antibody. The results suggested that our system will be useful for detecting FcεRIα-bindable IgE in human serum.

Key words FcεRIα; basophile; transfectant; human serum; IgE; degranulation

The binding of serum IgE antibodies to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils, and the cross-linking of the receptors by multivalent allergens are the first step in the degranulation where the pharmacologically active substances that cause type I allergy are released. Serum IgE level has been determined by using enzyme-linked immunoassay method, however, it has been reported that some factors are present in serum (especially in hyper-IgE sera), such as anti-IgE and/or anti-FcεRI antibodies.[1] And many persons are patch-test-negative even though their serum contains a high level of allergen-specific IgE.[2] As above, not all IgE antibodies in allergy patients' serum contribute to activation of allergic reactions. Thus, stable human cell lines of mast cells or basophils are essential to investigation of both the activation mechanisms and the evaluation of FcεRI-bindable serum IgE levels of allergic patients. However, since no useful cell lines are available we must every time prepare cells from freshly collected peripheral blood or develop mast cells from human cord blood $CD34^+$ progenitor cells.[3]

FcεRI is expressed in tetrameric form ($\alpha\beta\gamma_2$) on mast cells and basophils[4,5] and in trimeric form ($\alpha\gamma_2$) on eosinophils[6] and antigen-presentating cells.[7,8] The α-chain binds IgE, and the γ-chain transduces signals. The β-chain is an amplifier of signal strength and enhances the expression of unoccupied receptor at the cell surface.[9—11] Earlier studies showed that the transfected human α-chain of FcεRI associates with endogenously expressed β- and γ-chains in rat RBL-2H3 cells and that the human-rat complexes act as functional IgE receptors.[12—14] We therefore attempted to prepare a stable human FcεRIα-transfected RBL-2H3 cell line which can show human IgE-dependent degranulation, and evaluate FcεRI-bindable IgE antibody in human serum.

MATERIALS AND METHODS

Serum Specimens Sera of allergic patients and healthy volunteers were collected from 47 individuals from whom their informed consent was obtained and the ethical aspects of our experiments were approved by Institutional Review Board of National Institute of Health Sciences. The symptoms of the patients were as follows, bronchial asthma (39.1%), atopic dermatitis, urticaria, or eczema (32.6%), allergic rhinitis including pollenosis (30.5%), and so on. Total IgE antibodies in their sera determined by EIA method had been already known and some antigen-specific IgE antibodies were detected in their sera by radioallergosorbent test (RAST), they were pollen (67.4%), house dust (56.5%), mite (56.5%), food (17.4%), fungi (10.9%) and so on.

Allergen-specific sera were purchased from Plasma Lab International (Everett, WA, U.S.A.). The sera were stored at −80 °C, and they were heat-treated at 56 °C for 30 min before use.

Reagents Human myeloma IgE purified by dialysis against deionized water and gel filtration was obtained from Chemicon International, Inc. (Temecula, CA, U.S.A.), and affinity-purified anti-human IgE (Fc) antibody was purchased from American Qualex Antibodies (San Clemente, CA, U.S.A.). Murine anti-dinitrophenyl (DNP) monoclonal IgE antibody (IgE-53-569) and dinitrophenylated bovine serum albumin (BSA) (DNP_7/BSA) were prepared as described previously.[15] *Dermatophagoides farinae* (Df) extract was obtained from LSL Co., Ltd. (Tokyo, Japan).

Plasmid Construction pGEM-3-110B-1 plasmid containing human α-chain cDNA was purchased from ATCC (Manassas, VA, U.S.A.). The human α-chain cDNA was amplified using the pGEM-3-110B-1 plasmid as a template by PCR. The used primers were GAATTCGAAGAAGATG-GCTCCTGC and GTCGACTAAATCCTTGAGCACAGAC. The amplified cDNA was cloned into pCR2.1-TOPO vector

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(TOPO TA cloning kit, Invitrogen), and its sequence was confirmed. The pCR2.1-TOPO-human α-chain cDNA was exposed to *Eco*RI and *Sal*I and cloned into *Eco*RI–*Sal*I site of pCI-neo plasmid (Promega, Madison, WI, U.S.A.).

Cell Culture and Electroporation RBL-2H3 cells[16] were maintained in Dulbecco's modified Eagle Medium (Nissui Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, U.S.A.) and 60 μg/ml kanamycin. The transfection procedure was performed as described previously.[17] The cells were washed twice with HEPES buffered saline (50 mM HEPES buffer pH 7.4 containing 180 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose) and suspended in K^+PBS (10 mM PBS containing 30 mM NaCl, 120 mM KCl, and 10 mM $MgCl_2$) at 2×10^7 cells/ml. A total of 20 μg of human α-chain DNA in the pCI-neo vector was incubated with 1×10^7 RBL-2H3 cells in a gene pulse cuvette and transfected at 250 V, 950 μF with Gene Pulser (Bio-rad, Richmond, CA, U.S.A.). The cells were then incubated in the medium, and the next day the culture medium was replaced with fresh medium containing 500 μg/ml of G418 (Geneticine, Life Technologies, Rockville, MD, U.S.A.). The cells were cloned by limited dilution and the clones, which released β-hexosaminidase in response to the stimulation with anti-human IgE antibody after sensitization with human myeloma IgE, were selected.

Measurement of Degranulation β-Hexosaminidase is known as a component of the basophil and mast cell specific granule and the release by degranulation was parallel with histamine release in RBL-2H3 cells.[18] Therefore, degranulation of the transfectants was monitored by measuring released β-hexosaminidase activity.[19] The transfectants were sensitized with various concentrations of human myeloma IgE for 3 h or with diluted human serum for 18 h, and the sensitized cells were stimulated with anti-human IgE antibody (10 μg/ml) in PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose, 0.1% BSA and 10 mM PIPES, pH 7.4) for 30 min. And the medium was then collected, and 0.2% Triton X-100 was added to the cells. β-Hexosaminidase activities released into the medium and within the cells (Triton X-100 extract) were determined by a colorimetric assay using *p*-nitrophenyl-2-deoxy-β-glucopiranoside as a substrate. The β-hexosaminidase activity released into the medium is expressed as a percentage of total activity. For estimation of serum IgE, the RBL-hEIa-2B12 cells were cultured in 96-well plates, and the medium was replaced with diluted human serum. After incubation for 18 h at 37 °C, the cells were stimulated with anti-human IgE, and the β-hexosaminidase release was determined.

Measurement of Cytosolic Free Calcium Concentration ($[Ca^{2+}]_i$) The transfectants (6×10^5 cells/ml) were sensitized with 1 μg/ml human IgE or diluted human serum and loaded with 6 μM fura-2 AM (a fluorescent dye reagent for calcium ions, Dojindo, Kumamoto, Japan). After removing the free dye and unbound IgE by centrifugation, the cells were suspended in PIPES buffer (6×10^5 cells/ml) and stimulated with 100 μg/ml antigen or 10 μg/ml anti-human IgE antibodies. Fluorescence measurements were made in a 1 cm quartz cuvette with a Shimadzu RF-5000 spectrofluorophotometer (Shimadzu, Kyoto, Japan) as previously reported.[19]

Measurement of Total IgE Level by EIA Total IgE levels in human serum were determined by fluorescence enzyme immunoassay (Uni CAP system, Pharmacia Corporation, Peapack, NJ, U.S.A.) or colorimetric enzyme-linked immunosorbent assay (Mesacup IgE Test, MBL Co., Ltd, Aichi, Japan).

RESULTS AND DISCUSSION

Preparation of the Human FcεRIα-Expressing RBL Cells The FcεRIα-transfected RBL-2H3 cells were cloned by the limited dilution method, and the human-FcεRIα-expressing clones were selected by their β-hexosaminidase release following sensitization with human IgE and stimulation with anti-human IgE. Some of the clones released β-hexosaminidase in a human-IgE-dependent manner, and the results for three highly responsive clones, RBL-hEIa-2B12, RBL-hEIa-1G4, and RBL-hEIa-1C10, are shown in Fig. 1. Empty-vector-transfected RBL-2H3 cells were also cloned, but none of them showed β-hexosaminidase release in a human-IgE-mediated manner. The results for one of them (RBL-pCI-1C5) are also shown in Fig. 1. And Table 1 shows the β-hexosaminidase release by the three α-chain-transfected clones, an empty-vector-transfected clone, and wild-type RBL-2H3 cells in a human IgE/anti-human IgE mediated manner or a mouse anti-DNP-IgE/DNP-BSA mediated

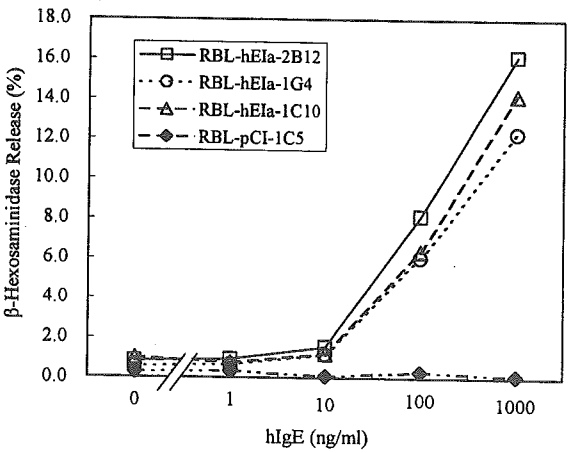

Fig. 1. Human IgE Dependent β-Hexosaminidase Release by Cloned Transfectants

After sensitization with various concentration of human myeloma IgE (0—1000 ng/ml) for 3 h at 37 °C, three clones of human-FcεRIα-transfected RBL-2H3 cells (RBL-hEIa-2B12, RBL-hEIa-1G4, and RBL-hEIa-1C10) and one clone of empty-vector-transfected cells (RBL-pCI-1C5) were stimulated with anti-human IgE (10 μg/ml). Values are the mean of the duplicate analysis. Similar results were observed in two other experiments.

Table 1. β-Hexosaminidase release (%) by transfectants and wild-type RBL-2H3 cells

Cell line	Control	Human IgE/ anti-human IgE	Mouse anti-DNP IgE/ DNP-BSA
RBL-hEIa-2B12[a)]	0.85	16.16	10.11
RBL-hEIa-1G4[a)]	0.56	12.29	20.15
RBL-hEIa-1C10[a)]	1.01	14.19	14.31
RBL-pCI-1C5[b)]	0.45	0.31	11.56
Wild-type RBL-2H3	0.77	0.54	21.08

a) Human α-chain cDNA transfected clone. *b*) Empty-vector transfected clone. After sensitization with 1.0 μg/ml human myeloma IgE or mouse monoclonal anti-DNP IgE for 3 h at 37 °C, cells were stimulated with 10 μg/ml anti-human IgE or DNP-BSA. Values are averages of duplicate analyses.

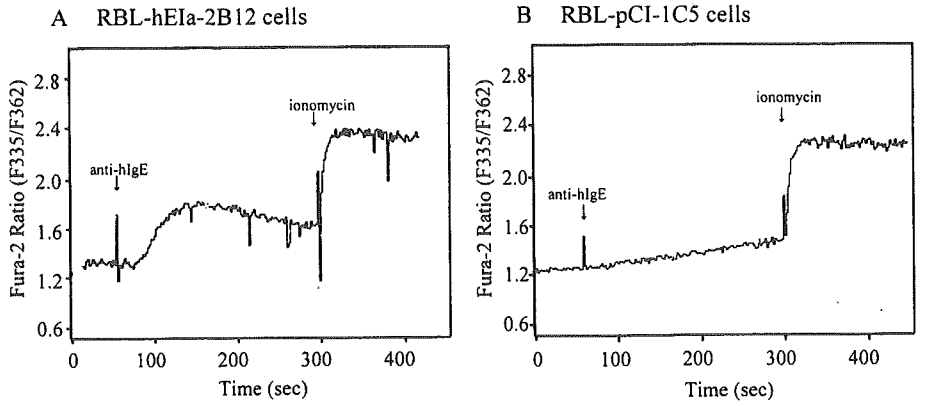

Fig. 2. Human IgE Dependent $[Ca^{2+}]_i$ Changes in the Transfectants

RBL-hEIa-2B12 cells (A) and RBL-pCI-1C5 cells (B) were sensitized with human myeloma IgE (1 μg/ml) for 3 h at 37 °C and suspended in PIPES buffer. Anti-human IgE (10 μg/ml) was added to the suspension followed by 0.5 μM ionomycin at the times indicated. Similar results were observed in two other experiments.

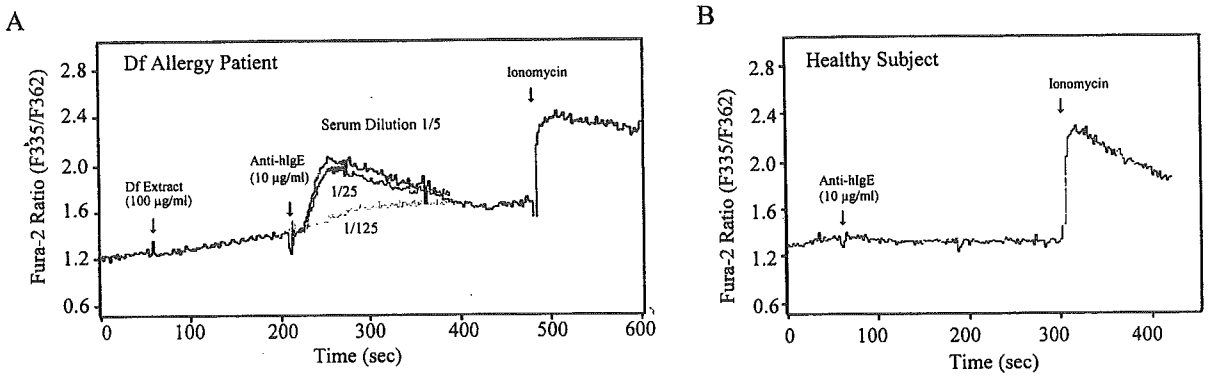

Fig. 3. $[Ca^{2+}]_i$ Changes in RBL-hEIa-2B12 Cells Sensitized with Human Serum

RBL-hEIa-2B12 cells were sensitized with serum from a Df allergy patient (5, 25, or 125 times diluted, A), or a healthy volunteer (50 times diluted, B) for 18 h at 37 °C and suspended in PIPES buffer. Df extract (100 μg/ml, A), anti-human IgE (10 μg/ml) and ionomycin (0.5 μM) were added to the suspension at the times indicated.

manner. Following sensitization with mouse IgE, both transfectants released β-hexosaminidase in response to the specific antigen, similar to the wild-type RBL-2H3 cells (Table 1). Mouse anti-DNP-IgE antibodies bind rat α-chain of FcεRI, however they do not bind human α-chain, so both transfectants seem to be expressing intrinsic rat FcεRI.

Human IgE-Induced Ca^{2+} Response in the Transfectants Then, intracellular $[Ca^{2+}]_i$ in the human-IgE-sensitized transfectants was determined after stimulation with anti-human IgE. The increase in $[Ca^{2+}]_i$ in the transfectants (6×10^5 cells) loaded with fura-2 AM was monitored with a spectrophotometer, and the results showed that anti-human IgE antibody increase $[Ca^{2+}]_i$ in RBL-hEIa-2B12 cells (Fig. 2A). The empty-vector transfectant RBL-pCI-1C5 did not show Ca^{2+} signaling (Fig. 2B).

Sensitization with mouse monoclonal anti-DNP IgE antibody and stimulation with the specific antigen DNP-BSA increased $[Ca^{2+}]_i$ in both the RBL-hEIa-2B12 cells and RBL-pCI-1C5 cells (data not shown). Thus, endogenous rat FcεRI is not lost by transfection.

Ca^{2+} Response to Using IgE in Allergic Patient Sera Instead of human myeloma IgE, we examined for IgE in human sera from allergic patients and healthy volunteers. A representative data was shown in Fig. 3, where the patient was allergic to mites (*Dermatophagoides farinae*, Df). The serum total IgE level of the patient measured by EIA was 6960 ng/ml, while that of normal serum was 624 ng/ml. The stimulation with anti-human IgE antibody induced Ca^{2+} response in RBL-hEIa-2B12 cells sensitized with diluted patient serum for 18 h at 37 °C (Fig. 3A). However, a longer sensitization period (18 h) is required for the human serum IgE than the myeloma IgE (3 h). The Ca^{2+} response for the normal serum was very weak (Fig. 3B).

In contrast to stimulation with anti-human IgE antibody, stimulation with the specific allergen Df extract, did not induce Ca^{2+} signaling in the sensitized cells (Fig. 3A).

The fact that both the Ca^{2+} response and β-hexosaminidase release were induced by sensitization with human myeloma and allergic patient IgE and stimulation with anti-human IgE suggests that the transfected human α-chains form a functional human IgE receptors on RBL-hEIa-2B12 cells. However, specific-allergen-induced response was not detectable. It is very likely that the cell surface density of human α-chain is still low to be cross-linked by specific allergens, and the ratio of specific IgE antibodies to the total IgE antibodies in a patient's serum seems to be very small and most of the expressed IgE receptors must be occupied with non-specific IgE antibodies, even if the patient shows allergic symptom to the allergen that is different with the case of the mouse monoclonal anti-DNP IgE.

Relationship between β-Hexosaminidase Release and Total IgE Concentration Measured by EIA Since β-hex-

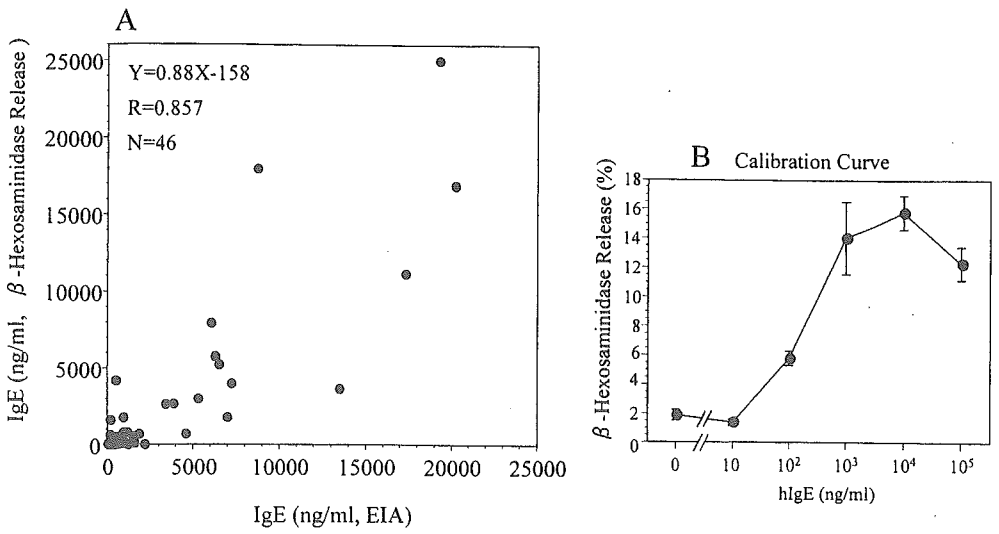

Fig. 4. Comparison of Serum IgE Levels Determined by Degranulation of RBL-hEIa-2B12 Cells and by the EIA Method

RBL-hEIa-2B12 cells were cultured in 96-well plates and sensitized with diluted human serum from 46 allergy patients for 18 h at 37 °C. The cells were then stimulated with 10 μg/ml anti-human IgE, and the β-hexosaminidase release was determined. Each IgE value was calculated based on the calibration curve of β-hexosaminidase release induced by human myeloma IgE sensitization followed by 10 μg/ml anti-human IgE stimulation (B), and compared with the values determined by the EIA method (A). Each value in the calibration curve represents the mean±S.D. of triplicate analysis and similar results were observed in two other experiments.

osaminidase release in the IgE/anti-IgE system is dependent on the IgE concentration as shown in Fig. 1, we calculated the IgE concentrations of patient sera using RBL-hEIa-2B12 cells and myeloma IgE as a standard.

When diluted patient sera (25-times or 125-times dilutions) was used, the release of β-hexosaminidase correlated with the IgE levels measured by EIA method. However, for 5-times diluted sera of some patients, enzyme release did not correlate with the IgE level determined by the EIA method. Even if IgE level of serum by EIA was less than 1000 ng/ml (linear part of calibration curve), β-hexosaminidase release was lower than the expected level. For this reason, we measured that β-hexosaminidase release from cells sensitized with serum diluted 5 times, 25 times, and 125 times, and the value at the highest release was used for calculation using calibration curve in Fig. 4B. As shown in Fig. 4A, β-hexosaminidase release correlated with the IgE concentrations determined by the EIA method (R=0.857).

As described above, a part of IgE antibodies in allergy patients' serum contribute to activation of allergic reactions and some of human sera were growth-inhibitory during the sensitization period at high concentrations.

In conclusion, our stable transfectant system made it possible to detect the level of the IgE in patient sera that binds receptors and causes degranulation under the conditions similar to physiological.

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Genomic DNA Fragmentation of Genetically Modified Corn during Food Processing

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Abstract

We studied the degradation and fragmentation of DNA molecules of corn during different food production processes. Grits prepared from the genetically modified (GM) corn varieties MON810 and GA21 were processed by frying or using an extruder, a device that treats a slurry of grits with both heating and pressure, followed by a sudden release of pressure to atmospheric conditions through a pinhole, to produce corn snacks. DNA fragments longer than 600 bp could be amplified by PCR for the genomic DNA prepared from fried processed corn grits at temperatures lower than 160℃ for 1 min, while those shorter than 400 bp could be detected from processed grits at 180℃. However, even at 128℃, extruder processing caused severe fragmentation of genomic DNA fragments shorter than 197 bp, and at much higher temperatures no amplified fragments longer than 100 bp could be detected. Grits derived from the GM corn varieties MON810 and GA21 were processed with an extruder at 128℃, and genomic DNAs were prepared from the processed grits. Each transgene of these GM corns and the cornstarch synthase IIb (*SSIIb*) gene, which is used as the control gene for the Japanese official quantification method, were quantified by quantitative TaqMan PCR. The copy number of the *SSIIb* gene in the processed grits was smaller than that of the transgenes of both MON810 and GA21, which indicated that the *SSIIb* gene would be easier to fragment than the transgenes with extruder processing. Therefore, the ratio of the copy number of the transgene per internal control *SSIIb* gene in the processed corn snacks using the extruder might be higher than in the raw materials of corn grits using quantitative TaqMan methods.

Key words: DNA fragmentation, extruder, genetically modified (GM) corn, processed food, quantitative PCR

I. Introduction

The Ministry of Health, Labor and Welfare (MHLW) revised the Food Sanitation Law in April 2000 and made safety assessment a legal obligation in order to prevent the distribution of genetically modified (GM) crops and processed foods made from GM crops that had not been assessed for safety[1),2)]. After a one-year extension, the import and sale of GM crops and processed foods containing GM materials that had not been assessed for safety were forbidden. It is well known that the GM corn variety, "StarLink™" (CHB351), which had not been authorized as safe for use in foods worldwide, was mixed into foods such as tortillas, shells, tostadas and chips sold in markets. These products were recalled in 2000 in USA. In Japan, the detection method for "StarLink™" (CHB351) was established by the laboratories of the Ministry of Agriculture, Forestry and Fisheries (MAFF) and MHLW[3)]. Using this method, contamination of "StarLink™" (CHB351) in corn in the cargo of a ship from the USA was detected and entry into Japan was prohibited on December 2002.

Several detection methods for GM corn, which were established and authorized by MHLW, use PCR[4)-10)]. These detection methods are useful for detecting GM corn unintentionally mixed into grains and processed foods. In April 2001, the Japanese government introduced an effective new labeling system, in which

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GM corn and soybean are referred to as either "genetically modified" or "not segregated from GM crops"[11]. Although GM potatoes were included in the labeling system in 2002, labeling is not enforced in the case of the unintentional mixing of GM crops that comprise less than 5% of the total weight of the crops. This means that the threshold level of mixed GM seed should be maintained at a level no greater than 5%. For this reason, the MAFF presented quantification methods for crops of GM corn and soybean and for ingredients prepared from them in order to monitor the adequacy of the labeling implementation[12)-14)]. Detection and quantification methods have since been revised and evaluated for further improvement[15), 16)].

Japanese quantification methods were established mainly to determine the level of mixing of GM corn and soybean in non-GM crops. Recently, two reports on the development of quantification methods for GM corn have been presented. However, their application was limited to grain and ground products[17), 18)], and they have not been verified to apply to processed foods. It is reasonable to assume that these methods might be difficult to apply to processed foods because genomic DNA would be damaged and degraded during food processing. Therefore, we studied the application of the Japanese official quantification methods on the processed food, "tofu", which had been made from seeds of GM and non-GM soybeans mixed in several ratios[19)]. During "tofu" processing, the drastic treatment that causes the degradation or fragmentation of genomic DNA is boiling. While genomic DNA fragmentation seemed to occur, the DNA did not fragment into lengths below approximately 600 bp. This indicated that the Japanese official quantification methods, in which quantitative TaqMan is used to amplify target DNA regions shorter than 500 bp of the transgene, could be applied to "tofu"[19)]. In products manufactured under much more severe conditions and with a higher degree of processing, such as "kombu-mame" and canned soybean, which are treated with heat and pressure for sterilization after processing, severe fragmentation of soybean genomic DNA was observed and fragments longer than 200 bp could not be amplified with qualitative PCR. As a result, the Japanese official quantification methods using TaqMan PCR could not be used[19)]. Since most GM crops are processed using various cooking methods under quite different conditions, and are supplied to markets as processed foods, the degree of degradation and fragmentation of genomic DNA of GM ingredients using these methods needs to be verified. It is to ensure that the Japanese official quantification methods using quantitative TaqMan PCR are applicable, and to demonstrate that the amount of unintentional mixing of GM crops in non-GM foods is kept below 5%, as required by the Japanese labeling system.

In this paper, grits prepared from the GM corn varieties MON810 and GA21 were cooked by frying at several temperatures, and also by using an extruder, which is commonly used in the manufacture of corn snacks. In the latter process, a slurry of corn grits is placed on a heating block under a screw, which provides the pressure. The pressure is suddenly released and at the moment grits are swollen to produce the corn snacks and the other food products. This process results in the severe fragmentation of corn genomic DNA, which is not observed in fry processing, a common method used to produce corn materials such as tortillas, shells, tostadas, and chips.

II. Materials and Methods

1. Corn grits prepared from non-GM corn and the GM corn varieties MON810 and GA21

Grits derived from the GM corn varieties MON810 and GA21 were provided by the National Institute of Health Sciences, MHLW; and those from non-GM corn were obtained from Nippon Flour Mills Co., Ltd. MON810 grits were mixed with non-GM corn up to 9.5% (w/w), while GA21 was unmixed and used as 100% GM corn grits. Commercially available corn snacks (A to E in Fig. 2, all of which might be processed using an extruder) and corn starch (Fig. 2 F) were purchased from markets in Osaka city.

2. Fry processing and heat and pressure extruder processing

Corn grits were wrapped with aluminum foil, and then dipped in a canola oil bath heated at 130℃, 160℃ or 180℃ for one minute. For extruder processing, grits were introduced into the twin-screw extruder LABORUDER MK II-S (Japan Steel Works Ltd, Japan) and mixed with water to make a slurry. The ratio of water to grits was 0.03-0.07 L/kg. This extruder could accurately regulate the block temperature of the screw. Processing conditions are shown in Table 1.

Table 1. Extruder (LABORUDER MK II-S (Japan Steel Works Ltd.) conditions for processed corn grits

Materials	Condition		
Temperature conditions for processing	Low	Optimum	High
Material temperature at front edge (℃)	128	162	179
Material feed (kg/h)	7.6	7.6	7.6
Addition of water (L/h)	0.56	0.24	0.24
Pressure at front edge (MPa)	6.3	6.1	5.3
Screw speed (r.p.m)	288	288	288
Main motor load (A)	9.2	9.3	9.1

3. DNA extraction from processed corn foods

Genomic DNA was extracted from corn snacks and other processed foods according to the Japanese official method[15), 16)]. For MON810 and GA21, an ion exchange resin-type DNA extraction kit (QIAGEN Genomic-tip) and a silica gel membrane-type kit (QIAGEN DNeasy Plant Mini Kit) were used, respectively.