Cells, Cell Culture, and Induction of Differentiation

The human promyelocytic leukemia cells, HL-60S, were provided by Dr. F. Takaku (Faculty of Medicine, The University of Tokyo) and cultured in suspension in RPMI1640 (Biomedicals Inc., Aurora, OH) supplemented with 5 % fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (Invitrogen,Carlsbad, CA) under 5 % CO_2 at 37 °C. HL-60WT cells were obtained from Japanese Collection of Research Bioresources Cell Bank and cultured same as HL-60S but with 10 % fetal bovine serum. Retinoids or equivalent concentration of ethanol (0.5 % of culture medium) were added to cells for 96 hours.

NBT Reduction Assay

After harvesting the cells, they were incubated for 20 min at 37 °C in RPMI1640 with 5% fetal bovine serum and an equal volume of PBS containing NBT (0.2 %) and TPA (200 ng/ml). The percentage of cells containing blue-black formazan was determined with a minimum of 200 cells under the microscope. All the measurements were done in triplicate.

Proteasome Inhibitor Treatment

MG132 was purchase from Sigma Chemicals Co. (St.Louis, MO). z-VAD-fmk (negative control) was purchased from BIOMOL International, LP (Plymouth, PA). Both were dissolved in ethanol. Cells were treated with z-VAD-fmk, MG132, or ethanol overnight. The final concentration of both inhibitors was 0.5 μM. Then cells were washed once with RPMI1640 medium and resuspended in RPMI1640 medium with fetal bovine serum. Suspended cells were divided into seven culture-wells and treated each well with Am80, Ch55, or TTNPB at the concentration of either 10 nM or 1 nM or with ethanol for 96 hours. Differentiated cells were counted by NBT reduction assay in triplicate.

Genomic DNA Preparation

Genomic DNA was prepared using a QIAmp DNA Blood Midi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Total RNA Preparation

After washing cells twice with PBS, total RNA was prepared with an RNeasy Mini total RNA Preparation Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA was prepared from HL-60WT cells and HL-60S cells six times independently.

Nuclear Extract Preparation

After washing cells twice with PBS, nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (PIERCE Biotechnology, Rockford, IL) with a Halt protease inhibitor cocktail kit (PIERCE Biotechnology, Rockford, IL) following the manufacturer's instructions. Concentrations of nuclear proteins were measured by the Bradford method available from BioRad (Hercules, CA).

Gene Expression Measurement

mRNA was transcribed into cDNA by TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Expression levels of genes were measured by the real-time PCR PRISM 7700, (Applied Biosystems, Foster City, CA) with specific TaqMan probes purchased from Applied Biosystems (Foster City, CA); RARα: Hs00230907_m1, RXRα: Hs00172565_m1. All measurements were done with the five independently prepared total RNAs in duplicate and normalized by the expressions of the β-actin gene.

Western Blotting

Western blotting was performed according to the standard protocols with 30 μg of nuclear extracts and detected by chemiluminescence with a proper secondary antibody. Antibodies used for the detection of proteins were as follows; RARα: anti human RARα mouse monoclonal antibody (PERSEUS PROTEOMIX, Tokyo, JAPAN), cdk-7: purified mouse anti-human CDK7 monoclonal antibody (BD Biosciences Pharmingen, San Jose, CA), cyclin H: cyclin H (D-10) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), MAT1: p36 (Transduction Laboratories, Lexington, KY). Recombinant RARα and RARβ expressed in a baculovirus system and purified were purchased from PROTEIN ONE (College Park, MD). Cdk7/cyclin H/MAT1 complex was purchased from Upstate (Lake Placid, NY). Each component of the complex was expressed by baculovirus in Sf21 cells as 6His-tagged full length (cdk7, MW: 40 kDa), untagged full length (cyclin H, MW: 38 kDa), or N-terminal GST-tagged full-length (MAT1, MW: 63 kDa) recombinant protein.

Array-based Comparative Genomic Hybridization

DNA prepared from HL-60WT and HL-60S were labeled with Cy3 and Cy5 respectively or Cy5 and Cy3 respectively (dye swap) according to the standard protocols provided by Agilent Technologies (Santa Clara, CA). Human Genome CGH 44B glass slides (Agilent Technologies, Santa Clara, CA) were hybridized with the labeled DNA for 40 hours, then washed and scanned with an Agilent Technologies Microarray Scanner (Agilent Technologies, Santa Clara, CA). Signal strength of each spot on the glass slide was measured by Feature Extraction ver. 8.1.1.1. (Agilent Technologies, Santa Clara, CA). Obtained

measurements were imported into the Agilent CGH Analytics Software, ver. 3.2.32, and analyzed by Z-score aberration algorithm with 2.0 Mb of window size. The results of aCGH are available on request to ishida@nihs.go.jp.

DNA Microarray Analysis

DNA microarray analysis was performed with human genome U95A GeneChip (Affymetrix, Santa Clara, CA) as described previously (Ishida 2003). GeneChip analyses were performed with six different preparations of HL-60WT and HL-60S total RNA. The results of DNA microarray analyses are available on request to ishida@nihs.go.jp.

Selection of Genes Expressed Differentially in HL-60WT and HL-60S

Selection of genes whose expressions were different between HL-60WT and HL-60S was done with two criteria, one was expression level and the other was statistical significance of the difference. First, the average of signals of twelve measurements, six from HL-60WT and six from HL-60S, was calculated and the genes whose averaged expression signals were more than 1,000 were selected. Then, the Student's *t*-test was conducted between the six measurements of HL-60WT and the six measurements of HL-60S. Genes which showed significance less than 0.01 were used for further analysis. The number of genes left at this step was 1,598. To visualize selected gene expressions on chromosomes, their chromosome location information were obtained from NetAffx, which was created on December 18, 2005.

Pathway Analysis

Pathway analyses of selected genes were conducted by Ingenuity Pathway Analysis (Ingenuity Systems, Inc. Redwood City, CA).

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FOOD COMPOSITION AND ADDITIVES

Interlaboratory Evaluation of Two Enzyme-Linked Immunosorbent Assay Kits for the Determination of Crustacean Protein in Processed Foods

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The labeling of foods containing material derived from crustaceans such as shrimp and crab is to become mandatory in Japan because of increases in the number of allergy patients. To ensure proper labeling, 2 novel sandwich enzyme-linked immunosorbent assay (ELISA) kits for the determination of crustacean protein in processed foods, the N kit (Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan) and the M kit (Maruha Nichiro Holdings, Inc., Ibaraki, Japan), have been developed. Five types of model processed foods containing 10 and/or 11.9 μg/g crustacean soluble protein were prepared for interlaboratory evaluation of the performance of these kits. The N kit displayed a relatively high level of reproducibility relative standard deviation (interlaboratory precision; 4.0–8.4% RSD_R) and sufficient recovery (65–86%) for all the model processed foods. The M kit displayed sufficient reproducibility (17.6–20.5% RSD_R) and a reasonably high level of recovery (82–103%). The repeatability relative standard deviation (RSD_r) values regarding the detection of crustacean proteins in the 5 model foods were mostly <5.1% RSD_r for the N kit and 9.9% RSD_r for the M kit. In conclusion, the results of this interlaboratory evaluation suggest that both these ELISA kits would be very useful for detecting crustacean protein in processed foods.

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In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults in these countries have some type of food allergy (1, 2). Burks et al. (3) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan as well, the number of patients with food allergies, especially among young children, is increasing (4). To prevent possible life-threatening reactions, the only effective measure is to strictly avoid the consumption of allergenic foods because of the lack of effective medical treatment for food allergies. However, various studies have shown that severe allergenic reactions can be induced by the accidental intake of food products containing allergenic materials (5, 6). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary.

The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label 8 kinds of foods that contain ingredients known to be allergens, including Crustacea (7). In Japan, the Ministry of Health, Labor and Welfare (MHLW) has enforced a labeling system for allergenic food materials since April 2002 in order to provide information about these foods to the allergic

consumer. According to the Japanese regulations, labeling for food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory, and is recommended for 20 other food materials such as shrimp and crab in light of the number of patients and the degree of seriousness. In a ministry notification in 2002 (8), the MHLW announced the official Japanese methods for the detection of allergens for mandatory labeling and the threshold value for labeling [10 μg/g (the corresponding allergenic ingredient soluble protein weight/food weight)]. The MHLW also described the intervalidation protocol criteria in the official guidelines in 2006 (9). The outline of the intervalidation protocol criteria is as follows: number of laboratories, ≥8; number of incurred samples, ≥5; number of dose level, ≥1, including 10 μg/g (the corresponding allergen protein weight/food weight); recovery, 50–150%; reproducibility relative standard deviation (RSD_R), ≤25%. In the guideline, reference material, the initial extract solution, and the extraction procedure from allergen were also specified and standardized.

Epidemiological investigations in Japan have shown that the number of patients with a crustacean allergy such as to shrimp and crab has increased (10). In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea as an ingredient and because the number of patients with allergies to Crustacea has been increasing, although crustacean allergy is still less prevalent than, for example, milk allergy in the food-allergic population (11, 12). Crustacean allergic reactions can be elicited by the intake of trace amounts of crustacean proteins, and anaphylaxis caused by Crustacea has been reported (13, 14). Considering current conditions, the MHLW will have a plan for crustacean labeling to be mandatory in 2008. Therefore, a reliable detection method for crustacean protein monitoring is necessary to ensure accurate labeling. We have developed 2 enzyme-linked immunosorbent assay (ELISA) kits capable of highly sensitive crustacean protein detection. This paper describes the results of an interlaboratory evaluation of the performance of these ELISA kits.

Experimental

Materials and Methods

Preparation of crustacean powder.—Crustacean powder was prepared by homogenization followed by freeze-drying of the tail muscle of the shrimp (black tiger prawn, *Penaeus monodon*). The crustacean soluble protein (CP) from crustacean powder was extracted using the extraction buffer phosphate buffered saline (PBS), pH 7.4, containing 0.5% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol. The CP content was then calculated using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). The amount of CP/1 g crustacean powder was approximately 684 mg.

Test Materials

Five model processed foods (fish sausage, freeze-dried egg soup, tomato sauce, creamy croquette, and chicken balls) containing CP were prepared and used as test materials. Crustacean powder was spiked at the ingredient stage before processing to establish a final level of 10 μg/g (CP weight/sample weight). In the case of freeze-dried egg soup, the CP content was calculated to be 11.9 μg/g (CP weight/sample weight). The prepared model processed foods were homogenized with a food processor (DLC-XG; Cuisinart, Stamford, CT) and sent to the participants as test materials.

Preparation of Model Processed Foods

All the model processed foods were prepared using procedures described by the manufacturers. To make a final protein concentration of 10 μg/g (CP weight/sample weight) in these foods, the amount of crustacean powder spiked in the foods at the ingredient stage was calculated, taking into account the protein content of the crustacean powder and the change in weight of the foods during preparation. Because the protein amount per 1 g crustacean powder was ca 684 mg, we spiked 14.6 mg crustacean powder to make 1 kg of each model processed food [the make-up of the final concentration is calculated for 10 μg/g (CP weight/sample weight)] because we estimated the ratio of CP weight to crustacean powder weight to be 68.4%.

The fish sausages were composed of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and crustacean powder. Lard, salt, sugar, ice water, and the crustacean powder were added to minced fish flesh and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into fish sausage casings. These were then heated at 121°C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The freeze-dried egg soup was composed of egg, potato starch, milk sugar (lactose), salt, and crustacean powder. Eggs, potato starch, milk sugar, salt, and the crustacean powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep-freezer at –80°C, and then lyophilized for 20 h.

Tomato sauce was composed of tomato, onion, olive oil, garlic, bay leaf, sugar, salt, black pepper, and crustacean powder. The measured raw materials were thoroughly mixed and placed in an aluminum pouch. The sauce was heated at 121°C for 1 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The creamy croquettes were composed of milk, butter, wheat, potato starch, salt, sugar, black pepper, flour, and crustacean powder. The raw materials were homogeneously mixed and kneaded for 20 min. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at –20°C until interlaboratory evaluation.

Table 1. Homogeneity test[a] results of processed food samples

Food	Mean, μg/g	RSD, %[b]	*n*	*F*-ratio	F_{crit}[c]
Fish sausage	6.5	4.5	6	1.6	4.4
Freeze-dried egg soup[d]	8.5	2.4	6	0.9	4.4
Tomato sauce	9.0	4.9	6	1.5	4.4
Creamy croquette	8.5	4.3	6	1.0	4.4
Chicken ball	7.1	5.5	6	3.8	4.4

[a] Homogeneity test was carried out with N kit.
[b] RSD% calculated from s_s (SD of sampling) and s_a (SD of analysis).
[c] F_{crit} = Critical *F*-value.
[d] CP was spiked at 11.9 μg/g.

The chicken balls were composed of white meat of chicken, lard, potato starch, sugar, and crustacean powder. Lard, potato starch, sugar, and the crustacean powder were added to ground white meat and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at –20°C until interlaboratory evaluation.

Each type of model processed food was also prepared as a blank sample which did not include CP for the purpose of confirming potential contamination, determination of the limit of detection (LOD), the limit of quantification (LOQ), false positive, interference, and matrix effects. All the values determined in the blank samples of the model processed foods using both kits were less than respective LOD in in-house validation (data not shown).

Homogeneity Tests of the Test Materials

The homogeneity of the test materials was verified by the coordinator before distribution following the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (15), except that the number of test materials was 6. Twelve test portions of each test material were analyzed using the N kit. The obtained concentrations of CP were submitted to one-way analysis of variance (ANOVA). Table 1 shows the average concentration, the relative standard deviation (RSD) percentages calculated from s_s (SD of sampling) and s_a (SD of analysis) as well as the *F*-ratios. The *F*-ratios for all test materials were below the critical *F*-value.

ELISA Kits

Crustacean kit "Maruha" (M kit; 16).—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (*Penaeus monodon*) are used in the M kit with the CP solution used as a calibrator. The outline of the procedure is as follows: Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at room temperature. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h. After the second washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added, and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by addition of 1 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The M kit was highly specific for the Decapoda group, apart from minor cross-reactivities to other Crustacea and Mollusks. We determined the LOD and LOQ according to International Standards Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC) guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 25 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 25 experiments. The LOD of the M kit determined using the dilution buffer was 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and the LOQ was 0.78 ng/mL, equivalent to a 0.31 mg/kg sample. Consequently, the practical determination range is between 0.78 and 50 ng/mL.

Food Allergen Test EIA Crustacean "Nissui" (N kit; 18).—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (*Penaeus monodon*) are used in the N kit, with the CP solution used as a calibrator. The detection procedure was performed according to the manufacturer's instructions. Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at 25°C. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h at 25°C. After the second washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by the addition of 0.5 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The N kit was also highly specific for the Decapoda group and showed the cross-reactivities to other Crustacea. We determined the LOD and LOQ according to ISO and IUPAC guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 36 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 36 experiments. The LOD of the N kit determined using the dilution buffer was 0.4 ng/mL, equivalent to 0.16 mg/kg samples, and the LOQ

Table 2. Results of the interlaboratory study for M kit: protein recovery content

	Fish sausage		Freeze-dried egg soup[a]		Tomato sauce		Creamy croquette		Chicken ball	
Lab	μg/g	%	μg/g	%	μg/g	%	μg/g	%	μg/g	%
A	11.4	114	12.2	103	11.6	116	10.0	100	13.2	132
B	11.6	116	11.8	100	10.8	108	10.2	102	10.8	108
C	10.1	101	8.6	72	11.6	116	8.7	87	8.0	80
D	8.6	86	11.0	93	8.7	87	7.5	75	8.8	88
E	13.3	133	14.0	117	9.9	99	9.0	90	9.6	96
F	10.0	100	9.5	80	7.7	77	6.4	64	10.4	104
G	6.8	68	9.7	81	7.5	75	6.0	60	8.9	89
H	11.8	118	13.4[b]	113	10.6	106	9.0	90	9.1	91
I	7.6	76	14.5	122	9.0	90	7.8	78	8.2	82
J	11.6	116	12.3	103	8.2	82	7.5	75	13.1	131

[a] CP was spiked at 11.9 μg/g.
[b] Value removed after Cochran's test.

was 0.78 ng/mL, equivalent to a 0.31 mg/kg sample. Consequently, the practical determination range is between 0.78 and 50 ng/mL.

Extraction

The extraction procedure was common to both ELISA series. A 1.0 g portion of the test material was extracted using 19 mL of the extraction buffer. This extraction buffer was also common to both ELISA kits. The mixture was shaken horizontally overnight (16 h) at room temperature, and then centrifuged at 3000 × *g* for 20 min after adjustment of the pH to 6.0–8.0. The supernatant was filtered if necessary, diluted 20 times using each kit dilution buffer, and subjected to ELISA.

Calibration Standard Solutions

The calibration standard solutions were identical in both ELISA kits except for the dilution buffer from the initial extract used for the calibration standard solution. The initial extract for calibration standard solution was prepared from crustacean powder as follows: A 0.1 g sample of the crustacean powder was added to 20 mL PBS (10 mM Na-phosphate, 154 mM NaCl, pH 7.4) containing 5 g/L SDS, 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M EDTA (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10 000 × *g* for 30 min and the supernatant was filtered through a 0.8 μm micro-filter paper (DISMIC 25 cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100°C for 10 min. The protein content of the initial extract was assayed using a 2-D Quant Kit (GE Healthcare). The initial extract was diluted with each kit dilution buffer to compose the calibration standard solution (50 ng/mL of extracted protein) for each kit. The calibration standard solutions of CP were provided by Nippon Gene Co., Ltd (Toyama, Japan).

Interlaboratory Study

Ten laboratories participated in the interlaboratory evaluation, organized by the National Institute of Health Sciences (Tokyo, Japan). The participants included manufacturing companies, public research institutes, local public inspection institutes, and private inspection institutes. The organizer sent each laboratory the 5 test materials (3 g each) and 2 ELISA kits, plus the extraction solution and the calibration standard solutions. The participants took 2 portions from each test material, extracted the protein using the extraction procedure, and assayed each extract using the ELISA kits. The calibration standard solution was diluted and assayed simultaneously with the test material extracts. Each sample extract was analyzed in triplicate (3 wells/sample extraction), and the average absorbance of 3 wells was used for the calculation. The obtained absorbance data of calibration solutions and test materials were reported to the coordinator.

The organizer calculated a 4-parameter logistic calibration graph based on the absorbance data of the calibration standard solution and calculated the concentrations of CP in the test material using the calibration graph. Each set of samples was extrapolated from the standard curve run in the same plate.

The study was performed from July to August 2006.

Statistical Analysis

The values reported by the participants are summarized in Tables 2 and 3. Twenty data items, as 2 portions from 10 laboratories, were fed into the calculation. Cochran's test and Grubbs' test were used to remove outlying data (P = 2.5%). Cochran's test was used to remove the laboratory that reported a significantly large variability between the results of

Table 3. Results of the interlaboratory study for N kit: protein recovery content

	Fish sausage		Freeze-dried egg soup[a]		Tomato sauce		Creamy croquette		Chicken ball	
Lab	μg/g	%	μg/g	%	μg/g	%	μg/g	%	μg/g	%
A	5.5[b]	55	7.7	65	7.7	77	7.0	70	6.4	64
B	6.4	64	9.0	76	8.7	87	8.2	82	7.3	73
C	6.4	64	9.8	82	9.7	97	7.9	79	7.6	76
D	5.1[b]	51	8.2	69	8.3	83	7.2	72	6.7	67
E	6.4	64	9.2	77	8.4	84	8.1	81	6.9	69
F	6.4	64	8.2	69	8.6	86	8.0	80	7.1	71
G	6.4	64	8.4	70	8.6	86	7.6	76	6.9	69
H	6.4	64	8.6	72	8.6	86	7.7	77	7.8	78
I	6.8	68	9.8	83	9.0	90	7.9	79	8.3	83
J	6.7	67	8.6	72	8.4	84	7.9	79	7.2	72

[a] CP was spiked at 11.9 μg/g.
[b] Value removed after Grubbs' test.

the 2 portions taken from the test material. Grubbs' test was used to remove the laboratory that reported the mean of the results of 2 portions significantly different from other laboratories. The use of statistical tests for outliers follows the AOAC protocol (19). The removed values are also shown in Tables 2 and 3. Recovery, repeatability, and reproducibility were calculated by one-way ANOVA using the remaining data after the removal of outliers.

Results and Discussion

Homogeneity of the Test Materials

The resultant *F*-ratios of the homogeneity test regarding fish sausage, freeze-dried egg soup, tomato sauce, and creamy croquettes were <1.6. The critical value of *F* was 4.4, and the homogeneity of the test materials was sufficient for the interlaboratory evaluation. The *F*-ratio from chicken balls was slightly higher than the others, but lower than the critical *F*. For most test materials, the RSD values among portions were <5.5% and smaller than the required RSD_R values (≤25%).

Calibration Graph

Figure 1 shows the calibration graphs of the 2 ELISA kits. Both graphs have good correlation between 0.78 and 50 ng/mL, and give sufficiently high absorbance at 25 ng/mL, corresponding to 10 μg/g CP in the test materials.

Recovery

The recovery, repeatability (RSD_r), and reproducibility (RSD_R) calculated using ANOVA are shown in Table 4 with the number of remaining laboratories after removing the outliers. As shown in Table 4, the recoveries of CP from 5 types of test materials using the M kit were found to be 82–103%, whereas those using the N kit were 65–86%. The recoveries of CP determined by the M kit displayed a relatively high degree of recovery compared with those of the

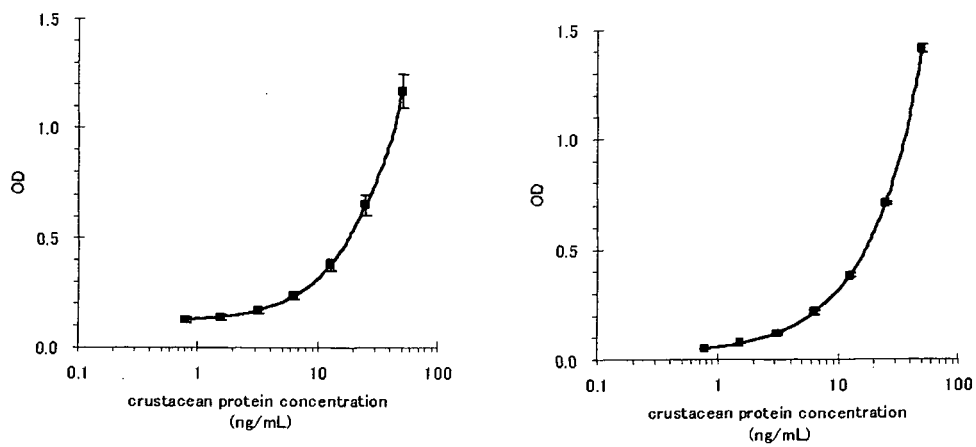

Figure 1. Crustacean protein calibration graphs of M kit (left) and N kit (right).

Table 4. Recovery, repeatability (RSD_r), and reproducibility (RSD_R) of the M kit and N kit for CP

Sample	No. of laboratories		Mean, μg/g		s_r, μg/g		s_R, μg/g		Recovery, %		RSD_r, %		RSD_R, %	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N
Fish sausage	10	8	10.3	6.5	0.5	0.3	2.1	0.3	103	65	4.9	4.0	20.5	4.0
Freeze-dried egg soup[a]	9	10	11.5	8.8	0.7	0.4	2.0	0.7	97	74	3.6	4.1	17.6	8.4
Tomato sauce	10	10	9.6	8.6	0.9	0.4	1.7	0.6	96	86	9.3	4.7	17.6	6.8
Creamy croquette	10	10	8.2	7.8	0.8	0.4	1.5	0.5	82	78	9.9	4.6	18.8	5.9
Chicken ball	10	10	10.0	7.2	0.6	0.4	1.9	0.6	100	72	6.1	5.1	19.2	8.4

[a] CP was spiked at 11.9 μg/g.

N kit for all test materials in the interlaboratory evaluation, being especially significant for fish sausage and chicken balls. We considered that the discrepancies in the recoveries of CP between the 2 kits could be due to some differences of the property of the antibody in each kit because the sample extract solutions from the test material were identical.

A 2-way ANOVA of the results of each kit and laboratory was performed for each test material. Significant differences were seen between the values determined by 2 kits. In addition, there appear to be differences among the results of the laboratories, although the differences are not statistically significant. Table 4 shows that all the values of the test materials determined by the M kit are higher than those determined by the N kits. These results suggest that the values determined by the M kit have a tendency to be higher than those of the N kits in identical materials.

Repeatability

Repeatability is a measure of the variance arising from the extraction and determination procedure in a particular laboratory. In intralaboratory evaluations, the RSD_r values of most cases were found to be <9.9%. The RSD_r values for the M kit materials in regard to tomato sauce and creamy croquette were approximately twice as large as those of the N kit. According to Horwitz theory, the RSD_r value generally is likely to be less than 2/3 of the RSD_R value (20). In this study, most RSD_r values for both kits were less than 2/3 of the corresponding RSD_R value, although some RSD_r values for the N kit were similar level to the corresponding RSD_R values due to the low RSD_R values.

Reproducibility

The reproducibilities, expressed by RSD_R values of CP from 5 types of test materials using the M kit, ranged between 17.6 and 20.5%, whereas those using the N kit ranged between 4.0 and 8.4%. The RSD_R values of the M kit were more than double those of the N kit. The N kit displayed a relatively high level of reproducibility compared with the M kit in the interlaboratory evaluation results.

With regard to repeatability and reproducibility, we considered that the differences between the 2 kits could be due to differences of the ruggedness of the assay performance in each kit, because the sample extract solution from the test material and calibration standard were almost identical except for the dilution buffer. Accordingly, the precision of the N kit performance is higher than that of the M kit in terms of the deviation effects of incubation times, reagent volumes, and reaction time with substrate.

Conclusions

The Japanese government MHLW established the interlaboratory validation protocol in the official guidelines published in 2006 (9). The outline of the interlaboratory validation protocol is as follows: number of laboratories, ≥8, number of incurred samples, ≥5; number of dose level, ≥1, including 10 μg/g (the corresponding allergen protein

weight/food weight); recovery, 50–150%; RSD_R, ≤25%. These criteria are based on ISO 5725 (JIS Z8402), which is mostly the same as that of AOAC (19). In the guidelines, the initial extract solution and the extraction procedure from allergen were specified and standardized.

The present study suggests that both test kits are reliable and precise methods to determine CP content, and the performance of both kits satisfies the validation criteria described in the official guidelines published by the Japanese government. Because food allergies can induce severe disease, the accuracy of the method is crucial. It is apparent from the data of interlaboratory evaluation that the M kit is more accurate than the N kit. On the other hand, the N kit produces much better repeatability and reproducibility measures than does the M kit because of the high precision of the assay performance of the N kit. The present interlaboratory evaluation was performed using 5 processed model foods, including highly processed foods such as fish sausage, freeze-dried egg soup, and tomato sauce, to ensure that these kits are able to detect CP from commercial foods and thereby guarantee accurate labeling. The creamy croquettes and chicken balls used in this study are not highly processed because they are generally marketed as frozen foods without undergoing heat processing in Japan. The present results demonstrate that both kits would be able to detect the CP contained in processed foods and thus support the food labeling system according to Japanese regulations.

Among the remarkable features of these kits are their unified extraction solutions and identical calibration standard solutions. These standardized features can provide a comparison of the assay performance of the 2 kits by comparing their results determined wih an identical calibrator as a common ruler without considering the deviation effects of the extraction and calibration standard.

In conclusion, the 2 ELISA kits provide rapid, precise, and reliable tools for the quantitative analysis of CP in processed foods. This proposed system is able to accurately monitor the labeling system in a reliable manner and can be useful for the inspection mandatory in the Japanese regulation.

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Original

Development of Event-Specific Quantitation Method for GA21 Maize, Which Is a GM Event without CaMV35S Promoter

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A real-time PCR detection method was developed for event-specific quantitation of Roundup Ready® maize, GA21. The developed PCR method was designed to amplify an artificial junction site between the native maize genome DNA and the recombinant DNA of GA21 maize, which provides only one target sequence per haploid of GA21 genome. Thus, the amplification efficiency of the event-specific target for GA21 became closely similar to the amplification of *SSIIb*, and the conversion factor (Cf) for the quantitation method was similar to the theoretical value. The developed method demonstrated better performance than the existing construct-specific method that has been used as a Japanese official method. The developed method can easily be combined with the real-time PCR targeting of the CaMV35S promoter, and the multiplexed method should be an effective screening method for GM maize.

Key words: genetically modified (GM); GA21 maize; Roundup Ready®; event-specific; real-time PCR

Introduction

Biotechnology has been used in modern farming since the initial commercialization of a genetically modified (GM) tomato in the USA in 1994. In fact, the global area of GM crops exceeded 100 million hectares in 2006[1)], and is expected to continue to rise. GM crops have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. However, consumers have been demanding appropriate information and labeling for foods derived from GM crops. Thus, labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia and other countries, and these systems are different from each other. For example, the threshold levels for the unintentional presence of GM materials in non-GM crops have been defined as 0.9% in the EU*[1], 3% in Korea*[2], and 5% in Japan[2)].

*[1] Regulation (EC) No. 1829/2003. Off. J. Eur. Union, L268, 1-23 (2003).

*[2] Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea. Seoul, Korea (2000).

For the monitoring of labeling systems, it has been necessary to develop methods for detecting GMOs in foods. Two approaches are available: detecting the protein produced by the introduced trait gene with a specific antibody, such as the enzyme-linked immunosorbent assay (ELISA) (reviewed in refs. 3 and 4); and detecting a specific DNA sequence used for gene modification by means of polymerase chain reaction (PCR) (reviewed in refs. 5-7). For the detection of GM maize in foods and food materials, PCR-based detection methods, which are able to detect even small amounts of transgenes in raw materials and processed foods, have been routinely used[8)-17)].

On the other hand, the cost and the duration of GM analyses could constitute one of the major bottlenecks for providing consumers with cost-effective labeling. The conventional or non-GM food supply chains, however, required to be secured by affordable detection methods. Thus, the development of time- and cost-effective detection methods is important to ensure freedom of choice of consumers.

Maize is a major agricultural crop which GM-

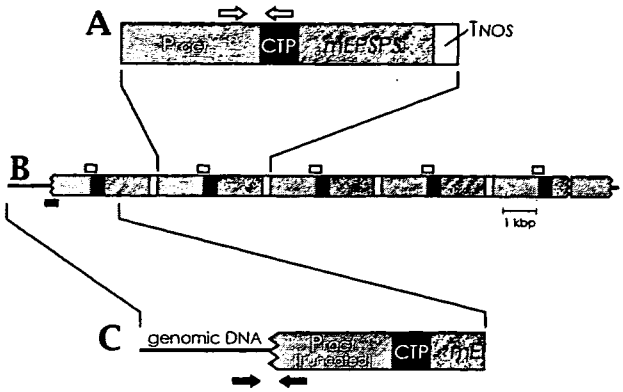

Fig. 1. Schematic models of the recombinant DNA of GA21

(A) The mEPSPS expression cassette consists of the rice actin1 promoter fragment including a part of non-coding region of rice *actin1* gene (indicated by a grey box), the artificial chloroplast transit peptide (CTP) sequence (filled box), the modified maize EPSPS gene (shaded box) and the nopaline synthase (NOS) terminator fragment (open Box). White arrows above the diagram indicate sites for the construct specific detection primer pair used in the currently available screening method. (B) The scheme of the overall recombinant DNA (r-DNA) fragment of GA21 event. The r-DNA consists of a truncated mEPEPS cassette including a truncated promoter, three complete mEPSPS cassettes, an mEPSPS gene-truncated cassette, and a truncated promoter fragment. The small open boxes above the model of r-DNA sequence indicate the detection target regions for the construct-specific method. The small filled box below it indicates the detection target region for the event-specific method developed in this study. (C) The scheme of the 5′ junction of maize genome (bar) and 5′ flanking region of r-DNA inserted in GA21 maize. Black arrows below it indicate sites for the event-specific detection primer developed in this study. These schemes are based on the developer's report[*5].

technology has been applied, and in fact, eight events of authorized GM maize, *i.e.*, Bt11, Event176, GA21, MON 810, MON863, NK603, T25 and TC1506, are currently used for commercial purposes in Japan. Analysis of the recombinant DNA (r-DNA) constructions of these eight GM maize events elucidated that all the events except GA21 maize contain the same promoter fragment, which originated from the cauliflower mosaic virus (CaMV) and is designated as P35S. The Ministry of Health, Labour and Welfare of Japan (MHLW) announced a combinational method for the quantitation of P35S and a construct-specific quantitation for GA21 maize. This method has been officially used as a screening method for GM maize[*3, 12], though it requires two independent quantitations. Thus, to improve the efficiency of the GM analysis method, we have been trying to develop a multiplex screening method for GM maize. For the multiplexing of PCR methods, it is preferable that the amplification efficiencies should be as similar as possible. From this viewpoint, the PCR system used in the already established construct-specific quantitation method for GA21 maize[12] was unsuitable for multiplexing with the PCR system for P35S. The reason for this is that the PCR system for P35S has one or two amplification target(s) in a set of the relevant GM maize genome, while the PCR system has four or five amplification targets in a set of GA21 maize genome[*4] (Fig. 1). Thus, in this study, we developed a new quantitation method for GA21 maize, which was event-specific and had one amplification target in a set of GA21 maize genome. In addition, we evaluated the developed method by means of a single-laboratory validation procedure.

Materials and Methods

Maize (Zea mays) and other cereal materials

Dry seeds of GM maize, *i.e.*, one progeny each of Bt11 and Event176 developed by Syngenta Seeds AG (Basel, Switzerland), a progeny of TC1507 developed by Dow Agrosciences LLC (Indianapolis, IN, USA), one progeny each of MON810, MON863, GA21, and NK603 developed by Monsanto Company (St. Louis, MO, USA), and a progeny of T25 developed by Bayer CropScience AG (Monheim am Rhein, Germany), were directly imported from the USA. Dry seeds of QC9651 maize (Quality Traders, Inc., Huntley, IL, USA) were also provided by the developer and used as a non-GM control. We used several other cereal materials as non-target controls, such as soy, rice, wheat and barley. Dry seeds of Roundup Ready® (RR) soy (Monsanto Company) were directly imported from the USA. Dry soybeans harvested in Ohio in 1998 were also directly imported and used as non-GM soy. Seeds of the rice (*Oryza sativa*) variety Kinuhikari, the wheat (*Triticum aestivum*) variety Haruyutaka and the barley (*Hordeum vulgare*) variety Harrington were obtained in Japan.

Preparation of test samples and DNA extraction

Dry seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany). The ground materials were freeze-dried for 24 hr in an FDU-540 freeze drier (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored at −20°C until use. The four levels of simulated GM-mixture samples containing 0.5, 1, 5, and 10% (w/w) of each of MON810 and GA21 maize in non-GM maize were made from the ground materials. DNA extraction was performed with an DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) as described in our previous report[12]. The DNA concentration of

[*3] Notification No. 110 (Mar. 27, 2001), Department of Food Safety, MHLW, Japan (2001).

[*4] Summary notification information format (SNIF) for products containing genetically modified higher plant (GMHP) —GA21 Roundup Ready maize—; based on Notifications for placing transgenic plants on the EU Market under Directive 2001/18/EC. Monsanto Europe S. A. (1998).

solutions was determined by measuring UV absorbance at 260 nm, and the quality was evaluated from the absorbance ratios at 260/280 nm and 260/230 nm. In the majority of maize varieties studied, the absorption ratio at 260/280 nm was between 1.7 and 2.0, and that at 260/230 nm was >1.7. These DNA samples were used for the subsequent experiment.

Oligonucleotide primers and probes

To design primers, we used Oligo™ primer analysis software (National Biosciences Inc., Plymouth, NM, USA) and Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer Express software was also used for the design of GA21 esp-Taq probe. The sequences of oligonucleotides were checked using a public database (DDBJ), and it was confirmed that they did not match unintended DNA sequences. The primer pairs used in this study are listed in Table 1. These primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and purified by HPLC. The TaqMan Probes were synthesized by Applied Biosystems, and labeled with 5-carboxyfluorescein (FAM) at the 5′-end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3′-end. Each oligonucleotide was diluted to the ape appropriate volume of sterilized water.

DNA sequencing

The fragment was amplified with GA21 genome 1 primer and GA21 3′-m primer, and subcloned into the pGEM®-T vector (Promega Co., Madison, WI, USA). The subcloned fragment was sequenced with a CEQ™2000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA) by the dideoxy sequencing method using a DTSC quick start kit (Beckman Coulter).

Qualitative PCR conditions

The reaction volume of 25 μL contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.5 μmol/L of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The reactions were buffered with the PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96-Well GeneAmp PCR System 9700 (Applied Biosystems) in Max mode, according to the following step-cycle program: pre-incubation at 95°C for 10 min; 40 cycles consisting of denaturation at 95°C for 0.5 min, annealing at 60°C for 1 min and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min.

Quantitative PCR conditions and calculation of GM contents

In general, the experimental procedures of quantitative PCRs followed the established methods we reported previously[12], that were also employed in Japanese official methods for the detection of GM food[*3]. For quantitative PCR, the reaction volume of 25 μL contained 12.5 μL of Universal Master Mix (Applied Biosystems), 50 ng genomic DNA as template, 0.5 μmol/L of the 5′ and 3′ primers, and 0.2 μmol/L of TaqMan® probe. Sample DNA was diluted to 20 ng/μL in TE buffer (pH 8.0), and 2.5 μL of the dilution was added to the reaction mixture. The real-time PCR reactions were performed with an ABI PRISM 7700 (Applied Biosystems) according to the following step-cycle program; pre-incubation at 50°C for 2 min and 90°C for 10 min, 40 cycles consisting of denaturation at 95°C for 0.5 min, annealing and extension at 59°C for 1 min. For the event-specific quantitation method, we constructed a plasmid molecule that is a pUC19 plasmid vector with two inserted fragments, *i.e.*, the fragment amplified from pMul5 plasmid[12] with SSIIb01-5′ primer and P35 S 1-5′ and the fragment amplified from GA21 maize genome with GA21esp5′-2 and GA21esp3′-1, and used it for experimental calibration. The plasmid was extracted from bacterial bulk culture using the QIAGEN® plasmid Mega kit (QIAGEN) and linearized by restriction digestion at sites located outside the inserted fragments. The linearized plasmid DNA was purified by CsCl density gradient centrifugation and the purified DNA solution was diluted with TE buffer including 5 ng/μL of ColE1 plasmid to five different concentrations, *i.e.*, 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μL, based on the quantitative copy numbers of *SSIIb* fragments.

Results and Discussion

Confirmation of genomic DNA sequence of GA21 maize

To design event-specific primers for GA21 maize, we first confirmed the DNA sequence of the artificial junction region between the native maize genome DNA and r-DNA of GA21 maize. A 112 bp part of the junction region was amplified by a primer pair, GA21 5′-m and GA21 3′-m[*5] (Table 1) and sequenced. Using this sequence, the genomic clone sequence (Accession# CG 765412) was found by BLAST search of the DDBJ database. Then, we designed a primer, GA21 genome1,

Table 1. List of primers

Name	Sequence	Specificity
GA21 5′-m (GA21 primer1)	5′-CTTCTCGTTATGCTATTTGCAACTTTAGA-3′	Maize genome
GA21 3′-m (GA21 primer2)	5′-TGGCTCGCGATCCTCCT-3′	Rice ACT1 5′-UTR[a]
GA21 genome1	5′-TCCCAGTCTCGACGATTCACG-3′	Maize genome
GA21esp 5′-1	5′-TGTAATGGGACCTTATCGTTATGC-3′	Maize genome
GA21esp 5′-2	5′-TGGGACCTTATCGTTATGCTATTTG-3′	Maize genome
GA21esp 3′-1	5′-CGATCCTCCTCGCGTTTCC-3′	Rice ACT1 5′-UTR[a]

[a] Rice *ACT1* 5′-untranslated region (UTR) sequence contains the promoter and 1st exon of rice *ACTIN1* gene.

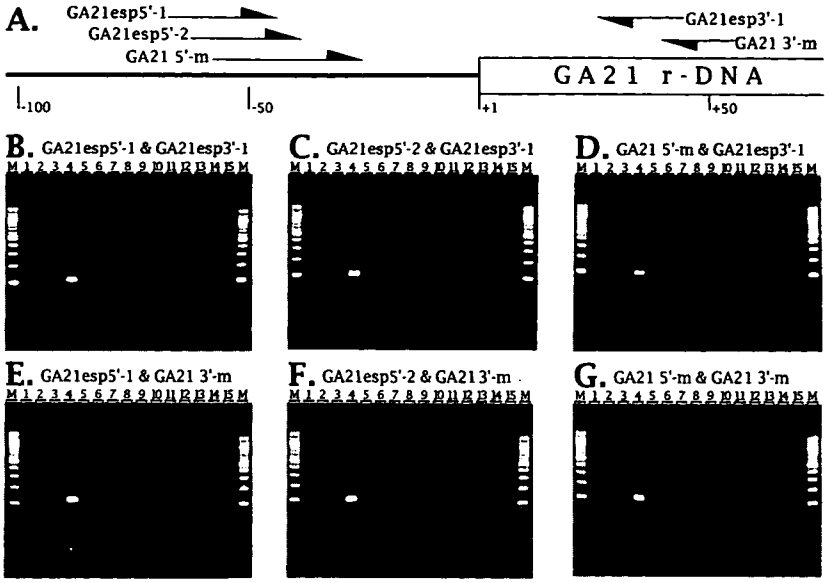

Fig. 2. (A) Schematic model of the positions for examined primers

The maize genome and GA21 r-DNA are indicated by a thick line and an open box, respectively. The arrows above the model indicate primers' position. The specificities of primer pairs were examined with GA21 maize.

(B–G) Specificities of examined primer pairs

Each photo shows the electrophoresis image for the amplification with the primer pair described at the top of each photo. Lanes 1–8, amplification of DNAs extracted from the seeds of NK603, Event176, T25, GA21, MON863, MON 810, TC1507 and Bt11, respectively; lane 9, non-GM maize; lane 10, non-GM soy; lane 11, Roundup Ready$^{(R)}$ soy; lane 12, rice; lane 13, wheat; and lane 14, barley. Lane 15 is the negative control (no template) and lane M shows 100 bp ladder size standards. Each electrophoresis was performed on 3% agarose gel.

on the flanking genome sequence, and used it for amplification with GA21 3′-m primer. Finally, we cloned a 325 bp fragment of the junction sequence that contained 268 bp of native maize genomic DNA and 57 bp of r-DNA of GA21 maize.

Design of the event-specific primers for GA21 maize

The design of the novel event-specific detection primer pair was based on the sequencing result of the clone described above. We designed two forward primers on the maize genomic fragment and a reverse primer on the r-DNA fragment. Then, we evaluated the amplification performance of six pairs of primers, including the primers released from Monsanto Co.*[5] (Fig. 2A). To harmonize with the methods developed previously, the experimental conditions followed those of the Japanese official PCR method for GM maize detection, including the concentrations of the reaction solution and the thermal cycle program. To evaluate the specificities of these primer pairs at the same time, DNAs extracted from the seeds of each of eight major events of GM maize authorized in Japan and from other cereal crops were used as the templates for PCR. The results indicated that all of tested primer pairs were specific for GA21 maize (Figs. 2B–G). However, some non-specific bands that were different in length were observed in reactions with GA21 3′-m primer. In addition, the length of this amplification product of this pair is nearly equal into that to the amplification product of the SSIIb03 primer pair (114 bp), which is the taxonomic control reaction of the real-time PCR quantitations of GM maize. As a result, we finally chose an event-specific primer pair, namely GA21 esp5′-2 and GA21 esp3′-1 that amplified a 111 bp of fragment across the artificial junction site (Fig. 1). This primer pair clearly amplified a PCR product using the DNA extracted from GA21 maize as a template (Fig. 2C). In contrast, no product was amplified from other events of GM maize, non-GM maize and other crops, *i.e.*, soy, rice, wheat and barley (Fig. 2C). Therefore, we concluded that the new primer pair was specific to GA21 maize, and demonstrated an equivalent performance with the established Japanese official method under the same experimental conditions.

Quantitation with GA21 maize event-specific primer pair

To apply the GA21 event-specific primer pair to the quantitation method based on real-time PCR system, we designed a TaqMan probe that contained 30 nucleotides of DNA fragment included in the amplification product. To develop the quantitation method following our previous methods[12], we also constructed a new plasmid molecule for analytical calibration. The calibrant plasmid contained the amplicon of GA21 maize event-specific primer pair and the amplicon of SSIIb03 primer pair in a plasmid vector originated from pUC19

*[5] Community reference laboratory (CRL) for GM food and feed, E.C. Event-specific method for the quantitation of maize line GA21 using real-time PCR (2005).

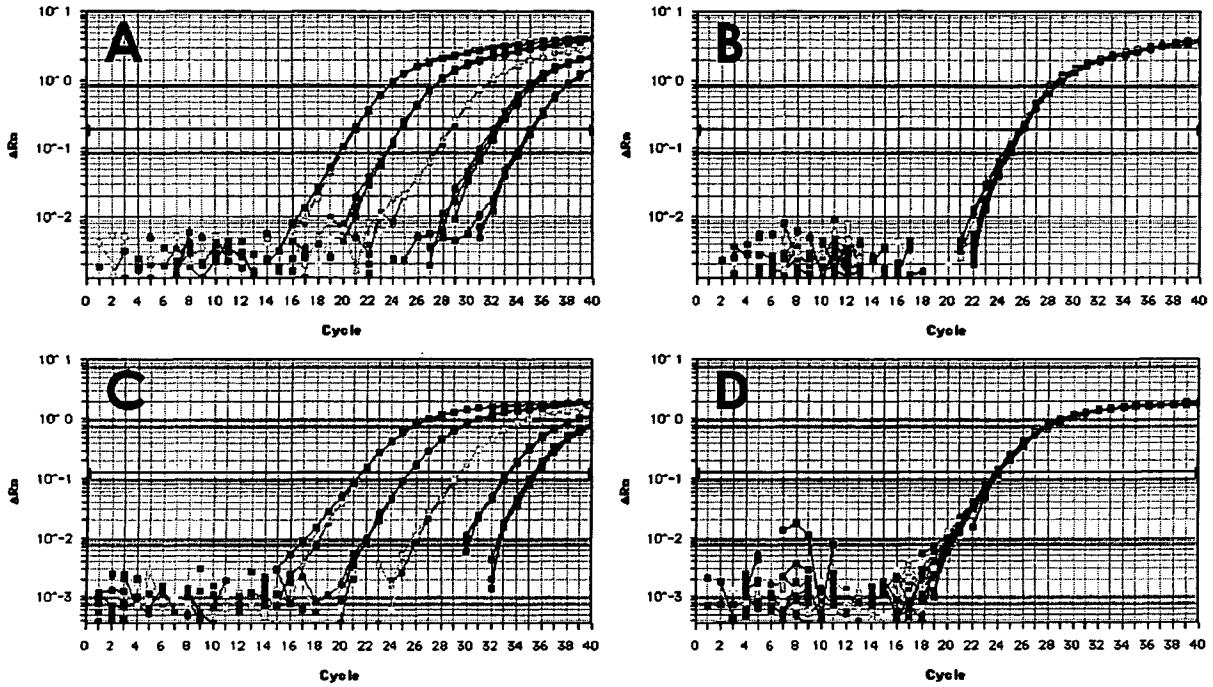

Fig. 3. Amplification plots of event-specific real-time PCRs (A and B) and construct-specific real-time PCRs (C and D)

The amplification plots for the plasmid standards are shown in A and C, and those for genomic DNA extracted from GA21 maize are shown in B and D. Horizontal axis indicates the cycle numbers of the thermal cycle reaction and vertical axis indicates the fluorescence intensity level (logarithmic scale).

plasmid, and was diluted to a concentration ranging from 20 to 250,000 copies per reaction, and used for the following experiments. Figure 3 shows typical amplification plots for a series of plasmid calibrants and the genomic DNA extracted from GA21 maize. Comparing amplification plots between the event-specific method and the established method that is construct-specific, no significant difference was found in plots for calibrant plasmids, because a single amplification target on each molecule was used (Figs. 3A and C). On the other hand, in comparison with amplification plots for the genomic DNA extracted from GA21 maize, logarithmic amplification phases of the event-specific method were sharper than that of the established method (Figs. 3B and D). Figure 4 shows the calculated copy numbers of the target in 50 ng of GA21 maize genomic DNA. The result revealed that the single copy of the event-specific target in a set of GA21 maize genome not only reduced the measurement values for the DNA extracted from genuine GM seeds, but also increased the precision of the measurements (Fig. 4). The stability of the measurements also contributed to the higher precision of the conversion factor (Cf). The Cf values were defined based on the measurement values for the DNA extracted from genuine GM seeds and were calculated by dividing the copy numbers of targets by those of the taxon-specific fragment (*SSIIb*)[12),18)]. In theory, the Cf value should be 0.5 for the event-specific method because of the heterogeneity of the single copy for the target and the homogeneity of the single copy for *SSIIb*, but is 2.5 for the construct-specific method because of the heterogenous five copies for the target and the homogenous single copy for *SSIIb*. In this study, we experimentally defined Cf for the event-specific method

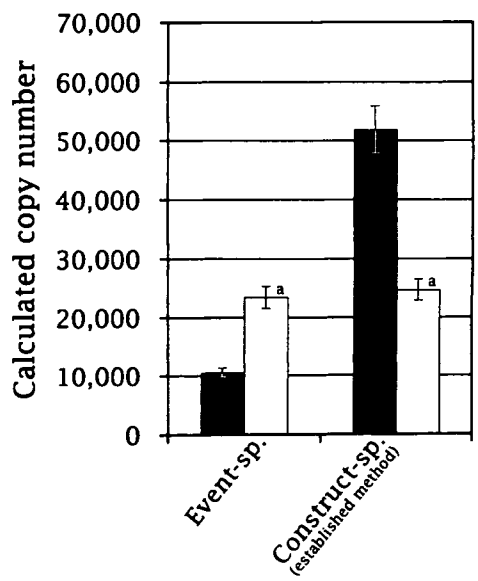

Fig. 4. Comparison of calculated copy numbers between the event-specific method and the construct-specific method

The calculated copy numbers of GA21 specific target sequence and the taxonomic control sequence, *SSIIb*, are indicated with solid bars and open bars, respectively. Ten lots of samples were measured in triplicate for each method. The difference of the copy numbers of *SSIIb* is not significant ($p=0.15$). The error bars indicate the standard deviation of the measurements.

Table 2. Homogeneity of simulated mixtures

Method[a]	Mixing level, %	Measured mean, %[b]	95% Confidence limit[b]		F-value	p-value
			Lower, %	Upper, %		
Event-specific	0.5	0.49	0.43	0.56	0.42	0.89
	1.0	0.94	0.88	1.01	1.97	0.15
	5.0	4.29	4.04	4.55	1.91	0.16
	10.0	10.47	9.92	11.03	0.11	1.00
Construct-specific	0.5	0.52	0.50	0.55	1.90	0.17
	1.0	1.01	0.95	1.07	0.43	0.89
	5.0	4.63	4.36	4.93	0.19	0.99
	10.0	9.34	8.91	9.78	1.54	0.25

[a] Measurements of ten lots with two replications were transformed using the logit transformation and were analyzed by one-way ANOVA.
[b] Mean and 95% confidence limits were calculated based on logistic valuses and transformed using the inverse logit transformation.

Table 3. Trueness and precision statistics for quantitation method

Methoda	Mixing level, %	Trueness		Precision	
		Measured mean, %	Bias, relative%	SD[b]	RSD[c]
Event-specific	0.5	0.50	−0.57	0.053	11
	1.0	0.95	−5.3	0.091	9.7
	5.0	4.30	−14	0.34	8.0
	10.0	10.50	5.0	0.28	2.6
Construct-specific	0.5	0.52	4.8	0.034	6.5
	1.0	1.01	1.4	0.057	5.6
	5.0	4.65	−6.9	0.17	3.6
	10.0	9.36	−6.4	0.55	5.9

[a] Statistical analyses were conducted for ten lots with two replications.
[b] SD; Sandard deviation
[c] RSD; Relative standard deviation

as 0.45, and that for the construct-specific method as 2.09. The similarity of experimental and theoretical Cf values may imply that the amplification efficiency of the event-specific target for GA21 maize is closely similar to that of *SSIIb*.

Single-laboratory evaluation

A single-laboratory evaluation for the event-specific quantitation method of GA21 maize was planned, referring to the harmonized guidelines of IUPAC, ISO, and AOAC[19]. For this purpose, we prepared four sorts of simulated maize mixtures with different commingled levels of GA21 maize as analytical samples. Each level of mixtures was divided into aliquots of about 1 g (about one or two hundred independent packets), and ten packets were chosen at random from each commingled level of samples for this experiment. All of the samples were subjected to quantitation with the event-specific method and the established construct-specific method. The measurement was done twice for each sample. To conduct these measurements, we first evaluated the homogeneity of each simulated mixture by the one-way ANOVA test. Table 2 shows the results of one-way ANOVA tests of the logit-transformed means for each duplicate quantitation. These results showed that the between-lot variations were not different from the within-lot variations in each sort of simulated mixture (Table 2). Thus, we concluded that the simulated mixtures used in the experiment were sufficiently homogenous within each sort. Then, using these measurements, we performed the preliminary method validation for the event-specific method, and compared the resuls with those for the existing construct-specific method. Table 3 shows the statistical analysis of the results for the event-specific method and the construct-specific method, respectively. The relative standard deviation (RSD) scores for the event-specific method and the construct-specific method ranged from 2.6% (10% mixture) to 11% (0.5% mixture) and from 3.6% (5% mixture) to 6.5% (0.5% mixture), respectively. The RSD scores of these two methods were found below 20% (Table 3). The results of single-laboratory evaluation tests suggested that the precision of the event-specific method was sufficient for the quantitation of GA21 maize, although interlaboratory collaborative studies should be conducted to confirm this.

In conclusion, the event-specific quantitation method for GA21 maize developed in this study demonstrated better performance than the established construct-specific method that is a Japanese official method. This newly developed method has the advantage that the detection target is present as a single copy per set of GM genome. Based on this study, we are trying to develop a simple and rapid detection method for GM crop

screening.

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RETINOIC ACIDS ACTING THROUGH RETINOID RECEPTORS PROTECT HIPPOCAMPAL NEURONS FROM OXYGEN-GLUCOSE DEPRIVATION-MEDIATED CELL DEATH BY INHIBITION OF C-JUN-N-TERMINAL KINASE AND p38 MITOGEN-ACTIVATED PROTEIN KINASE

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Abstract—Retinoic acids (RAs), including all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), play fundamental roles in a variety of physiological events in vertebrates, through their specific nuclear receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR). Despite the physiological importance of RA, their functional significance under pathological conditions is not well understood. We examined the effect of ATRA on oxygen/glucose-deprivation/reperfusion (OGD/Rep)-induced neuronal damage in cultured rat hippocampal slices, and found that ATRA significantly reduced neuronal death. The cytoprotective effect of ATRA was observed not only in cornu ammonis (CA) 1 but also in CA2 and dentate gyrus (DG), and was attenuated by selective antagonists for RAR or RXR. By contrast, in the CA3 region, no protective effects of ATRA were observed. The OGD/Rep also increased phosphorylated forms of c-jun-N-terminal kinase (P-JNK) and p38 (P-p38) in hippocampus, and specific inhibitors for these kinases protected neurons. ATRA prevented the increases in P-JNK and P-p38 after OGD/Rep, as well as the decrease in NeuN and its shrinkage, all of which were inhibited by antagonists for RAR or RXR. These findings suggest that the ATRA signaling via retinoid receptors results in the inhibition of JNK and p38 activation, leading to the protection of neurons against OGD/Rep-induced damage in the rat hippocampus.

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Abbreviations: ATRA, all-*trans* retinoic acid; CA, cornu ammonis; DG, dentate gyrus; ERK, extracellular signal-regulated kinase; Hanks' BSS, Hanks' balanced salt solution; JNK, c-jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; OGD, oxygen and glucose deprivation; PBS-T, phosphate-buffered saline with 0.3% Triton X-100, pH 7.6; P-ERK, phosphorylated extracellular signal-regulated kinase; PI, propidium iodide; P-JNK, phosphorylated c-jun-N-terminal kinase; P-p38, phosphorylated p38; RA, retinoic acid; RAR, retinoic acid receptor; Rep, reperfusion; RXR, retinoid X receptor; ssDNA, single-stranded DNA; TBS/T, Tris-buffered saline containing 0.1% Tween-20; 9-*cis* RA, 9-*cis* retinoic acid.

Key words: retinoic acid, RAR, RXR, MAPK, ischemia.

Retinoids, including vitamin A (retinol) and its derivatives, regulate a wide range of biological processes, such as cell growth and differentiation, development, and carcinogenesis (Chambon, 1996; Maden, 2001). Retinoic acids (RAs) regulate the expression of a large number of genes upon binding and activation of the nuclear retinoid receptors, retinoic acid receptors (RARα, RARβ, and RARγ) and retinoid X receptors (RXRα, RXRβ, and RXRγ) (Chambon, 1996; Maden, 2001). RARs are activated by all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), whereas RXRs are activated by 9-*cis* RA (Chambon, 1996) and other non-retinoid lipid ligands such as docosahexaenoic acid (de Urquiza et al., 2000). In the presence of RA, these receptors act as transcription factors in forms of RAR/RXR heterodimers or RXR homodimers, which bind to retinoic acid response elements (RARE) in the promoter of target genes (Chambon, 1994; Kastner et al., 1997). RA has multiple effects on physiological functions in the adult brain, such as long-term potentiation and long-term depression (Chiang et al., 1998; Misner et al., 2001) and neurogenesis (Haskell and LaMantia, 2005; Jacobs et al., 2006). RA also plays significant roles under pathological conditions. For example, in mesangial cells and fibroblast, ATRA has a protective effect against H_2O_2 via receptor-mediated mechanisms (Konta et al., 2001; Xu et al., 2002). Although several lines of evidence link RA to some psychiatric pathogenesis (Strahan and Raimer, 2006), defects in retinoid signaling have been associated with neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis (Corcoran et al., 2002; Goodman and Pardee, 2003). However, the role of RA signaling in other pathological conditions such as ischemia in the brain remains unclear.

The mitogen-activated protein kinase (MAPK) family, which plays an essential role in the transduction of environmental stimuli to the nucleus, consists of three commonly recognized subgroups: extracellular signal-regulated kinase (ERK), c-jun-N-terminal kinase (JNK), also known as the stress activated protein kinase (SAPK) and p38 kinase. JNK and p38 kinase are activated in response to cellular stresses like ischemia in the heart, kidney and brain (Hu and Wieloch, 1994; Mizukami et al., 1997; Yin et al., 1997; Herdegen et al., 1998; Walton et al., 1998) and have been associated with neuronal cell death (Xia et al., 1995; Watson et al., 1998; Namgung and Xia, 2000). JNK

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and p38 kinase activate downstream molecules such as caspase-3 (Kuan et al., 2003; Lee and Lo, 2003), Bax (Okuno et al., 2004), MAPK-activated protein kinase 2 (MAPKAP2) (Wang et al., 2002) and activator protein-1 (AP-1) (Ishikawa et al., 1997; Yokoo and Kitamura, 1997; Behrens et al., 1999) thereby leading of neuronal cell death. In contrast, ERK1/2 is mainly activated by various neurotransmitters, hormones and growth factors, controlling transcription factor activity to induce various physiological responses, such as cell proliferation or differentiation (Boulton et al., 1991; Marshall, 1995; Segal and Greenberg, 1996). However, ERK1/2 is also activated by various types of stress such as oxidative or shear stress, controlling the survival of cells (Xia et al., 1995; Guyton et al., 1996; Wang et al., 1998). Recent studies have shown that ATRA protects neurons from amyloidβ (Sahin et al., 2005) and staurosporine (Ahlemeyer and Krieglstein, 1998). Based on the findings above, we hypothesized that RA signaling would be inversely associated with neuronal cell death under pathological conditions. To test our hypothesis, we examined the effect of ATRA on hippocampal neurons against oxygen and glucose deprivation/reperfusion (OGD/Rep)-induced neuronal damage, and found that ATRA protected hippocampal neurons against cell death. We also found that the neuroprotective effect of ATRA was mediated by inhibition of OGD/Rep-induced activation of JNK and p38 kinase.

EXPERIMENTAL PROCEDURES

Materials

Propidium iodide (PI) and anti-β-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The sources of the following chemicals are shown in parentheses: ATRA and 9-*cis* RA (Wako Pure Chemicals, Osaka, Japan), U0126, SB203580, SP600125 and anti-NeuN antibody (Calbiochem Biosciences, Inc., San Diego, CA, USA). LE135 (Li et al., 1999) and HX531 (Ebisawa et al., 1999) were synthesized and graciously provided by Dr. Koichi Shudo (Research Foundation Itsuu Laboratory, Tokyo, Japan). Antibodies against phosphorylated extracellular signal-regulated kinase 1/2 (P-ERK1/2), phosphorylated p38 (P-p38), phosphorylated c-jun-N-terminal kinase (P-JNK), and active caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The sources of the following antibodies are shown in parentheses: single-stranded DNA (ssDNA) antibody (DAKO Cytomation, Glostrup, Denmark), RAR and RXR antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Organotypic hippocampal slice culture

Organotypic slice cultures of the hippocampus were prepared using the culture method described by Skrede and Westgaard (1971) All animals were treated in accordance with the laboratory animal care guidance of the National Institute of Health Sciences at Tokyo and the guidelines of the International Council for Laboratory Animal Science (ICLAS; http://www.iclas.org/). Every effort was made to minimize the number of experimental animals used and their suffering. Eleven-day-old Wistar rats were decapitated, and the brains were rapidly dissected and placed in a Petri dish containing ice-cold 1× Hanks' balanced salt solution (Hanks' BSS, from Gibco, Rockville, MD, USA). Both right and left hippocampi were isolated and sectioned into 300 μm transverse slices with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Goose Green, UK). The slices were then carefully separated and transferred onto porous membrane inserts of six-well culture plates (two or three slices per insert) (Millicell-CM, Millipore, Billerica, MA, USA). To reach the level of insert membrane, 800 μL culture medium was added to the lower compartment of each well, and the culture plates were then placed in a 37 °C humidified incubator enriched with 5% CO_2. The cell culture medium consisted of 50% Minimum Essential Medium with 25 mM Hepes, 25% Hanks' BSS and 25% heat-inactivated horse serum, which were supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. On the next day, the culture medium was replaced with fresh medium and from then on changed once every 2 days. Hippocampal slices were used for each experiment after incubation for 12 days.

OGD

Hippocampal slices were exposed to OGD using an anaerobic chamber. The slices were transferred in dishes with 800 μl glucose-free DMEM containing (mg/L): 200 $CaCl_2$, 400 KCl, 97.67 $MgSO_4$, 6400 NaCl, 3700 $NaHCO_3$, 125 NaH_2PO_4 and then placed into an airtight chamber containing 95% N_2, 5% CO_2 (37 °C) for 40 min. After OGD, the slices were placed back in the incubator in normal culture medium and incubated for 24 h. RAs were added to the culture medium 24 h before OGD and during Rep. Antagonists for RAR and RXR were added to the medium 30 min before and during the RA treatment. Inhibitors of MAPKs were added to the slices simultaneously at Rep.

Immunohistochemistry

The hippocampal slices were fixed with 4% paraformaldehyde for 1–2 h and rinsed two times (each 10 min at room temperature) with PBS-T (phosphate-buffered saline with 0.3% Triton X-100, pH 7.6). After blocking with 3% normal goat serum in PBS-T for 2 h at room temperature, the slices were incubated with the primary antibody (1/1000-fold dilution in PBS-T with 3% goat serum) for 48 h at 4 °C, followed by incubation with the Alexa Fluor-conjugated secondary antibody (1/1000-fold dilution, Molecular Probes, Eugene, OR, USA) for 3 h at room temperature. Images were collected with a MRC-1024 laser-scanning microscope (Bio-Rad, Richmond, CA, USA) using ×4 or ×20 objective lenses. For comparisons of double-stained patterns, images were processed using Photoshop CS (Adobe Systems, Mountain View, CA, USA). High magnification images of the NeuN/active caspase-3/ssDNA/P-p38/P-JNK staining are shown for the cornu ammonis 1 (CA1) region, unless otherwise indicated.

Western blotting analysis of slices

Slices were homogenized in 100 μl lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 1% deoxycholate, and 10 μg/mL each aprotinin, bestatin, pepstatin A, leupeptin), using Tissue Lyser (Qiagen, Hilden, Germany). The proteins were separated in 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBS/T) and 5% non-fat dry milk at room temperature. Then the membranes were incubated with the primary antibody (1/1000-fold dilution in TBS/T containing 5% BSA) overnight at 4 °C. After three washes with TBS/T, the membranes were incubated with a horseradish peroxidase–conjugated anti-rabbit antibody (1/2000 dilution in TBS/T containing 5% non-fat dry milk) for 1 h at room temperature. The membranes were washed with TBS/T three times, and the proteins were visualized by chemiluminescence.