Table 2. PCR primer list for the *SSIIb* gene

Primer name	Sequence	Length of amplified DNA fragment (bp)
F1	5'-ACGGTGGTTGGCCTACCGCCTACGTCG-3'	112
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	
F2	5'-GTCGCAGGACCTGGACGTTATTTAGAA-3'	197
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	
F3	5'-CTGGGACCACGCCGCCGTGCTGTATGA-3'	289
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	
F4	5'-CCGCGCGGAGGCGAACCGGATGATCGA-3'	403
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	
F5	5'-CGGGACCGTGCCCGTGGTGCACGCCGT-3'	499
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	
F6	5'-CGGGACCGTGCCCGTGGTGCACGCCGT-3'	602
R1	5'-CATCGCCGTCGGTTTCATATCATACTA-3'	

4. Qualitative PCR to determine the degree of fragmentation of the genomic DNA of processed corn grits.

The primer pairs for the corn starch synthase IIb (*SSIIb*) gene, which is used as the internal standard gene of the Japanese official quantification methods, were designed and synthesized. These primer pairs would give PCR products at an interval of approximately 100 bp over a range from 100 bp to 600 bp (Table 2). The reaction mixture of 50 μL consisted of 5 μL of 10 x reaction buffer, 0.25 mmol/L of dNTP, 0.25 μmol/L of each primer, one unit of *Taq* DNA polymerase (TaKaRa Biochemicals, Japan) and about 50 ng of extracted corn genomic DNA. The reaction mixtures were set in the PCR machine (the GeneAmp PCR System 9600 (Applied Biosystems, Japan)) and heated at 94℃ for 2 min, then 40 cycles of the reaction at 94℃ for 30 sec, 55℃ for 1 min and 72℃ for 1 min.

5. Quantification of copy numbers of *SSIIb* and transgenes in the corn genomic DNAs prepared from processed grits by TaqMan PCR

Quantification of copy numbers of the *SSIIb* gene was according to the Japanese official method[15), 16)]. The primers shown in the Japanese official method were used in this study. The reaction mixture of 25 μL/well consisted of 12.5 μL of 2 x Universal PCR Master Mix, 0.5 μmol/L of each primer, 0.2 μmol/L of TaqMan probe and about 50 ng of the extracted corn genomic DNA. The reaction plate was set in the ABI PRISM™ 7700(Applied Biosystems, Japan) and heated at 50℃ for 2 min, followed by heating at 95℃ for 10 min, then 40 cycles of the shuttle reaction at 95℃ for 30 sec and 59℃ for 1 min.

III. Results and Discussion

1. Amplification of DNA fragments by PCR with corn genomic DNAs prepared from fry and extruder processed foods.

To elucidate genomic DNA fragmentation by heating (fry processing) or heating with a pressure treatment (extruder processing), genomic DNA was extracted from processed corn grit products, and target DNA fragments of the *SSIIb* gene were amplified by PCR using several designed primer pairs to give lengths from about 100 bp to 600 bp with a 100 bp interval. With the heating treatment at 130℃ or 160℃, DNA fragments up to 602 bp could be amplified, while fragments shorter than 403 bp could be amplified even at 180℃. These results indicated that heating at temperatures lower than 160℃ for 1 min did not cause the fragmentation of corn genomic DNA shorter than 600 bp. However, heating at 180℃ caused significant fragmentation less than 400 bp in size (Fig. 1). In our previous report[19)], the heat process used to prepare "tofu" from soybean seeds by boiling at 100℃ did not cause the fragmentation of soybean genomic DNA shorter than 600 bp. Our results presented here indicate that genomic DNA fragments of 400 bp could remain intact by heating at the much higher temperatures (130, 160, 180℃) used in fry processing than that with boiling processing. In contrast to fry processing, extruder processing even at 128℃ caused genomic DNA fragmentation shorter than 197 bp (Fig. 1), while at much higher temperatures, no DNA fragments could be amplified (Fig. 1). These results suggest that severe genomic DNA fragmentation occurred under both a heating and pressure treatment.

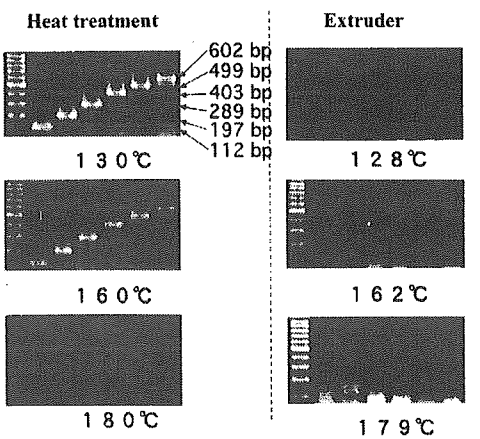

Fig. 1. Fragmentation of the *SSIIb* gene of processed corn grits. Agarose gel electrophoresis of the amplified products derived from the genomic DNAs extracted from MON810 corn grits processed with heat (left) and the extruder (right) using primer pairs for the detection of the *SSIIb* gene shown in the Table 2.

2. Detection of DNA fragments by qualitative PCR for DNAs prepared from the corn-processed foods in markets.

Since the model processing experiments above showed that significant fragmentation of corn genomic DNA occurred during processing, five corn snacks (A to E) were purchased from markets and their genomic DNAs were extracted to assess how fragmentation of corn genomic DNA occurred in processing (Fig. 2). DNA fragments up to about 500 bp could be amplified by PCR for the genomic DNAs prepared from corn snacks of A, B and C, whereas no fragments could be detected from D and E. All of the corn snacks from A to E might be cooked using an extruder, but the processing conditions were thought to be different. These differences were mainly the pressure, which was regulated by the screw speed and the extruder temperature. The screw introduces the slurry of corn grits and the block heats them, and then a pinhole in the extruder instantly releases the pressure to atmospheric conditions. The procedure results in a sudden release of water as vapor from the grit slurry and the production of the snacks. When both the screw speed and block temperature are low, the slurry receives low pressure and the processed snacks are soft and damp, while high speed screwing at high temperature gives high pressure, which results in hard and dry snacks. The critical difference of screw speed and block temperature might be the most important cooking step that differentiates one snack from another at the markets. It might also give rise to the range and severity of fragmentation of genomic DNA in the corn grits.

The target DNA fragment could not be amplified in the case of corn starch (Fig.2F). This might be caused by the absence of genomic DNA, which is removed during producing the starch.

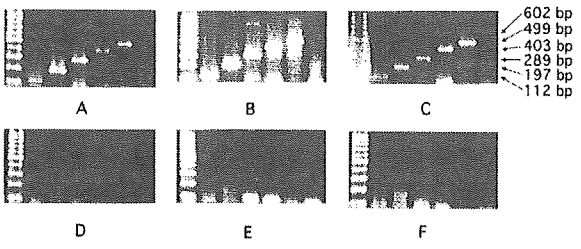

Fig. 2. Fragmentation of the *SSIIb* gene of snacks and starch made from corn in markets. Agarose gel electrophoresis of the amplified products derived from the DNAs extracted from corn snacks (A-E) and corn starch (F) purchased from markets using *SSIIb* primers shown in Table 2.

3. Quantification of the *SSIIb* gene in the processed corn grits by frying or heat/pressure processing with an extruder using the TaqMan PCR method.

Corn grits were processed by heating using an oil bath, and genomic DNA extracted from the processed grits. The copy number of the *SSIIb* gene was then determined by quantitative TaqMan PCR (Table 3). Compared to non-heated grits, only 14% of the copy numbers decreased by heating at 130℃. However, the copy numbers decreased to about 40% with heating at 160℃, furthermore it dropped to less than 10% in the control at 180℃. These results indicated that higher temperatures gave rise to a much more severe fragmentation of genomic DNA (Table 3). Since the best cooking temperature for frying corn processed foods is around 180℃, the results of our model experiments indicated that about one tenth of the genomic DNA with fragments longer than 100 bps remained in the fried foods. The remained DNA fragments should become the target template DNA for quantitative TaqMan PCR of the Japanese official methods.

When pressure was added with heating in extruder processing, over 99% of the genomic DNAs in the grits were fragmented into small pieces. This fragmentation was observed even at 128℃, and resulted in DNA copies rarely being detected by quantitative TaqMan PCR (Table 4). At over 162℃, few copies of the *SSIIb* gene were detectable by quantitative TaqMan PCR. These results suggested that the extruder processing gave the physical shearing force on the genomic DNAs and led a drastic decrease in copy numbers.

4. Quantification of grit amounts of the GM corn varieties, MON810 and GA21, processed with heat and pressure using an extruder

The grits derived from MON810 were mixed with non-GM corn grits to 9.5%, and then processed using an extruder. The genomic DNAs were extracted from processed grits and the copy number of the MON810 transgenes quantified by quantitative TaqMan PCR according to the Japanese official method (Table 5). According to the official methods, in which the ratio of the transgene of MON810 to the *SSIIb* gene was compensated for by a factor of 0.38, the calculated mean value of the five indepen-

Table 3. Changes in the copy numbers of the *SSIIb* gene detected from MON810 corn grits fry-processed with heating at several temperature (n = 3)

Temperature condition in corn grits	Temperature (℃)	*SSIIb gene*					Average of each temperature condition	Residual ratio of DNA (%)
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5		
Control (untreated)	Room temp.	630000	570000	430000	410000	570000	522000	100.0
Low	130	610000	630000	120000	520000	380000	452000	86.6
Optimum	160	340000	330000	50000	170000	160000	210000	40.2
High	180	39000	32000	6100	44000	90000	42220	8.1

Table 4. Changes in copy numbers of the *SSIIb* gene detected from MON810 corn grits processed with an extruder (n = 3)

Temperature condition in corn grits	Temperature (℃)	*SSIIb gene*					Average of each temperature condition	Residual ratio of DNA (%)
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5		
Control (untreated)	Room temp.	680000	760000	760000	630000	570000	680000	100.0
Low	128	300	50	480	680	1100	522	0.1
Optimum	162	66	0	18	28	71	37	0.0
High	179	0	0	0	N.T.	N.T.	0	0.0

* N.T. indicates "not tested".

dent quantifications of the content of MON810 in the untreated grits was 9.9% (Table 6). This value was close to 9.5% in the expected mixed content. These results indicated that the official quantification method using quantitative TaqMan PCR worked quite well for the untreated corn grits. Similarly to the internal control gene, *SSIIb*, the copy number of the transgene was heavily decreased by extruder processing (Table 5). When the mixed grits were processed with both heat and pressure using an extruder, the calculated content of MON810 corn was 65.5%, even at 128℃. This was markedly different from 9.5%, the real content of the GM-material (Table 6). If fragmentation occurred evenly in both the transgene and *SSIIb* gene, regardless of the difference in the nucleotide sequences or the locations of the genes in the genome of MON810, the calculated ratio of the transgene to the *SSIIb* gene should theoretically be close to 9.5%, the true value of the unprocessed grit material used as the control. The content of 65.5% obtained here indicated that the copy number of the MON810 transgene, which could be amplified by quantitative TaqMan PCR, was much higher than that of the *SSIIb* gene. The DNA of the *SSIIb* gene might fragment into much smaller pieces than the transgene at 128℃. As such, they could not be amplified by quantitative TaqMan PCR. However, the transgene might have been less fragmented and more of it remained to be the target template for quantitative TaqMan PCR after processing. When the grits were processed with an extruder at more than 162℃, the copy number of the transgene, which might have been completely fragmented so as not to be amplified by quantitative TaqMan PCR, was under the detectable level (Table 5). As a result, the content of MON810 in the mixed grits was calculated as 0% (Table 6).

However, unlike MON810 corn, when the copy number of the *SSIIb* gene was measured in the grits prepared from the other GM corn variety, GA21, less than seven copies of the *SSIIb* gene, which was almost below the detection level, were found at 128℃ (Table 7). In spite of similar processing conditions for both varieties of GM corn (Tables 4 and 7), about 500 copies on average could be detected from the grits of MON810 after processing at 128℃ (Table 4). These results suggested that the degree of DNA fragmentation of the *SSIIb* gene was different between the processing models prepared from the grits of MON810 and GA21 corn. The reason why such a difference was observed in the same gene is unknown. One possibility is small differences in the water content of grits between MON810 and GA21, which might have arisen from A188xB73 and DK626, the host inbred corn lines of the two GM varieties, respectively. These differences could cause significant ones in the physical shearing forces following the sudden release of water as vapor with extruder processing.

However, unlike the *SSIIb* gene, the transgene introduced into GA21 could be detected, even though severe DNA fragmentation might have occurred and the copy number was extremely low in the grits processed with an extruder at 128℃ (Table 8). The ratio of the transgene of GA21 (Table 8) to the *SSIIb* gene (Table 7) could not be calculated because the copy number of the *SSIIb* gene, which is the denominator of the ratio, was not reliable at 128℃, and zero at 162℃ and 179℃. These results were compatible in the case of MON810, in that the transgene might be considerably less fragmented than the internal control gene, *SSIIb*, during extruder processing at 128℃.

Our results suggested that the degree of fragmentation by heat and pressure processing using an extruder differed in the DNAs between transgenes and the *SSIIb* gene. Two possibilities for these results are: (1) the DNA of the transgene might be harder to fragment by processing than the *SSIIb* gene or other internal host genes, and (2) the DNA of the *SSIIb* gene might be more fragmented than both the transgene and other internal host genes. From our results, we could not conclude which possibility was correct. We consider that further experiments should be undertaken to determine the degree of DNA fragmentation by processing on other internal host genes to compare with the transgenes and *SSIIb* gene. Regardless of the reason, our results showed that both transgenes of MON810 and GA21 were less fragmented than the *SSIIb* gene for the target template regions for quantitative TaqMan PCR. This fact indicates that the ratio of the copy number of the transgene per internal control *SSIIb* gene in corn snacks processed with an extruder might be higher than the real ratio in the raw materials of corn grits using quantitative TaqMan methods.

It is probable that not only the length, but also the nucleotide sequence of the DNA, or even the location and position of the gene in the whole corn genomic DNA, could contribute to the difference in the degree of fragmentation or degradation of the template DNA region for quantitative TaqMan PCR following processing with heat and pressure. The factor(s) inducing the differences should be elucidated in future studies. It is known that the high GC content of the genomic DNA in thermophilic bacte-

Table 5. Changes in copy numbers of the transgene detected from MON810 corn grits processed with an extruder (n = 3)

Temperature condition in corn grits	Temperature (℃)	MON810 gene					Average of each temperature condition	Residual ratio of DNA (%)
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5		
Control (untreated)	Room temp.	25000	24000	25000	32000	22000	25600	100.0
Low	128	120	0	120	150	260	130	0.5
Optimum	162	0	0	0	0	0	0	0.0
High	179	0	0	0	N.T.	N.T.	0	0.0

* N.T. indicates "not tested".

Table 6. Percentages of the content of MON810 grits calculated from the copy numbers of the transgene (Table 5) and *SSIIb* gene (Table 4) by quantitative TaqMan PCR (n = 3)

Temperature condition in corn grits	Temperature (℃)	MON810/*SSIIb* (%)					Average of each temperature condition
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5	
Control (untreated)	Room temp.	9.7	8.3	8.7	13.4	10.2	9.9
Low	128	105.3	0.0	65.8	58.0	62.2	65.5
Optimum	162	0.0	0.0	0.0	0.0	0.0	0.0
High	179	0.0	0.0	0.0	N.T.	N.T.	0.0

* N.T. indicates "not tested".

Table 7. Changes in copy numbers of the *SSIIb* gene detected from GA21 corn grits processed with an extruder (n = 5)

Temperature condition in corn grits	Temperature (℃)	*SSIIb gene*					Average of each temperature condition	Residual ratio of DNA (%)
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5		
Control (untreated)	Room temp.	20000	18000	15000	17000	23000	18600	100.0
Low	128	7	6	6	5	6	6	0.03
Optimum	162	0	0	0	0	0	0	0.0
High	179	0	0	0	0	0	0	0.0

Table 8. Changes in copy numbers of the GA21 transgene detected from GA21 corn grits processed with an extruder (n = 5)

Temperature condition in corn grits	Temperature (℃)	GA21 gene					Average of each temperature condition	Residual ratio of DNA (%)
		Sample 1	Sample 2	Sample 3	Smaple 4	Sample 5		
Control (untreated)	Room temp.	37000	36000	28000	32000	37000	34000	100.0
Low	128	52	52	45	46	55	50	0.1
Optimum	162	0	0	0	0	0	0	0.0
High	179	0	0	0	0	0	0	0.0

ria gives rise to an increased thermal stability of the hydrogen bonds between double strands. If such stabilization mechanisms are clarified, it might be possible to estimate the degree of fragmentation of genomic DNA following treatment with both heat and pressure.

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論　　文

遺伝子組換えトウモロコシの食品加工過程における核DNAの断片化

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キーワード: DNA断片化、エクストルーダー、遺伝子組換えトウモロコシ、加工食品、定量PCR

概　要

我々は、食品の加工過程におけるトウモロコシの核DNAの分解と断片化を調べた。遺伝子組換えトウモロコシのMON810、GA21から得られたグリッツに、フライ加工およびエクストルーダー加工処理を行った。エクストルーダーは、加熱加圧工程後に急激に大気圧に戻す加工法であり、グリッツのスラリーを処理することによりコーンスナックが製造される機械である。フライ加工においては160 ℃、1分間以下であれば600 bp以上のDNA断片がPCR法によって増幅されるが、180 ℃の加工では400 bp以下に断片化されることが明らかになった。また、エクストルーダー加工においては、128 ℃においても、すでに197 bp以下にDNAは断片化されており、より高い温度では100 bp以下にまで断片化されていることが明らかになった。また、MON810およびGA21のグリッツをエクストルーダーによって128 ℃で加工したものについて、導入された遺伝子と公定法における標準遺伝子であるスターチ合成酵素IIb (*SSIIb*) 遺伝子の定量をTaqMan PCR法によって行ったところ、導入された遺伝子よりも*SSIIb*遺伝子のコピー数の方が小さくなり、*SSIIb*遺伝子の方が断片化されやすいことが明らかになった。エクストルーダーによって加工されたコーンスナックにおいて、コントロールの内在性*SSIIb*遺伝子に対する導入遺伝子のコピー数の比率は、定量TaqMan PCR法を用いると原料としたコーングリッツよりも高くなることが示された。

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Highlighted paper selected by Editor-in-chief

New Qualitative Detection Methods of Genetically Modified Potatoes

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In Japan, 8 lines of genetically modified (GM) potato (2 lines of NewLeaf® potato; NL, 3 lines of NewLeaf Plus® potato; NLP, and 3 lines of NewLeaf Y® potato; NLY) have already been authorized as safe for use in foods and feeds. We have developed polymerase chain reaction (PCR) methods for the qualitative detection of the GM potatoes for the screening and the identification of NL, NLP and NLY. The gene encoding uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase) was used as a taxon specific gene. We designed the primer pair to detect the cryIIIA genes as a screening method for GM potatoes because the gene should be inserted in all 8 lines of the GM potatoes. For identification of NL, NLP and NLY, we further designed three specific primer pairs for the different recombinant DNAs (r-DNA) specifically introduced into NL, NLP, or NLY. In addition, to identify the 3 lines of NLY that have been introduced with the same r-DNA, the three line-specific primer pairs for the border sequence between the r-DNA and genomic DNA of NLY 3 lines were designed. Six lines of GM potato used as the test material were specifically identified using the each primer pair under the same PCR condition. The detection limits of all the GM potatoes should be approximately 0.1%. Furthermore, the specificity and reproducibility of the methods were confirmed in a six-laboratory collaborative study.

Key words genetically modified potato; detection method; NewLeaf; NewLeaf Plus; NewLeaf Y; uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase)

Many kinds of genetically modified (GM) crops, which include GM soy, maize, rapeseed, cotton and potato, have already been developed and the cultivated acreage of these crops has continued to grow year by year. It was reported that the global area of GM crops for 2003 was 67.7 million hectares with a growth rate of 15% compared to that in 2002. This growth is estimated to rapidly increase, since the planting has been spread all over the world in addition to the nations such as United States (U.S.) and Canada.[1] On the other hand, public concern has been raised in terms of food safety and environmental effects of the GM crops. Especially, consumers are concerned about the negative effects of GM food on their health by their consumption and scientific information has been strongly required.[2] Therefore, many governments have now been considering regulations for the use and implementing a labeling system for GM crops as food and feed. Thus, new labeling systems have been introduced for GM foods in the European Union (EU), Australia, Korea, Japan and other countries.

The commercialization of fifty-five lines of safety-assessed GM crops including soy, maize, potato, rapeseed, cotton and sugar beet, have already been approved by the Ministry of Health, Labour and Welfare (HMLW) in Japan. To monitor the labeling system, it is necessary to develop reliable and practical methods for the detection and identification of GM crops. The polymerase chain reaction (PCR) is one of the widely used systems for the quantitative or qualitative detection of GM crops and we also have previously reported PCR methods for the detection of GM soy, maize, papaya and potatoes.[3—10]

The tetraploid cultivated potato (*Solanum tuberosum*) is one of the world's four major crops and an important feedstuff, but it is easily infected by many kinds of pests and pathogens.[11] Therefore, molecular biology techniques has been attempted to improve the potato varieties which ended with the breeding of GM potatoes commercialized by the Monsanto Co. (St. Louis, MO, U.S.A.). In Japan, two lines of NewLeaf® potato (NL), three lines of NewLeaf Plus® potato (NLP) and three lines of NewLeaf Y® potato (NLY) have been approved by HMLW for human food consumption by July 2003. The information provided by the Monsanto Co. showed that (a) two lines of NL (Bt-6 and SPBT02-05 line) were transformed with the same plasmid vector, PV-STBT02, which contains cryIIIA derived from *Bacillus thuringiensis* subsp. *tenebrionis*, providing of the trait of Colorado potato beetle (CPB) resistance, (b) three lines of NLY (RBMT15-101, SEMT15-15 and SEMT15-02 line) were transformed with the same plasmid vector, PV-STBT02, which contains cryIIIA, Potato virus Y coat protein (PVYcp) gene providing of the traits of CPB and PVY resistance, (c) two of three lines of NLP (RBMT21-129 and RBMT21-350 line) were transformed with the same plasmid vector, PV-STMT21, which contains cryIIIA, the potato leaf role virus replicase (PLRVrep) gene, providing of the traits of CPB and PLRV

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resistance, and (d) the other one (RBMT22-82) was transformed with the plasmid vector, PV-STMT22, which contains cryIIIA, PLRVrep, and CP4-epsps genes, providing of the traits of CPB, PLRV resistance and herbicide tolerance, respectively. The detection methods based on PCR for GM potatoes have been reported.[9,10,12] To verify the labeling and identify of the lines, however, further reliable and practicable detection methods for the GM potatoes are required.

In the present study, we designed seven primer pairs for screening, construct-specific, and line-specific detection and developed the condition of PCR to perform the test under the same conditions using all the primer pairs. To design the line-specific primer pairs, we focused on the junction sequences between the recombinant DNA (r-DNA) and potato genomic DNA as the target sequence to distinguish the specific one from the GM limes that was transformed with the same plasmid vector. The specificities and sensitivities were examined using reference materials provided by the Monsanto Company. The specificity and reproducibility of the methods were then confirmed by an inter-laboratory study.

MATERIALS AND METHODS

Reference Potato Materials and Other Crops Freeze-dried powder of six lines of GM potato (NL potatoes; Bt-6 and SPBT02-05, NLP potato; RBMT21-350, NLY potatoes; RBMT15-101, SEMT15-02 and SEMT15-15 line) and three varieties of non-GM potato (Shepody, Russet Burbank and Superior), which were conventional varieties used for the transformation, were kindly provided by the Monsanto Co. through the Department of Food Safety, Pharmaceutical and Food Safety Bureau of MHLW. Japanese conventional potato varieties (Dansyaku and May queen), egg plant (Solanum melongena), tomato (Lycopersicum esculentum), maize (Zea mays), soy (Glycine max) and wheat (Triticum aestivum) were purchased from a local market.

Preparation of Test Samples Dehydrated Japanese conventional potatoes, the provided powders of the non-GM potato varieties and GM potatoes were ground with the 0.2 mm sieve ring using an Ultra-Centrifugal Mill ZM100 (Retsch GmbH, Haan Germany) and freeze-dried for 24 h using an FD-81 freeze dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). To evaluate our screening and construct-specific methods, three mixing levels of the potato-powder samples containing 0, 0.1 and 1.0% of each NL-Bt-6, NLP-RBMT21-350, and NLY-SEMT15-15 lines were prepared, according to a previous study.[13] In this study, we selected the Bt-6, RBMT21-350, and SEMT15-15 lines as the representative line for each NL, NLP and NLY by referring to the acreage in the U.S. in 2000 and 2001. Six mixing levels of potato-genomic DNA samples containing 0, 0.05, 0.1, 0.5, 1.0, and 5.0% of each SEMT15-15, SEMT15-02, and RBMT15-101 line were also prepared to evaluate our line-specific methods. Because the amount of the three lines of NLY, which was provided by Monsanto, was not enough to prepare the powder samples, the genomic DNA samples were used for the evaluation. For the preparation of both the powder and genomic DNA samples, Dansyaku was used as a non-GM material. Other crops were also well ground by the Ultra-Centrifugal Mill ZM100 or Grindomix GM · 100 (Retsch) and used for the test samples.

Extraction and Purification of Genomic DNA Genomic DNAs were extracted from soy and maize using a silica-gel membrane-type kit (DNeasy Plant Maxi; QIAGEN GmbH, Hilden, Germany) according to a previous study.[6] Genomic DNAs were extracted from the other crops including potatoes using a silica-gel membrane-type kit (DNeasy Plant Mini; QIAGEN) according to a previous study.[9] The DNA concentration in the solutions was determined by measuring the UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE). The purity of the extracted DNA was evaluated using the ratio of 260/280 nm and the ratio was between 1.7 and 2.0 for most of the test samples. The extracted DNA was diluted with an appropriate volume of DW to a final concentration of 10 ng/μl, and stored at -20 °C until used. These DNA samples were used for the subsequent PCR analysis.

Oligonucleotide Primers Eight primer pairs, in which the primer pair to detect a potato taxon specific gene was included, were designed for the qualitative detection of the GM potatoes (Table 1). The primer pair UGPase 01-5′/UGPase 01-3′ to detect a gene encoding uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase) that was reported as a single copy gene of potato,[14] was designed on the first exon by referring to GenBank Accession No. U20345 which generated a 111 bp amplified fragment.

The cryIIIA gene from *Bacillus thuringiensis* should be introduced into all 8 lines of the GM potatoes conferring resistance to the CPB pest.[15] The primer pair CryIIIA 01-5′/CryIIIA 01-3′ to detect this gene as screening for the GM potatoes was designed by referring to GenBank Accession No. X70979 in order to generate a 117 bp amplified fragment. To specifically detect NL, the NL construct-specific primer pair NL 01-5′/NL 01-3′ was designed in the junction between the enhanced 35S promoter (e-p-35S) and cryIIIA by referring to GenBank Accession No. AF078810 (e-p-35S), and the primer pair generated an amplified fragment of 113 bp. In previous studies,[9] p-FMV02-5′/PLRV 01-3′ had been designed in the junction between e-p-35S and cryIIIA for the construct-specific detection of NLP and generated a 234 bp amplification fragment. Moreover, p-FMV05-5′/PVY 02-3′ had been designed in the junction between the 35S promoter sequence of the figwort mosaic virus (P-FMV) and PVYcp for the construct-specific detection of NLY and generated a 225 bp amplified fragment.[10] NLP and NLY were specifically detected using these two primer pairs, respectively, but the efficiencies of the PCR should be low because the amplified fragments were weakly detected in the potato-powder samples containing 0.1% of both the NLP and NLY.[13] In this study, we aimed to develop more reliable and practical detection methods by re-designing the primer pairs for the construct-specific detection of NLP and NLY. The primer pairs NLP 01-5′/NLP 01-3′ and NLY 01-5′/NLY 01-3′ were designed in the internal sequences of the PCR products generated by the primer pairs, p-FMV02-5′/PLRV 01-3′ and p-FMV05-5′/PVY 02-3′, to increase the PCR efficiencies while keeping the specificities. These primer pairs generate a 125 bp and 123 bp of amplified fragments, respectively.

To identify three lines of NLY that have been transformed with the same plasmid vector, the NLY line-specific primers (NLY15-01-5′/NLY15-01-3′; NLY15-15 line specific,

NLY02-01-5′/NLY02-01-3′; NLY15-02 line specific and NLY101-01-5′/NLY101-01-3′; NLY15-101 line specific) were designed in each of the border sequences between r-DNA and the genomic DNA by referring to the DNA sequence information described in the safety assessment document submitted from the developer. These primer pairs generate 164 bp, 86 bp and 150 bp amplified fragments, respectively. The locations of each targeted sequence are shown in Fig. 1.

The primers were synthesized and purified on a reversed-phase column by FASMAC Co., Ltd., (Atsugi, Japan), then diluted with an appropriate volume of DW to a final concentration of 50 μmol/l, and stored at −20 °C until used. The sequences of the designed oligonucleotides in this study are listed in Table 1.

PCR Conditions The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 25 ng of genomic DNA, 0.2 mmol/l dNTP, 1.5 mmol/l $MgCl_2$, 0.5 μmol/l of 5′ and 3′ primers and 0.625-units AmpliTaq Gold (Applied Biosystems, Foster City, CA, U.S.A.). The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95 °C for 10 min, denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min.

Agarose Gel Electrophoresis After PCR amplification, agarose gel electrophoresis of the PCR product was carried out according to previous studies.[5,9] The amplification products (7.5 μl) of each specific PCR were submitted for electrophoresis at a constant voltage (100 V) on a 3% TAKARA L03 agarose gel (TAKARA Co., Ltd., Tokyo, Japan) in the TAE (40 mmol/l Tris–HCl, 40 mmol/l acetic acid, and 1 mmol/l EDTA, pH 8.0) buffer solution. After electrophore-

Table 1. List of Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	UGPase 01-5′ UGPase 01-3′	5′-CTC TCC ATA CTC TCT GCT CCT CG-3′ 5′-CGG CAT CAG CAG GAG AAA G-3′	Potato *UGPase*/sense Potato *UGPase*/anti-sense	Potato	111 bp
B:	CryIIIA 01-5′ CryIIIA 01-3′	5′-GAA AGC CTA CAA GCT GCA ATC TG-3′ 5′-TCA GGT GTC ACG TAG ATA GTA G-3′	*CryIIIA*/sense *CryIIIA*/anti-sense	*CryIIIA*	117 bp
C:	NL 01-5′ NL 01-3′	5′-CCT TCG CAA GAC CCT TCC TC-3′ 5′-CGG TGT TGT TGT CTG CAG TCA-3′	p35S/sense *CryIIIA*/anti-sense	NeaLeaf	113 bp
D:	NLP 01-5′ NLP 01-3′	5′-CCC ATT TGA AGG ACA CAG AAA CA-3′ 5′-AGC GGC ATA TGC GGT AAA TC-3′	pFMV-*GmHsp*/sense *PLRV-rep*/anti-sense	NewLeaf Plus	125 bp
E:	NLY 01-5′ NLY 01-3′	5′-CAA AAT CCC AGT ATC AAA ATT CTT-3′ 5′-TGG TTT TGT ATC TTT CTT GTT GCT TC-3′	*GmHsp*/sense *PVY-cp*/anti-sense	NewLeaf Y (3 lines)	123 bp
F:	NLY15-01-5′ NLY15-01-3′	5′-AAC GCT GCG GAC ATC TAA ATT CA-3′ 5′-TAC CGT TAC CAC TAG CTA CAC T-3′	pNOS/sense Potato genomic DNA/anti-sense	NewLeaf Y (SEMT15-15)	164 bp
G:	NLY02-01-5′ NLY02-01-3′	5′-TGA AAT TCG ACT AAT TAC AAG TTG A-3′ 5′-GCA TCG ATC GTG AAG TTT CTC AT-3′	Potato genomic DNA/sense pNOS/anti-sense	NewLeaf Y (SEMT15-02)	86 bp
H:	NLY101-01-5′ NLY101-01-3′	5′-ATG GCT CGT ACC TTG TTG ATT G-3′ 5′-AGG TCA AAG TTA AAA TGA AAC ATG-3′	pFMV/sense Potato genomic DNA/anti-sense	NewLeaf Y (RBMT15-101)	150 bp

A; for confirmation of validity of the DNA extracted from potato for PCR, B; for screening detection of GM potatoes, C; for detection of NL, D; for detection of NLP, E; for detection of NLY, F; for identification of NLY-SEMT15-15 line, G; for identification of NLY-SEMT15-02 line, H; for identification of NLY-RBMT15-101 line.

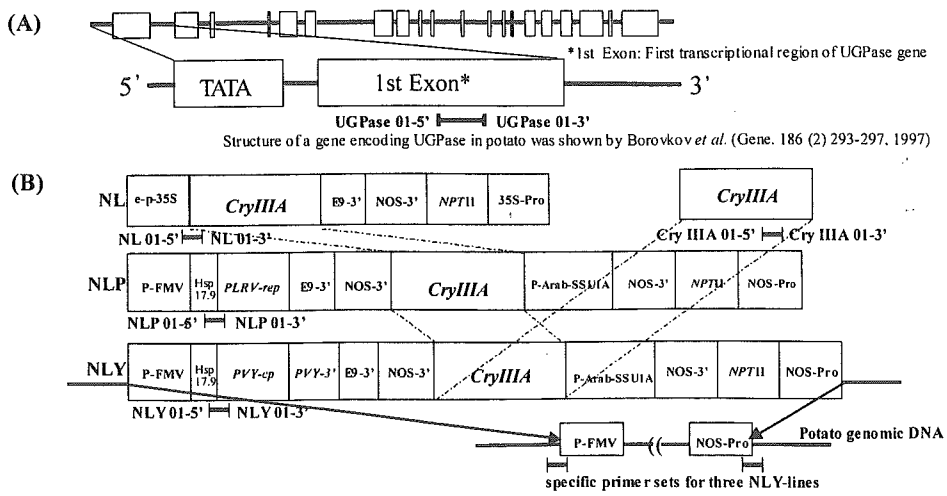

Fig. 1. Schematic Diagrams of PCR Primers Designed for Three Kinds of Genetically Modified Potatoes

(A) The structure of a gene encoding UGPase is shown. The primer pair was designed on the first exon of UGPase. (B) Schematic representation of recombinant DNA (r-DNA) introduced into three kinds of genetically modified potatoes (NL, NLP and NLY). The primer pairs were designed in the junction between the integrated r-DNA and potato genomic DNA for the specific detection of the 3 lines of NLY. Further information on the location of primers is shown in Table 1.

sis, the gel was stained in DW containing 0.5 μg/ml ethidium bromide for 30 min and then washed in DW for 30 min. The gel was photographed using a Chemi-lumi Image Analyzer (Chemi-lumi Image Analyzer with "Diana" system as the analytical software, Raytest, Germany).

Inter-Laboratory Studies Inter-laboratory studies, in which 6 laboratories participated, were organized by the National Institute of Health and Sciences (NIHS) to verify the detection methods. We prepared 24 separate tubes containing 200 mg test-samples for two Japanese varieties, and the 6 lines of GM potato described above. The homogeneities of the samples were confirmed by the methods using specific primer pairs with the 3 tubes selected randomly for each sample at the NIHS. The blind samples were designed as a pair of blind duplicates including 0 and 100% NL-Bt-6, NL-SPBT02-05, NLP-RBMT21-350, NLY-RBMT15-101, NLY-SEMT15-02, and NLY-SENT15-15. The blank samples, two Japanese conventional varieties, were used to confirm whether the tests were performed correctly without false-positive results. A total of 16 tubes containing blind samples, a solution of 8 primer pairs (5 μmol/l each), and the experimental protocol were supplied to the 6 labs from the NIHS.

RESULTS AND DISCUSSION

Evaluation of the Validity of Extracted DNA for PCR To avoid a false-negative result, it is important to confirm the validity of the extracted DNA for PCR. Therefore, the primer pair, UGPase 01-5′/UGPase 01-3′, was designed as the analytical control to evaluate the validity of the potato DNA for PCR. We performed the PCR with DNAs extracted from 11 kinds of potatoes including the GM and non-GM potatoes to examine the conservation of the UGPase gene among the potato varieties. As shown in Fig. 2A, all of the potato DNAs generated a 111-bp amplified fragment with almost the same intensity, whereas no fragments were amplified from the tomato, egg plant, maize, wheat and soy DNAs. These results suggested that PCR using the primer pair should specifically detect the DNA extracted from potato. The method to identify the potato DNA has been reported by Jaccaud *et al.*[12] They designed the primer pairs to detect the patatine gene which is encoded by a multigene family. In their method, however, the amplified fragments were generated from the potato, tomato and tobacco using the primer pair for identification of the patatine gene. In contrast, we showed that PCR using the designed primer pair, UGPase 01-5′/UGPase 01-3′, generated no amplified fragments in the tomato and egg plant which belong to the Solanaceae taxonomic family (Fig. 2A, lanes 13 and 14). These results suggest that potato DNA should be more stably detected with high specificity and the validity of the extracted potato DNA for PCR would be assessed by PCR using the UGPase 01-5′/UGPase 01-3′ primer pair.

Specificity of Screening Method for the Detection of GM Potatoes Screening methods for the detection of the GM crops including soy and maize have already been reported.[5,16] The DNA sequences of the 35S promoter and the terminator sequence of the nopaline synthase gene from *Agrobacterium tumefaciens* (NOS terminator), which were commonly and frequently used to regulate the expression of a gene in the various GM crops, were selected to design the primer pairs for the screening detection. The primer pair designed for the sequence of the NOS terminator could be used to detect GM potatoes for the screening purpose because the DNA sequence of the NOS terminator was commonly introduced in the NL, NLP and NLY (Fig. 1). However, the GM potatoes and the other GM crops, such as GM maize and GM soybean, should be undistinguishable using the primer pair for the NOS terminator because GM maize and GM soybean could generally contain the NOS terminator. On the other hand, a gene encoding cryIIIA should be introduced into all lines of NL, NLP and NLY and it has not been reported that the cryIIIA gene was introduced in the other GM crops. Therefore, the CryIIIA 01-5′/CryIIIA 01-3′ primer pair was designed on the cryIIIA gene for the screening detection of the GM potatoes. An amplified fragment (117 bp) was specifically detected from all 6 lines of GM potato used in this study (Fig. 2B). In contrast, no amplified product was detected when the DNAs extracted from tomato, egg plant, maize, wheat, soy, and non-GM potatoes were used as the template DNA. Two lines of NLP (RBMT21-129 and RBMT22-82) were not provided from the developer and could not be used for this study, but the same results would be expected, because the same target sequence should be introduced into the two lines according to the information described in the safety assessment document.

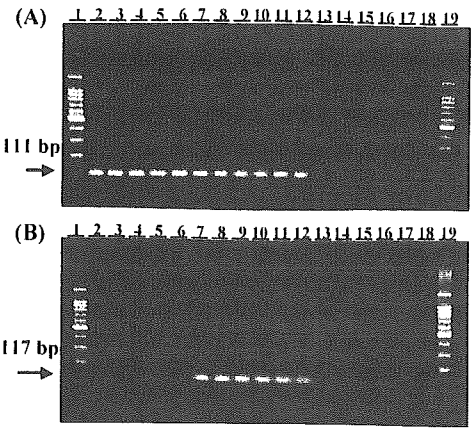

Fig. 2. Specificity of Potato and GM Potato Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs UGPase 01-5′/UGPase 01-3′ (A) and CryIIIA 01-5′/CryIIIA 01-3′ (B). Lanes 1 and 19, 100-bp ladder size standard; lane 2, non-GM potato (Russet Burbank); lane 3, non-GM potato (Superior); lane 4, non-GM potato (Shepody); lane 5, non-GM potato (Dansyaku); lane 6, non-GM potato (May queen); lane 7, NL (SPBT02-05); lane 8, NL (Bt-6); lane 9, NLP (RBMT21-350); lane 10, NLY (SEMT15-15); lane 11, NLY (RBMT15-101); lane 12, NLY (SEMT15-02); lane 13, tomato; lane 14, egg plant; lane 15, maize; lane 16, soy; lane 17, wheat; lane 18, negative control (no template DNA).

Specificity of Construct-Specific Methods for NL, NLP and NLY In NL, the e-p-35Ssequence[17] is used to regulate the expression of the cryIIIA gene, whereas the riblose-1,5-bisphosphate carboxylase small subunit ats1A promoter (P-Arab-SSU1A)[18] is used to control the cryIIIA gene expression in NLP and NLY. Therefore, the construct-specific primer pair, NL 01-5′/NL 01-3′, was designed in the junction between e-p-35S and cryIIIA to specifically detect NL. As shown in Fig. 3A, two lines of NL, which were transformed with the same plasmid vector PV-STBT02, generated 113 bp amplified fragments by PCR. On the other hand, no fragment was detected in any DNAs including the non-GM and other GM potatoes. These results suggest that the 2 lines of NL should be specifically detected using the NL 01-5′/NL 01-3′

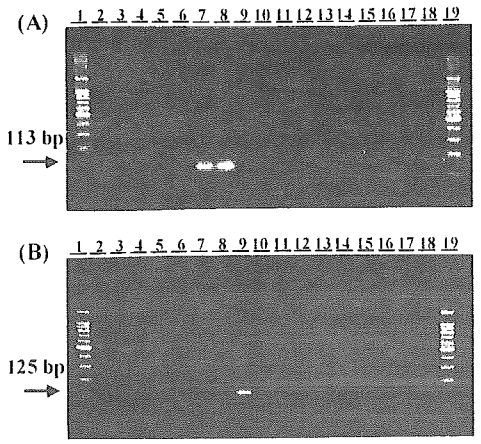

Fig. 3. Specificity of NL and NLP Construct-Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs NL 01-5′/NL 01-3′ (A) and NLP 01-5′/NLP 01-3′ (B). See footnotes for Fig. 2.

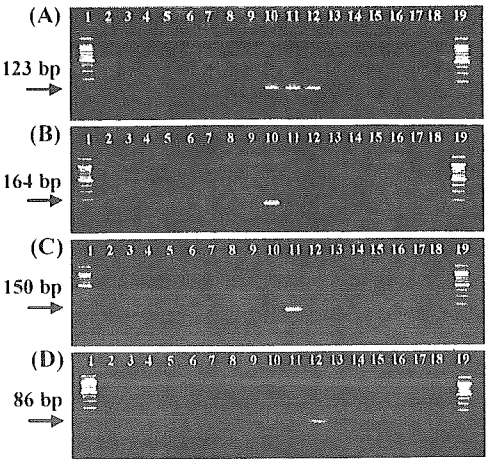

Fig. 4. Specificity of NLY Construct- and Line-Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs NLY 01-5′/NLY 01-3′ (A), NLY15 01-5′/NLY15 01-3′ (B), NLY101 01-5′/NLY101 01-3′ (C) and NLY02 01-5′/NLY02 01-3′ (D). See footnotes for Fig. 2.

primer pair.

NLP and NLY contain the 35S promoter sequence of the Figwort mosaic virus (P-FMV),[19] and P-FMV regulates the expression of PLRVrep in NLP and that of PVYcp in NLY. If the primer pairs were designed in the gene encoding PLRVrep or PVYcp for the detection of NLP and NLY, false-positive results might be obtained in the potatoes infected by the viruses. To avoid the false-positive results that might be caused in such samples, primer pairs were designed in the specific sequence available only at the junction region derived from different organisms. Therefore, the region between the leader sequence of heatshock protein isolated from Glycine max (Hsp 17.9)[20] and the sequence of the PLRVrep was selected to design the NLP construct-specific primer pair, NLP 01-5′/NLP 01-3′. To design the NLY construct-specific primer pair, NLY 01-5′/NLY 01-3′, the region between Hsp 17.9 and PVYcp was selected as the target sequence. An amplified fragment (125 bp) was obtained from only NLP using the primer pair, NLP 01-5′/ NLP 01-3′ (Fig. 3B). Furthermore, an amplified fragment (123 bp) was generated from only 3 lines of NLY during the specificity examination of the primer pair, NLY 01-5′/NLY 01-3′ (Fig. 4A). For assessment of the screening method, two lines of NLP could not be used for the examination, but the same results would be obtained because the same target sequence should be introduced into the other two lines of NLP.

Specificity of Line-Specific Methods for NLY In Japan, two lines of NLY (NLY-SEMT15-15 and RBMT15-101 line) were approved in April 2003 and the NLY-SEMT15-02 line was approved in July 2003. Therefore, it was necessary to identify the unapproved NLY-SEMT15-02 line until the safety assessment was finished. As shown in Figs. 3A and 4A, the same size PCR-amplified fragment was obtained from the three lines of NLY, which were transformed with the same plasmid vector, using the construct-specific methods. To identify the three lines, the specific region of each NLY should be selected to design the primer pair. Therefore, the border sequences between the r-DNA and potato genomic DNAs were selected as the NLY line-specific region. The NLY line-specific primers, NLY15-01-5′/NLY15-01-3′, NLY 02-01-5′/NLY02-01-3′ and NLY101-01-5′/NLY101-01-3′, were specifically designed on the NLY15-15, NLY15-02 and NLY15-101 line, respectively, and PCR using the corresponding primer pairs generated a 164 bp, 86 bp and 150 bp of amplified fragment from the specific lines, respectively (Figs. 4B, C, D). These results suggested that the border sequence between the integrated r-DNA and plant genomic DNA should be critical for examining the line-specific detection methods. The line-specific methods for GM potatoes have not been reported yet, though the line-specific methods for other GM crops have been reported.[21,22] When some GM crops, which contain the same r-DNA, might be bred in the near future, these findings will help to develop the detection method with high specificity and reliability.

Sensitivities of the Methods We examined the sensitivities of the screening and construct-specific detection methods with the potato-powder mixing samples containing 0, 0.1 and 1.0% of the NL-Bt-6, NLP-RBBT21-350 and NLY-SEMT15-15 line, respectively. Twenty-five ng of extracted genomic DNAs from the samples were amplified by PCR, and all of the target sequences were clearly detected in the 0.1% and 1.0% powder samples (Fig. 5A). It has been reported that the amount of unreplicated haploid genome (*i.e.*, the 1C value) of potato is 0.88 pg.[23] On the basis of the 1C value, 28.4 copies of the haploid GM potato genome were calculated to be present in the reaction tube prepared for a 0.1% potato-powder sample, since 25 pg of genomic DNA derived from each GM potato should be contained in the reaction tube. Furthermore, one or more copies of the target sequences are presented in each tetraploid genome of the NL-Bt-6, NLP-RBBT21-350 and NLY-SEMT15-15 line according to the information provided by Monsanto. Therefore, at least, 7.1 copies of the target sequences should be contained in the reaction tube. Theoretically, a copy of the target sequence could be amplified by PCR. In fact, the specific amplified fragments were detected in the 0.05% samples prepared by twice diluting the DNAs extracted from the 0.1% powder samples with non-GM potato DNA (data not shown). The potato-powder samples with a concentration less than 0.1% were not used for the examination since it was difficult to correctly prepare the samples. However, GM-potatoes might be detected in the lower concentrated samples using screening and construct-specific detection methods. We further ex-

amined the sensitivity of the NLY line-specific detection methods using potato-DNA samples containing 0, 0.05, 0.1, 0.5, 1.0 and 5.0% DNAs extracted from the three lines of NLY. As shown in Fig. 5B, by using the NLY-SEMT15-15 and RBMT15-101 line-specific methods, the specific amplified fragments were detected in the 0.05% sample. On the other hand, the specific amplified fragment for the NLY-SEMT15-02 was detected in the 0.5% sample. These results suggested that the detection limits of NLY-SEMT15-15, RBMT15-101 should be 0.05%, whereas the detection limit of the NLY-SEMT15-02 line-specific method was 0.5%. The sensitivity of the PCR methods would be affected by various factors including the copy numbers of the target sequence, physical character of the primer pair and PCR conditions. For the line-specific methods, the copy number should not be correlated with the sensitivity because a single target sequence is presented in each line. To increase the sensitivity of the NLY-SEMT15-02 line-specific method, we would have to increase the amount of DNA used for the template, to change the PCR condition suitable for the method, or to redesign the primer pair.

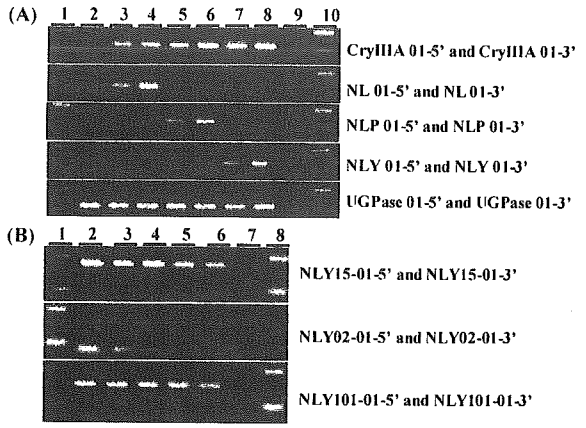

Fig. 5. Sensitivity of the PCR Methods Using the Specific Primer Pairs

Genomic DNAs extracted from the mixed samples of potato powder containing the 0.1 or 1.0% of some GM potatoes (A) or mixed samples of genomic DNAs containing various amounts of genomic DNAs extracted from some GM potatoes (B) were amplified. (A) Lanes 1 and 10, 100- and 200-bp size standard bands of ladder marker; lane 2, non-GM potato; lanes 3 and 4, potato containing 0.1 or 1.0% of NL; lanes 5 and 6, potato containing 0.1 or 1.0% of NLP; lanes 7 and 8, potato containing 0.1 or 1.0% of NLY; lane 9, negative control (no template DNA). (B) Lanes 1 and 8, 100 and 200 bp size standard bands of ladder marker; lanes 2 to 7, genomic DNAs containing 5.0, 1.0, 0.5, 0.1, 0.05% of genomic DNAs extracted from NLY-SEMT15-15, SEMT15-02, or RBMT 15-101, respectively; lane 7, negative control (DNA extracted from non-GM potato was used as template).

Inter-laboratory Study To confirm the specificity and reproducibility of the methods, we conducted inter-laboratory studies using 100% pure non-GM and GM potato-powder materials as blind samples. The blind samples were prepared as blind duplicates including two Japanese conventional varieties and 6 lines of GM potatoes (NL-Bt-6, NL-SPBT02-05, NLP-RBMT21-350, NLY-RBMT15-101, NLY-SEMT15-15 and NLY-SEMT15-02). All participants received the protocol, primer solutions, and 16 blind-sample tubes containing different potato powders. Six laboratories participated in the studies and analyzed a total of 96 samples. The methodology of the experiment was designed as described below. For the screening of the GM potato, a first PCR was performed to detect the potato taxon specific gene and cryIIIA gene. When the screening result was positive, a second PCR was performed to identify NL. If the sample was NL-negative, a third PCR was performed to identify NLY. A fourth PCR was performed for the NLY positive samples to identify the line of NLY. On the other hand, the NLY negative-samples was identified as NLP by performing an NLP construct-specific PCR. Typical results reported by a participant are shown in Table 2. The results showed that the test was correctly performed following the designed flow of the experiment, and no false-negative and -positive results were

Table 2. Typical Results of Inter-laboratory Study

Targets	Primer sets	Sample															
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
UGPase	UGPase 01-5′ UGPase 01-3′	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CryIIIA	CryIIIA 01-5′ CryIIIA 01-3′	+	+	+	+	+	−	−	+	−	+	+	+	−	+	+	+
NL	NL 01-5′ NL 01-3′	+	−	−	−	−	/	/	+	/	+	−	−	/	+	−	−
NLP	NLP 01-5′ NLP 01-3′	/	+	/	/	/	/	/	/	/	/	+	/	/	/	/	/
NLY	NLY 01-5′ NLY 01-3′	/	−	+	+	+	/	/	/	/	/	−	+	/	/	+	+
SEMT 15-02	NLY02-01-5′ NLY02-01-3′	/	/	+	−	−	/	/	/	/	/	/	−	/	/	+	−
SEMT 15-15	NLY15-01-5′ NLY15-01-3′	/	/	−	+	−	/	/	/	/	/	/	−	/	/	−	+
RBMT 15-101	NLY101-01-5′ NLY101-01-3′	/	/	−	−	+	/	/	/	/	/	/	+	/	/	−	−
Judgments		NL	NLP	15-02	15-15	15-101	Negative	Negative	NL	Negative	NL	NLP	15-101	Negative	NL	15-02	15-15

+; positive, −; negative, /; no test shown. A first PCR was performed with UGPase 01-5′/UGPase 01-3′ and CryIIIA 01-5′/CryIIIA 01-3′. When the result was positive, a second PCR was performed with NL 01-5′/NL 01-3′. If the PCR product was not obtained from the second PCR, a third PCR was performed with NLY 01-5′/NLY 01-3′. A forth PCR was performed for the NLY-positive samples with NLY15 01-5′/NLY15 01-3′, NLY101 01-5′/NLY101 01-3′ and NLY02 01-5′/NLY02 01-3′, respectively. On the other hand, the NLY-negative samples were identified by the PCR with NLP 01-5′/NLP 01-3′. Two Japanese conventional cultivars were judged as negative.

Table 3. Judgments and Percentage of Correct Results for All Test Samples in Inter-laboratory Study

Laboratory	Judgement						
	NL	NLP	NLP15-15	NLY15-02	NLY15-101	Dansyaku	Mayqeen
I	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
II	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
III	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
IV	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
V	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
VI	++++/++++	++/++	++/++	++/++	++/++	−−/−−	−−/−−
Percentage of correct results	100	100	100	100	100	100	100

++/++; both of two positive samples were judged correctly, −−/−−; both of two negative samples were judged correctly. For NL, ++++/++++ showed that all of four positive samples were judged correctly, because NL samples were included the two lines of NL as blind duplicates, which can not be identified using the construct-specific method.

reported. In all results reported from the six laboratories, specific amplified fragments were completely detected in all test samples and no misjudgments were reported from any laboratories (Table 3). These results suggest that the proposed methods should be specific and reproducible for the detection of each line of GM potatoes.

In summary, detection methods using PCR were developed for screening and identifying eight lines of GM potato approved in Japan for human consumption. The highly specific PCR methods reported in this study are simple and useful for identifying GM potatoes.

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A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins

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Abstract

Rationale. Evaluation of the potential allergenicity of proteins derived from genetically modified foods has involved a weight of evidence approach that incorporates an evaluation of protein digestibility in pepsin. Currently, there is no standardized protocol to assess the digestibility of proteins using simulated gastric fluid. Potential variations in assay parameters include: pH, pepsin purity, pepsin to target protein ratio, target protein purity, and method of detection. The objective was to assess the digestibility of a common set of proteins in nine independent laboratories to determine the reproducibility of the assay when performed using a common protocol.

Methods. A single lot of each test protein and pepsin was obtained and distributed to each laboratory. The test proteins consisted of Ara h 2 (a peanut conglutin-like protein), β-lactoglobulin, bovine serum albumin, concanavalin A, horseradish peroxidase, ovalbumin, ovomucoid, phosphinothricin acetyltransferase, ribulose diphosphate carboxylase, and soybean trypsin inhibitor. A ratio of 10 U of pepsin activity/μg test protein was selected for all tests (3:1 pepsin to protein, w:w). Digestions were performed at pH 1.2 and 2.0, with sampling at 0.5, 2, 5, 10, 20, 30, and 60 min. Protein digestibility was assessed from stained gels following SDS–PAGE of digestion samples and controls.

Results. Results were relatively consistent across laboratories for the full-length proteins. The identification of proteolytic fragments was less consistent, being affected by different fixation and staining methods. Overall, assay pH did not influence the time to disappearance of the full-length protein or protein fragments, however, results across laboratories were more consistent at pH 1.2 (91% agreement) than pH 2.0 (77%).

Conclusions. These data demonstrate that this common protocol for evaluating the in vitro digestibility of proteins is reproducible and yields consistent results when performed using the same proteins at different laboratories.

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1. Introduction

Historically, the evaluation of the potential allergenicity of novel gene products (that is, proteins) derived from genetically modified (GM) foods has involved the use of a decision-tree strategy that incorporated an evaluation of protein digestibility using pepsin (Metcalfe et al., 1996). The current assessment strategy, as outlined by the Ad Hoc International Task Force on Foods Derived from Biotechnology (*Codex Alimentarius* Commission, 2003), focuses on a weight-of-evidence approach that recognizes that no single endpoint can be used to predict human allergenic potential. In this context, the following factors are considered: the source of the gene, the similarity of the amino acid sequence of the protein of interest to that of known allergens, the stability of the protein in an in vitro pepsin digestibility assay, and, when necessary, in vitro human sera testing or in vivo clinical testing. The pepsin digestibility assay was first conceived as a means to determine the relative stability of a protein to the extremes of pH and pepsin protease encountered in the mammalian gastric environment. It was originally developed and utilized as a method to assess the nutritional value of protein sources by predicting amino acid bioavailability (Marquez and Lajolo, 1981; Nielson, 1988; Zikakis et al., 1977). For the purpose of safety evaluation, it seems reasonable that highly digestible proteins would be expected to have reduced potential for systemic exposure.

Before the use of recombinant technology became widely available, pepsin and other proteases were used by several investigators to identify the segments of proteins that bind IgE (Budd et al., 1983; Kawashima et al., 1992; Lorusso et al., 1986). In general, proteolytic digests of allergens retain the ability to bind IgE. The first systematic application of an in vitro pepsin digestion assay to evaluate food allergen digestibility was reported by Astwood et al. (1996). The relative resistance to pepsin digestion of some of the major allergenic proteins of peanut, soybean, egg and milk, and common non-allergenic plant proteins (for example, ribulose diphosphate carboxylase, RUBISCO), was determined in a pepsin digestion assay using simulated gastric fluid (SGF) prepared according to the US Pharmacopoeia (1995). Generally, the allergens and lectins examined in these experiments were resistant to pepsin digestion whereas the other proteins were more rapidly and completely digested. Since this initial report, there have been several studies examining the stability of a variety of proteins in pepsin digestion assays in which distinctions between allergens and non-allergenic proteins were less clear (Asero et al., 2001; Besler et al., 2001; Buchanan et al., 1997; del Val et al., 1999; Fu, 2002; Fu et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002; Tanaka et al., 2002). Variability in the digestibility measured in these studies is likely to be due in part to the lack of a standardized protocol to assess the digestibility of proteins using SGF. Variations include differences in the pH of the assay, the purity of the pepsin, the pepsin to target protein ratio, target protein purity and structural conformation, and the method of detection. The objective of this study was to assess the reproducibility of a common digestion protocol in nine different laboratories with a standard set of proteins.

2. Materials and methods

2.1. Participating laboratories

The laboratories participating in this test represented industry: Bayer CropScience (Sophia Antipolis, France); The Dow Chemical (Midland, MI); DuPont, Haskell Laboratory (Newark, DE); Monsanto (St Louis, MO); Syngenta Biotechnology (Research Triangle Park, NC); and Syngenta Central Toxicology Laboratory (Alderley Park, UK); federal researchers: US Food and Drug Administration, National Center for Food Safety and Technology, (Summit-Argo, IL); National Institute of Health Sciences, Japan (Tokyo, Japan); and a private research foundation, Sanquin Research (Amsterdam, The Netherlands). All laboratories received the same lot of each test protein, used the same lot of pepsin, and performed the pepsin digestion assay using the same conditions.

2.2. Pepsin activity

A ratio of 10 U of pepsin activity/μg of test protein was used throughout the study. This pepsin to target protein ratio was selected based on an evaluation of the average activity of pepsin recommended in the US Pharmacopoeia (2000) and used in previous studies in some of the participating laboratories. Pepsin (Cat. #P-6887) was purchased from Sigma Chemical (St. Louis, MO) in a single lot having 3460 U/mg of protein as analyzed by Sigma, and was provided to each of the participating laboratories as a lyophilized powder. For this lot of pepsin, the pepsin/protein ratio in the digestion reaction was approximately 3:1 (w:w).

2.3. Test proteins

Ten milligrams of the following test proteins were supplied to each laboratory as lyophilized powders: conglutin-like peanut allergen Ara h 2 (IUIS Allergen

Nomenclature, 1994) that was purified from peanuts in the presence of the reducing agent dithiothreitol by Arkansas Children's Hospital Research Institute, (Little Rock, AR) for Monsanto, (St. Louis, MO), bovine β-lactoglobulin (BLG; Sigma, Cat. #L0130), bovine serum albumin (BSA; Sigma, Cat. #A0281), concanavalin A from Jack beans (ConA; Sigma, Cat. #C2010), horseradish peroxidase (HRP; Sigma, Cat. #P6782), chicken ovalbumin (Ova; Sigma, Cat. #A5503), chicken egg-white ovomucoid (Ovm; Sigma, Cat. #T2011), phosphinothricin acetyltransferase (PAT; provided by Syngenta Biotechnology, Research Triangle Park, NC), ribulose 1,5-diphosphate carboxylase from spinach leaves (RUBISCO; Sigma, Cat. #R8000), and Kunitz soybean trypsin inhibitor (STI; Sigma, Cat. #T9003). All proteins had been isolated from their native source (plant or animal) with the exception of PAT, that was produced as a recombinant protein in *Escherichia coli* by over-expression of the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987). All proteins were dissolved at a concentration of 5 mg protein/ml in water or in 50 mM Tris–HCl, pH 9.5 (PAT and RUBISCO due to solubility constraints). All proteins were stable when dissolved at this concentration and stored at −20 °C. Each protein used in this study was at least 85% pure as determined by gel electrophoresis and densitometry.

2.4. Pepsin digestion assay conditions

For each protein, a single tube containing 1.52 ml of simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2 or 2.0, and 4000 U of pepsin) was pre-heated to approximately 37 °C prior to the addition of 0.08 ml of test protein solution (5 mg/ml).

The tube contents were mixed by mild vortexing and the tube was immediately placed in a 37 °C water bath. Samples of 200 µl were removed at 0.5, 2, 5, 10, 20, 30, and 60 min after initiation of the incubation. Each 200 µl sample was quenched by addition of 70 µl of 200 mM $NaHCO_3$, pH 11, and 70 µl 5× Laemmli (1970) buffer (40% glycerol, 5% β-mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8). Quenched samples were immediately heated to >75 °C for approximately 10 min and analyzed directly, or stored at −20 °C. The zero time point protein digestion samples were prepared by quenching the pepsin in the solution before adding the test protein. Control samples for pepsin auto-digestion (pepsin without test protein) and test protein stability (reaction buffer with test protein but without pepsin) were included. These control reactions were treated exactly as described above except samples were prepared only for the 0 and 60 min time points. Digestion samples were analyzed by visually inspecting stained protein bands following electrophoresis by one of the methods summarized in Table 1 and as described below.

2.5. SDS–PAGE electrophoresis

Samples (15 µl) from each time point and control reactions were subjected to SDS–PAGE electrophoresis in reducing conditions, according to the method of Laemmli (1970), using either 15-well, 1 mm thick, 10–20% polyacrylamide Tricine gels (7 laboratories used gels from Invitrogen, Carlsbad, CA, one laboratory (# 3) used gels from BioRad, Richmond, CA) or 10–20% polyacrylamide Tris–glycine gels (laboratory #2, Bio-Rad Laboratories) [Table 1]. Mark12 standard markers (Invitrogen, Cat. # LC5677) containing 12 proteins

Table 1
Laboratory, gel type, fixative, and staining type

Laboratory #	Gel type Buffer	Gel Manuf.	Fix	Stain
1	Tricine Tris/Tricine/SDS	Invitrogen	No	Colloidal Coomassie blue (G-250) Invitrogen
2	Tris–HCl 10–20% Tris/glycine/SDS	Bio-Rad	2% HOAc	Coomassie Blue (R-250) Bio-Rad
3	Tricine Tris/Tricine/SDS	Bio-Rad	No	GelCode Blue (G-250) Pierce
4	Tricine Tris/Tricine/SDS	Invitrogen	MeOH/HOAc	Colloidal Coomassie blue (G-250) Sigma
5	Tricine Tris/Tricine/SDS	Invitrogen	TCA	Coomassie Blue (R-250)
6	Tricine Tris/Tricine/SDS	Invitrogen	TCA	Coomassie Blue (R-250)
7	Tricine Tris/Tricine/SDS	Invitrogen	TCA	Coomassie Blue (R-250)
8	Tricine Tris/Tricine/SDS	Invitrogen	No	Colloidal Coomassie blue (G-250) Invitrogen
9	Tricine Tris/Tricine/SDS	Invitrogen	No	Colloidal Coomassie blue (G-250) Invitrogen

ranging in size from 2500 to 200,000 MW were included on all gels.

2.6. Gel staining

Investigators choose to use one of four different staining procedures in the course of this study (Table 1). Three laboratories (#5, 6, and 7) fixed the gels in 5% trichloroacetic acid (TCA) for less than 5 min and then washed them in 45.5% methanol/9% glacial acetic acid for 1 h. The gels were then stained with 0.1% Coomassie brilliant blue R-250 (Sigma #B-5133) for 10 min and destained in 25% methanol with 7.5% glacial acetic acid. One laboratory (# 2) stained the gels with Coomassie brilliant blue R-250 (Bio-Rad) following fixation in 2% acetic acid. Gels were destained in Bio-Rad destaining solution (Cat. #161-0438). One laboratory (# 4) stained the gels with colloidal Coomassie brilliant blue G-250 (Sigma Cat. # B-2025) for 2 h following 30 min of fixation in a solution of 40% methanol with 7% acetic acid. The gels were destained for 60 s in a solution of 25% methanol with 10% acetic acid, then overnight in 25% methanol. Three laboratories (# 1, 8, and 9) stained the gels using the colloidal Coomassie brilliant blue G-250 (Invitrogen, Cat. # LC6025) without pre-fixation. Gels were stained for 3–15 h in a mixture containing phosphoric acid, ammonium sulfate, methanol, and Coomassie blue G-250, prior to destaining with deionized water for 7 h to 3 days. One laboratory (# 3) stained with Gelcode Blue (Pierce, Rockford, IL, USA), a colloidal blue stain that includes phosphoric acid, methanol, and dimethyl sulfoxide with Coomassie blue G-250, without pre-fixation. Gels were washed three times with deionized water prior to staining for 2 h. Excess stain was removed by multiple washes in deionized water. Images of stained gels were captured in participating laboratories using a variety of scanners or digital cameras and image capture programs. Whole image brightness and contrast adjustments were made during capture, but not altered thereafter. Gel images were adjusted to comparable size and merged into common Microsoft PowerPoint figures for each of the pH conditions (pH 1.2 and 2.0).

2.7. Data evaluation

Due to some inter-individual variation in the interpretation of gel images, a panel of 8 scientists representing 5 participating laboratories jointly viewed and scored the images from all 9 laboratories to provide a consensus view regarding the final time a full length protein or fragment bands were visible for each gel.

3. Results

Proteins used in this study were from plant, bacterial, or animal sources, and included allergens, non-allergens, and proteins with and without enzymatic activity (Table 2). Five proteins were isolated from plant sources: Ara h 2, ConA, HRP, RUBISCO, and STI, while four proteins were of animal origin: BLG, BSA, Ova, and Ovm. Phosphinothricin acetyltransferase (PAT) was produced as a recombinant protein in *E. coli*. Six of the proteins: Ara h 2 (Sen et al., 2002), BLG (Jarvinen et al., 2001), BSA (Tanabe et al., 2002), Ova (Honma et al., 1996), Ovm (Yamada et al., 2000), and STI (Gu et al., 2000), are known to be food allergens, while PAT, HRP, and RUBISCO have not been reported to be allergenic. One protein, ConA, while not considered to be an allergen, is

Table 2
Biochemical properties of test proteins

Protein	Source	Apparent size (MW)	Allergen/major or minor	Function	Relative abundance
Ara h 2	Plant-peanut	20,000	Yes/major	Seed storage protein	High[a]
		18,000			
		13,000			
BLG	Mammal-bovine	17,000	Yes/major	Milk protein	High
BSA	Mammal-bovine	65,000	Yes/minor	Blood protein	High
ConA	Plant-jack bean	30,000 (main)	No	Seed protein	High
		13,000			
		11,000			
HRP	Plant-horseradish	48,000	No	Enzyme	High
Ova	Avian-chicken egg	45,000	Yes/major	Storage protein	High
Ovm	Avian-chicken egg	42,000	Yes/major	Storage protein	High
PAT	Bacterium-*E. coli*	23,000	No	Enzyme	Low[b]
RUBISCO	Plant-spinach leaves	54,000	No	Enzyme	High
		20,000			
STI	Plant-soybeans	19,000	Yes/minor	Inhibitor trypsin	Low

[a] Protein is present at greater than 1% of the protein content of source material.
[b] Protein is present at less that 1% of the protein content of source material.

a lectin and a T cell mitogen and is considered to be an anti-nutrient (Mendez et al., 1998). Three proteins: HRP, PAT (the selectable gene marker product in multiple transgenic seed products), and RUBISCO are enzymes. The proteins included in this study have diverse biochemical properties and all are consumed in foods commonly eaten in some cultures, albeit in widely different quantities.

Results from one of the test proteins, Ovm, were not included in the analytical summaries presented below, as there was no consensus among testing laboratories regarding detection. Ovm co-migrated with the pepsin enzyme as a protein-staining smear (Fig. 3C) due to its heavy glycosylation pattern making it difficult to definitively determine when the full-length protein was completely digested. In addition, the Ovm preparation was contaminated with a strongly staining protein band that was identified as lysozyme (sequence confirmed by Monsanto, data not shown). Lysozyme is stable to digestion by pepsin, making it difficult to determine when fragments of Ovm were last observed. This illustrates the importance of using well-characterized test proteins in this assay.

3.1. Test proteins exhibit different pepsin digestion characteristics

The susceptibility of the test proteins to the in vitro pepsin digestion assay utilized in this study varied significantly depending on the protein tested. Some proteins (for example, HRP) were rapidly and completely digested, with neither the full-length protein nor fragments of the protein detectable at the first time point (Fig. 1A). Other full-length proteins (for example, BSA) were undetectable 0.5 min after the start of digestion but fragments were observed up to 60 min (Fig. 1B). In another group of proteins (for example ConA), both the full-length protein and fragments were detectable for at least 30 min after the start of the assay (Fig. 1C). Finally, there were proteins (for example, STI) that were resistant to digestion and relatively unchanged after 60 min of digestion (Fig. 1D). These results demonstrate that the proteins chosen for testing exhibited a wide range of susceptibility to pepsin digestion.

3.2. Comparison of the digestibility of the test proteins in pepsin as performed in the participating laboratories

In order to evaluate the results from all participants in a manner that would indicate whether substantive differences in protein digestibility were observed between laboratories, each participant's results were assessed for the final time point for which the full-length protein or fragment was detectable. The results from this analysis are depicted in Fig. 2. There was 91% agreement among

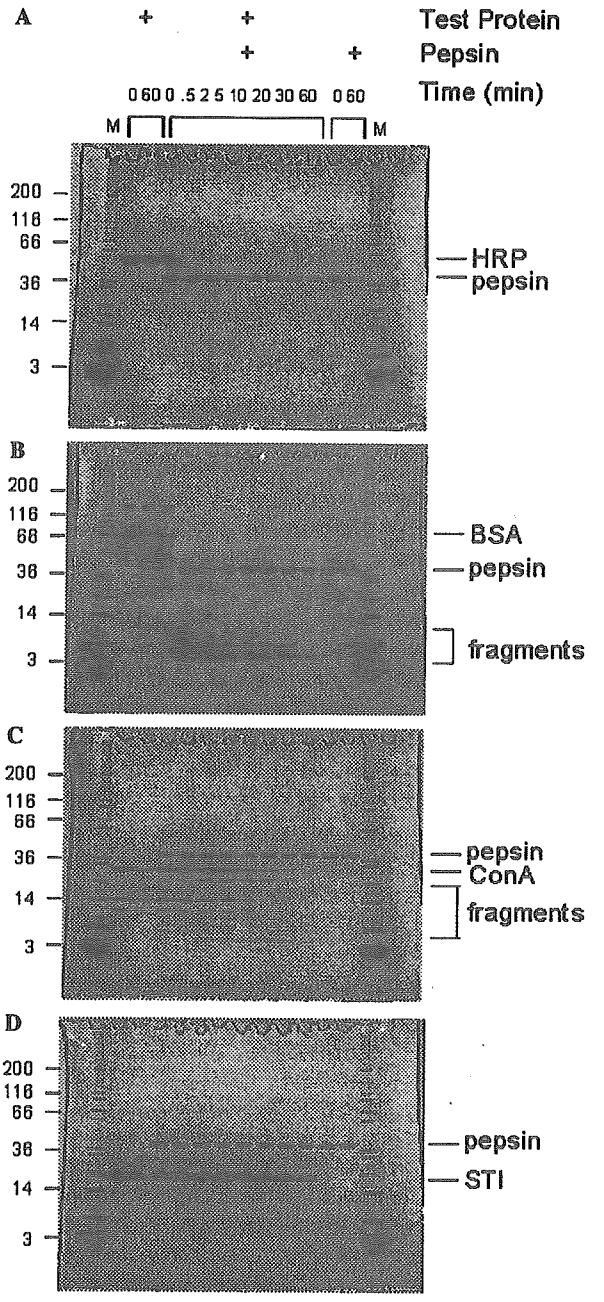

Fig. 1. Test proteins digested at different rates in a standardized in vitro pepsin digestion assay. Four patterns of digestion results were observed in this study. Some proteins were rapidly digested without visible fragments (A, HRP), some were rapidly digested, but produced resistant protein fragments (B, BSA), some were relatively resistant and also produced resistant fragments (C, Con A), and some were apparently fully resistant to digestion (D, STI). All gels in the study were loaded in the same order with the same amount of digestion reaction mix or markers. Mark12 markers, molecular weights ($\times 10^{-3}$) are indicated for lanes labeled "M." All other lanes contained 15 μl of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). These 10–20% polyacrylamide, Tricine gels are representative of reactions conducted at pH 1.2 from a single laboratory.

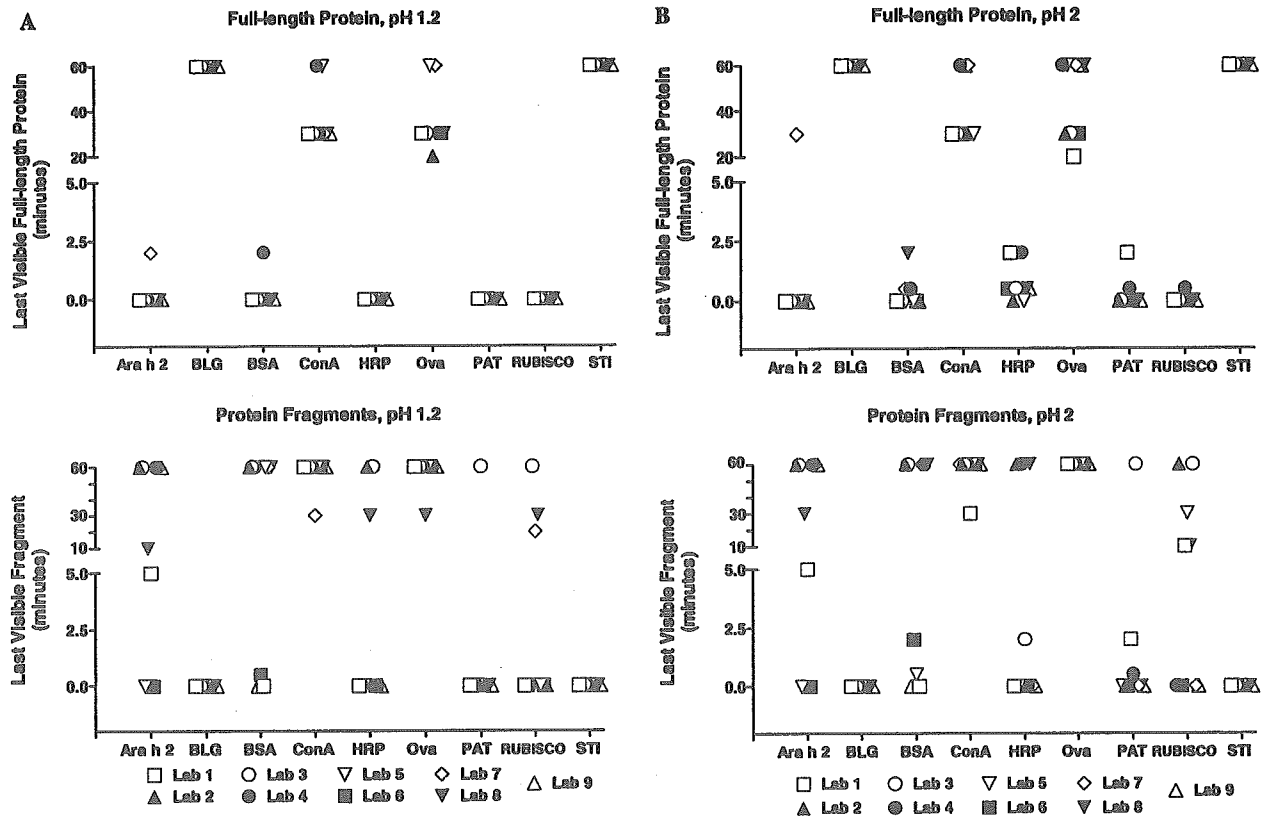

Fig. 2. Comparison of the digestibility of the test proteins across laboratories at pH 1.2 and 2.0. The last time-point each of the full-length test proteins or fragments were clearly visible in gels after staining in colloidal Coomassie blue G-250 or Coomassie brilliant blue R-250 from each of the nine laboratories are plotted. Protein samples digested in pH 1.2 pepsin buffer are shown in (A), samples digested in pH 2.0 buffer are shown in (B). Data from each laboratory are plotted.

laboratories regarding the digestibility of full-length proteins at pH 1.2 (Table 3, Fig. 2A) and 77% agreement on these proteins when digested at pH 2.0 (Table 4, Fig. 2B). For example, at pH 1.2 the PAT protein was undetectable after time zero in all laboratories. In contrast, at pH 2.0 the PAT protein was undetectable after time zero in 7/9 laboratories but in one laboratory this protein was last detected at the 0.5-min time point and in another laboratory it was last observed at the 2-min time point.

There was 80% agreement between laboratories regarding the detection of protein fragments at pH 1.2 (Table 3) and 75% agreement at pH 2.0 (Table 4). For example, at pH 1.2 the Ova protein fragments were detected at the 60-min time point in all labs. However, at pH 2.0 only 5/9 laboratories detected Ova fragments at the 60-min time point while 3/9 laboratories last detected them in the 30-min time point and 1/9 laboratories last detected them in the 20-min time point. The lower concordance between laboratory results for protein fragments compared to full-length proteins may be attributed to differences in staining procedures and gel electrophoresis methods as described below.

3.3. Effects of the gel electrophoresis system on detection of proteins and protein fragments

Laboratory # 2 used 10–20% polyacrylamide Tris–glycine gels while all other laboratories used 10–20% polyacrylamide Tricine gels. Overall, the two gel systems did not yield significantly different results. However, in some cases, the resolution of either the full-length protein or protein fragments was affected by the gel system used. For example, resolution of the full-length HRP and Ova proteins from the pepsin enzyme were substantially better in Tricine gels than that observed for these same proteins on Tris–glycine gels (Figs. 3A and B). In contrast, the full-length Ovm protein was more readily distinguished from the pepsin enzyme on Tris–glycine gels compared with Tricine gels (Fig. 3C).

3.4. Effects of the gel stain on detection of protein fragments

Several different gel fixing and staining procedures were used in the study (Table 1). Four laboratories used Coomassie brilliant blue R-250 staining procedures, while the other five laboratories used one of three

Table 3
Concordance of digestibility between laboratories, pH 1.2

Last time detected	0	0.5	2	5	10	20	30	60
Full-length Protein[a] *pH 1.2*								
Ara h 2	8[b]		1					
BLG								9[b]
BSA	8[b]		1					
ConA							7[b]	2
HRP	9[b]							
Ova						1	6[b]	2
PAT	9[b]							
RUBISCO	9[b]							
STI								9[b]
Sum of labs in agreement[b]	74 (out of 81 possible)							
Percent agreement	91%							
Protein Fragment(s)[a] *pH 1.2*								
Ara h 2	2			1	1			5[b]
BLG	9[b]							
BSA	2	1						6[b]
ConA							1	8[b]
HRP	6[b]						1	2
Ova							1	8[b]
PAT	8[b]							1
RUBISCO	6[b]					1	1	1
STI	9[b]							
Sum of labs in agreement[b]	65 (out of 81 possible)							
Percent agreement	80%							

Results of the number of laboratories reporting the indicated time (minutes) as the last observed time-point for each protein at pH 1.2.
[a] Ovomucoid (OVM) was not included in this evaluation due to the interference caused by the lysozyme contamination and the poor resolution of the full-length protein.
[b] The maximum number of laboratories in agreement regarding the last time each specific protein, or fragments of the specific protein were visible was summed in each experiment to calculate the percent agreement based on 81 (nine laboratories times 9 proteins).

versions of colloidal blue staining with Coomassie blue G-250. One difference noted among some laboratories that to some extent appears related to these staining procedures was the ability to detect protein fragments in digestion samples of Ara h 2, BSA, HRP, PAT, and RUBISCO (summarized in Fig. 2 and Tables 3 and 4). Even in examining data collected using a single staining method, some differences were found among laboratories, most notably for the detection of fragments of Ara h 2 (Fig. 4). In the stained gel images of seven laboratories, a proteolytic fragment of ~10,000 MW was visible through the first several digestion time points. However, two of the three laboratories that used Coomassie brilliant blue R-250 staining after fixing in TCA were unable to detect this band at any time point. This ~10,000 MW band has been identified as a fragment of Ara h 2 by N-terminal sequencing and MALDI-TOF (Monsanto, data not shown). These results suggest that the fixing and staining methods are likely to impact the ability to detect smaller molecular weight bands, and that factors that were not identified, such as time of fixing, staining or destaining may also be important. Detection of full-length proteins was markedly less variable as demonstrated by the closer agreement among laboratories on the final time of detection of the full-length proteins relative to the fragments (Tables 3 and 4).

4. Discussion

There have been several studies reported in the literature that have examined pepsin digestibility of a variety of allergens and non-allergens (Asero et al., 2001; Astwood and Fuchs, 1996; Astwood et al., 1996; Besler et al., 2001; Buchanan et al., 1997; del Val et al., 1999; Fu, 2002; Fu et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002; Sen et al., 2002; Tanaka et al., 2002). These studies did not always come to the same conclusions regarding the susceptibility of proteins to pepsin digestion. Reported differences in digestibility may be attributable to variations in the pepsin digestion assay parameters including pH of the assay buffer, the purity of the pepsin, the ratio of pepsin to target protein, the target protein purity and structural conformation, and finally, the method of detection. Although the kinetics of protein degradation (that is, rate) can be assessed,the more simple "time to disappearance" was evaluated in this study addressing the reproducibility of the pepsin digestion assay in different laboratories using a common protocol and test proteins.

4.1. Effect of pH on the in vitro pepsin digestion assay

The optimal pH range for porcine pepsin activity is 1.2–3.5, depending to some extent on the substrate