表1　コチニール色素とカルミンの用途，使用対象，成分表示および使用基準

	コチニール色素	カルミン
用　途	着色料	着色料
使用対象	飲料，菓子，デザート，あん，乳飲料，水・畜産加工品など	医薬品，医薬部外品，化粧品(口紅，アイシャドー，チークなど)，石鹸など
成分表示	コチニール色素，カルミン酸色素，着色料(コチニール)など	コチニール，カルミン，カルミン被覆雲母チタン，カルミン・コンジョウ被覆雲母チタン
使用基準	こんぶ類，食肉，鮮魚介類(鯨肉を含む)，茶，のり類，豆類，野菜およびわかめ類に使用してはならない	食品の着色に使用してはならない

表2　コチニール色素とカルミンの成分規格の対比

	コチニール色素		カルミン				
	食品添加物公定書	医薬部外品原料規格	医薬部外品原料規格	JECFA	EU	FDA	FCC
定義	カルミン酸が主成分	カルミン酸が主成分	カルミン酸のアルミニウムまたはアルミニウム・カルシウムレーキ	カルミン酸の水酸化アルミニウムキレート(1：2)	カルミン酸およびその塩	カルミン酸のアルミニウムまたはアルミニウム・カルシウムレーキ	カルミン酸のアルミニウムまたはアルミニウム・カルシウムレーキ
性状	赤～暗赤色の粉末，塊，液体またはペースト	赤紫色の粉末	赤～赤紫色または暗赤色の粉末	赤～暗赤色の固体または粉末	赤～暗赤色の固体または粉末	レーキ	赤～暗赤色の固体または粉末
含量	色価の規定	規格なし	規格なし	カルミン酸として50%以上	カルミン酸として50%以上	カルミン酸として50%以上	カルミン酸として50%以上
乾燥減量	規格なし	規格なし	20%以下	20%以下	規格なし	20%以下	20%以下
タンパク質	2.2%以下	規格なし	規格なし	25%以下	規格なし	2.2%以下	25%以下
重金属	Pbとして40 μg/g以下	20 ppm以下	規格なし	規格なし	40 mg/kg以下	規格なし	規格なし
鉛	10 μg/g以下	規格なし	20 ppm以下	2 mg/kg以下	10 mg/kg以下	10 ppm以下	2 mg/kg以下
カドミウム	規格なし	規格なし	規格なし	規格なし	1 mg/kg以下	規格なし	規格なし
水銀	規格なし	規格なし	規格なし	規格なし	1 mg/kg以下	規格なし	規格なし
ヒ素	As_2O_3として4 μg/g	2 ppm以下	5 ppm以下	規格なし	3 mg/kg以下	1 ppm以下	1 mg/kg以下
サルモネラ	規格なし	規格なし	規格なし	認めない	規格なし	認めない	認めない

れている．また，EUでは連合内で使用する食品添加物にはE番号を付与しており，コチニール色素とカルミンを区別することなくE120として管理し，食品ごとに使用が許可され，医薬部外品や化粧品などへの使用も許可されている．

表2にコチニール色素とカルミンの成分規格の対比表を示した．コチニール色素については食品添加物公定書[3]および医薬部外品原料規格[4]，カルミンについては医薬部外品原料規格[4]，また諸外国の規格としてJECFA[2]，EU[5]，米国食品医薬品局(FDA)[6]，米国食品化学物質規格(FCC)[7]を示した．食品添加物公定書[3]と医薬部外品原料規格[4]を比較すると，重金属やヒ素などの規格値に若干の違いがある．医薬部外品原料規格[4]にはタンパク質の規格値が設定されていないが，食品添加物公定書[3]にはタンパク質の規格値が設定されており，また，諸外国の規格においても，EU[5]を除いてタンパク質の規格値が設定されているという状況になっている．

コチニール色素によるアレルギー

コチニール色素による即時型アレルギーは，3

タイプに分けられると考えられている．1つ目のタイプは，職業性吸入曝露のタイプである．このタイプは，コチニールカイガラムシからの色素抽出に従事したり，化粧品工場でカルミンを取り扱うなど，職業的にコチニール色素やカルミンの吸入曝露をする労働者がアレルギーを発症するものであり，多くは男性症例である．発症する症状は喘息，鼻炎から結膜炎などといった気道症状が多い．2つ目のタイプは，コチニール色素およびカルミンを含んだ化粧品による皮膚症状を呈するタイプである．3つ目のタイプは，コチニール色素を含んだ食品の経口摂取により生じるタイプである．この経口摂取によるタイプは，気道症状，蕁麻疹・血管性浮腫などの皮膚症状，消化器症状などいろいろな症状を引き起こし，その多くは多臓器が同時に発症するアナフィラキシー症状を起こす．

豊永ら[8]はコチニール色素の経口摂取による即時型アレルギー症例に関して，2011年に既報告症例19例を集計しており，全例が23～52歳の成人女性の症例であったと報告している．Kägiら[9]は，カルミンE120で着色したカンパリオレンジを摂取して重篤なアナフィラキシー症状を起こした例を報告している．この例ではカンパリ，カルミンE120を用いた皮膚プリックテストで陽性を示し，また，カルミンが配合された口紅，マスカラ，アイシャドーなどの化粧品にも陽性反応を示したとしている．その他，女性症例が多く報告されていることから，コチニール色素が配合された化粧品を使用することによって，経皮的あるいは経粘膜的にコチニール色素に対するアレルギー感作が成立し，カルミンあるいはコチニール色素を添加した食品を摂取して，アナフィラキシー症状を生じるという仮説が有力視されている[9,10]．

図1に，コチニール色素の主色素成分のカルミン酸の構造式を示したが，分子量は492であり，低分子量の化合物である．食物による即時型アレルギーの場合，一般に低分子化合物が発症の原因となることはまれである．Chungら[11]は，カルミン摂取後とアレルギー症状発現の関連性を解析し，市販カルミンにはコチニールカイガラムシ由来のタンパク質が残存し，これらのタンパク質がIgEを介するアレルギー症状の発現に関与していると考察している．その他にも，コチニール色素またはカルミン中の夾雑タンパク質が原因であると考察する報告もある．さらに，アレルゲンの探索・同定を目的としたOhgiyaら[12]の報告によれは，コチニールカイガラムシ中のホスホリパーゼ様のタンパク質CC38Kが，主要夾雑アレルゲンタンパク質であると推定している．

このような研究報告から，コチニール色素のアレルギーの原因は，主色素成分のカルミン酸によるものではなく，製造工程で取り除くことができないコチニールカイガラムシ由来のタンパク質によるものと考えられきた．しかし，Sugimotoら[13]は，コチニール色素のアレルギー患者の好塩基球細胞を用いた*in vitro*の実験において，タンパク質がほとんど含まれていない高純度のカルミン酸でもヒスタミン遊離が起こることを報告している．したがって，カルミン酸をハプテン抗原としたアレルギー発症も考えられ，複数の原因の可能性も示唆されている．

コチニール色素によるアレルギーへの対策

JECFAでは第55回会合(2000年)において，コチニール色素およびカルミンのアレルギー誘発の可能性について検討を行い，これらは特定の個人に対しアレルギー反応を引き起こすと結論しており，これらが含まれる食品と飲料について注意を喚起する必要があるとし，成分規格の改正が実施されている[14]．食品表示に関するEU指令(EU-Directive 2000/13/EC)において，「食品添加物であるカルミンを食品の原材料リストに含めること」を要求しており，他の添加物と同様に表示義務が課せられている．また，2006年に米国FDAは，コチニール抽出物およびカルミンを評価し，一部のグループに対するアレルゲンを含有すると判断している．その後，食品および化粧品を対象に，コチニール抽出物とカルミンの表示を施すよう要求し，当該規制は，2011年1月5日に施行されている．すなわち，これらの注意喚起

や規制は，コチニール色素とカルミンによるアレルギー発症の可能性を認め，表示によってアレルギー発症やアナフィラキシーのリスク回避を消費者の判断で行うことを促しているものと思われる．

一方，わが国においてもコチニール色素によるアレルギー誘発の可能性についてはかなり前から懸念されており，その対策は既に講じられている．食品添加物として用いられるわが国のコチニール色素のタンパク質含量の規格値は，諸外国と比べて低く設定されている(**表2**)．また，国内メーカーでは「低アレルゲンコチニール色素」として，タンパク質含量を低減化したコチニール色素を開発し販売している．これは主色素成分のカルミン酸の精製度を上げて高純度化し，結果としてカイガラムシ由来のタンパク質含量を従来製品よりさらに低減化したものである．「低アレルゲンコチニール色素」については，マウス膝窩リンパ節測定法(popliteal lymph node assay：PLNA)による抗原性試験法を用いて評価したとき，通常のコチニール色素より明らかに抗原性が低下したことも報告されている[15]．今般の消費者庁のコチニール色素に関する注意喚起により，国内の食品用に用いられるコチニール色素は，「低アレルゲンコチニール色素」に切り替えられる方向になると予想される．また，医薬部外品や化粧品などに用いられるコチニール色素やカルミンの場合は，医薬部外品原料規格にタンパク質の規格が定められていないため，不純物やタンパク質の除去が十分でない製品の存在は否定できない．また，海外の化粧品等についても同様な問題が懸念される．したがって，医薬品部外品や化粧品などにおいても規格の改定が必要と考えられる．

おわりに

コチニール色素によるアレルギー発症は，他の食物アレルギー発症と比較して極めて頻度の低いケースであると思われるが，国内外におけるアレルギー発症の実態や原因を完全に特定するための十分な知見がないのも事実である．このような背景から，厚生労働省は，成分表示・仕様上の注意への記載の徹底を呼びかけている[16]．特にコチニール色素に対して既にアレルギー症状の既往歴がある方の場合は，特に注意が必要だろう．

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Development and evaluation of a novel DNA extraction method suitable for processed foods

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Development and evaluation of a novel DNA extraction method suitable for processed foods

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Abstract

For easy and rapid DNA extraction from processed foods, we developed a new silica membrane-based DNA extraction method. DNA extraction conditions suitable for processed foods were examined based on an existing DNA extraction kit for raw grain materials, GM quicker 2. Twenty microliters of proteinase K solution (20 mg/ml) was used for cell lysis and the digestion was carried out at 65°C for 30 min. In addition, 200 μl for wet processed foods or 400 μl for dry processed foods of 2.0 M potassium acetate (pH 3.7) and 600 μl of 8.0 M guanidine hydrochloride were adopted as buffers to achieve good DNA recovery from cell lysates. The novel method was compared to four conventional methods using six kinds of processed foods as analytical samples, i.e., roasted soybean flour, soy milk, miso, canned whole kernel sweet corn, corn snack and dried soup mix. The developed method showed wide applicability to various process foods and it gave sufficient amounts of DNA with high purity. Also, the method was highly user-friendly because of the short handling time, the small number of pipette operations and non-use of toxic organic solvents. The method would be practically used for food testing to detect genetically modified organisms, allergens, pathogenic microorganisms and so on.

Keywords : processed foods, DNA extraction, guanidine hydrochloride, silica membrane, genetically modified organism

I Introduction

DNA analyses based on molecular biological techniques, such as polymerase chain reaction (PCR), are widely performed for food testing to detect genetically modified organisms (GMOs), allergens, pathogenic microorganisms and so on[1-3]. PCR analysis is generally comprised of four steps, i.e., sample grinding as pretreatment, DNA extraction, PCR and electrophoresis analysis. Of these steps, the DNA extraction step tends to be the most labor-intensive. An easy and fast DNA extraction method is highly desirable for efficient food testing. So far, methodologies enabling DNA extraction and purification from biological materials have been established. The Cetyltrimethylammonium bromide (CTAB)-based method[4, 5], the anion exchange resin-based method[6], and the silica membrane-based method[7] are practical, and a variety of DNA extraction kits based on these methods are commercially available.

From the viewpoint of consumer protection, it is important to test end products in food supply chain, many of which are processed foods. DNA in processed foods appears to be fragmented or degraded by physical, chemical, and/or biological factors during processing[8]. Additionally, processed foods are composed of numerous materials and/or ingredients. Hence, successful DNA extraction from processed foods is difficult. In fact, the currently existing DNA extraction methods have several drawbacks, including unstable yield, long handling time, complex operation, and/or use of toxic

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organic solvents such as phenol and chloroform. So, we attempted to develop a DNA extraction method for processed foods that is easier to use and faster.

Previously, we developed a silica membrane-based DNA extraction kit for raw grain materials, named GM quicker 2 kit[9]. The kit is recognized as simple and reliable, and was adopted as part of the Japanese official testing method for GMO in food[10, 11]. In this study, we developed a DNA extraction method suitable for processed foods based on the GM quicker 2 kit. Comparative evaluation of the developed method and the currently existing DNA extraction methods was also performed.

Ⅱ Materials and Methods

1. Samples

Three kinds of processed soybean products (soy milk, roasted soybean flour, miso) and three kinds of processed maize products (canned whole kernel sweet corn, corn snack, dried soup mix) were purchased at a local market in Toyama, Japan. As the pretreatment for DNA extraction, miso and canned whole kernel sweet corn were combined with an equal weight of distilled water and ground using a knife mill Grindomix GM200 (Retsch, Dusseldorf, Germany). Corn snack was ground using the knife mill Grindomix GM200 directly. Soy milk, roasted soybean flour and dried soup mix were used without pretreatment.

2. Optimization of buffer condition for DNA recovery from processed foods

Soy milk and roasted soybean flour samples were employed in determining the optimal buffer condition for DNA recovery from processed foods.

GM quicker 2 kit (NIPPON GENE, Tokyo, Japan) was used as the base technology for developing the new DNA extraction method. One gram of sample was transferred to a 50 ml polypropylene centrifuge tube (Sarstadt, Nuembrecht, Germany), and 1.0 ml (for soy milk) or 4.0 ml (for roasted soybean flour) of GE1 buffer, 20 μl (for soy milk) or 40 μl (for roasted soybean flour) of proteinase K solution (20 mg/ml), 2 μl of α-amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed. The mixture was incubated at 65°C for 30 min, with vortexing every 10 min during incubation. Then, 200 μl (for soy milk) or 400 μl (for roasted soybean flour) of 0.3 or 1.0 M potassium acetate (pH 3.5, 4.5 or 5.5) or 2.0 M potassium acetate (pH 3.7, 4.5 or 5.5) was added to the mixture, and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780 (KUBOTA, Tokyo, Japan) and 800 μl of supernatant was transferred to a 2 ml microcentrifuge tube. Six hundred microliters of 8.0 M guanidine hydrochloride (condition A), or 300 μl of 8.0 M guanidine hydrochloride and 300 μl of 2-propanol (condition B) was added to the supernatant and mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and first, half of the supernatant (about 700 μl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, 600 μl of GW buffer was added to the spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g and placed in a correction tube. The DNA was eluted by the addition of 50 μl of TE (10 mM Tris-HCl, pH8.0, 1 mM ethylenediaminetetraacetate (EDTA)) buffer (pH 8.0), incubation for 3 min at room temperature and centrifugation for 1 min at 10,000 × g. The DNA extraction was subjected to real-time PCR assay as described below.

3. Optimization of proteinase K treatment for lysing processed foods

Soy milk and roasted soybean flour samples were employed in determining the optimal amount of proteinase K for DNA extraction from processed foods. One gram of sample was transferred to a 50 ml polypropylene centrifuge tube and 1.0 ml (for soy milk) or 4.0 ml (for roasted soybean flour) of GE1 buffer, 0, 10, 20, 40, 80 μl of proteinase K solution (20 mg/ml), 2 μl of α-amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated at 65°C for 0, 15, 30, 45, 60 min with vortexing every 15 min during incubation. Then, 200 μl (for soy milk) or 400 μl (for roasted soybean flour) of 2.0 M potassium acetate (pH 3.7) was added to the mixture, and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780, and 800 μl of supernatant was transferred to a 2 ml microcentrifuge tube. Six hundred microliters of 8.0 M guanidine hydrochloride was added to the supernatant and mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and half of the supernatant (about 700 μl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin-column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, 600 μl of GW buffer was added to the spin column, the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g, and placed in a correction tube. The DNA was eluted by the addition of 50 μl of TE (pH 8.0), incubation

for 3 min at room temperature and centrifugation for 1 min at 10,000 × g. The DNA extracts were subjected to real-time PCR assay as described below.

4. Development of a novel DNA extraction method for processed foods

1) DNA extraction from wet processed foods (soy milk, miso, canned whole kernel sweet corn)

One gram of sample (ground and mixed with water) was transferred to a 50 ml polypropylene centrifuge tube, then 1.0 ml of GE1 buffer, 20 µl of proteinase K solution (20 mg/ml), 2 µl of α-amylase solution (60 units/µl) and 10 µl of RNase A solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated at 65°C for 30 min, with vortexing every 5 min during incubation. Then, 200 µl of 2.0 M potassium acetate (pH 3.7) was added to the mixture and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780, and 800 µl of supernatant was transferred to a 2 ml micro centrifuge tube. Six hundred microliters of 8.0 M guanidine hydrochloride was added to the supernatant and mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and half of the supernatant (about 700 µl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin-column, and centrifuged for 1 min at 10,000 × g. After removed the filtrate, 600 µl GW buffer was added to the spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g, and placed in a correction tube. The DNA was eluted by the addition of 50 µl of TE (pH 8.0), incubation for 3 min at room temperature and centrifugation for 1 min at 10,000 × g.

2) DNA extraction from dry processed foods (roasted soybean flour, corn snack, dried soup mix)

One gram of sample was transferred to a 50 ml polypropylene centrifuge tube, and 4.0 ml of GE1 buffer, 20 µl of proteinase K solution (20 mg/ml), 2 µl of α-amylase solution (60 units/µl) and 10 µl of RNase A solution (100 mg/ml) were added and vortexed. The mixture was incubated at 65°C for 30 min, with vortexing every 5 min during incubation. Then, 400 µl of 2.0 M potassium acetate (pH 3.7) was added to the mixture and mixed well by inverting. Afterwards, the same procedure as 4.1) was performed.

5. Conventional DNA extraction methods for processed foods

1) Silica membrane-based method

The silica membrane-based method was performed with the DNeasy® Plant Maxi kit (QIAGEN, Hilden, Germany) as described in the Japanese Agricultural Standard (JAS) analytical test handbook. According to the handbook, protocols A and B were applied to processed food made from soybean and maize, respectively[12].

2) CTAB-based method

The CTAB-based method was performed as described in the JAS analytical test handbook[12].

3) CTAB/Silica membrane-based method

The CTAB/Silica membrane-based method was performed with the DNeasy® *mericon*™ Food kit (QIAGEN) as described in the manufacturer's protocol for small DNA fragments.

4) Anion exchange resin-based method

The anion exchange resin-based method was performed with the Genomic-tip 20/G kit (QIAGEN) as described in the JAS analytical test handbook[12].

6. Estimation of amount and quality of extracted DNA

The concentration of DNA solutions was determined by both UV absorption and fluorescence detection of DNA intercalation. The UV absorbance at 260 nm was measured using a DU-800 spectrophotometer (Beckman Coulter, FL, USA). Fluorescence intercalator detection was carried out using the PicoGreen ® dsDNA Quantitation Kit (Molecular Probes, OR, USA) according to the manufacturer's protocol. The fluorescence of DNA sample was measured at 520 nm after excitation at 480 nm using a Infinite™ F200 (Tecan, Mannedorf, Switzerland).

7. Agarose gel electrophoresis of extracted DNA

Agarose gel electrophoresis was carried out with 1% (w/v) Agarose S gel (NIPPON GENE) in Tris-acetate-ethylenediaminetetraacetate (TAE) buffer with 0.5 µg/ml of ethidium bromide. Ten microliters of solution including 250 ng of DNA was mixed with 2 µl of 6 × loading buffer, and the samples were subjected to electrophoresis at a constant voltage (100 V) for approximately 30 min in TAE buffer. After the electrophoresis, the gel was photographed under UV radiation using a densitograph system (ATTO, Tokyo, Japan).

8. Real-time PCR assay of extracted DNA

For processed soy foods, the copy number of the taxon-specific gene encoding the soy lectin1 (*Le1*) was analyzed by real-time PCR. For processed maize foods, the taxon-specific sequence encoding the maize starch synthase IIb gene (*SSIIb*) was analyzed. The PCR amplification was carried out in 25 µl total reaction volume containing 2.5 µl of a DNA extract, 12.5 µl of TaqMan® Universal PCR Master Mix (Life Technologies, CA, USA), 0.5 µM of the primer pair and 0.2 µM of double dye-labeled probe. PCR amplifications were performed using the following program: preincubation at 50°C for 2 min and

95°C for 10 min, 45 cycles of denaturation at 95°C for 30 sec, and annealing and extension at 59°C for 1 min. The GM maize detection SSIIb03 (for endogenous gene) oligonucleotide set and the GM soybean detection Le1 (for endogenous gene) oligonucleotide set (NIPPON GENE) were used as primers and probes. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5' and 3' ends, respectively. The ABI PRISM™ 7500 Fast Sequence Detection System (Life Technologies) was used as the real-time PCR instrument.

Ⅲ Results and Discussion

1. Development of new DNA extraction method for processed foods

1) Optimization of buffer condition for DNA recovery from processed foods

To mix a sample sufficiently and recover supernatant, adequate volumes of GE1 buffer were different for dry and wet processed foods. Therefore, processed foods fell into two categories, dry and wet materials, based on its water content. We chose roasted soybean flour and soy milk as representative samples of dry and wet materials, respectively, in order to determine the optimal condition for efficient DNA extraction and purification from both kinds of processed food. First, several different buffer conditions were compared concerning binding efficiency of DNA to the silica membrane (Fig. 1). Copy numbers of *Le1* measured using real-time PCR were used as an indicator of DNA yield. For extraction of proteins and saccharides, potassium acetate buffer of various concentrations and pH were prepared. To boost DNA binding to the silica membrane, guanidine hydrochloride was used as a chaotropic agent. For condition A, 8.0 M of guanidine hydrochloride was used in accordance with the original report of the silica membrane-based method by Boom R. et al[7]. For condition B, a mixture of equal volumes of 8.0 M of guanidine hydrochloride and 2-propanol was used, the same as for the GM quicker 2 kit. The results indicated that extracted DNA bound to the silica membrane most efficiently under condition A with using 2.0 M potassium acetate buffer (pH 3.7). Thus 2.0 M potassium acetate (pH 3.7) and guanidine hydrochloride (condition A) were selected as buffers for the developed method. Adoption of condition A, which does not require 2-propanol, is expected to simplify the DNA extraction process.

2) Optimization of proteinase K-treatment condition

Optimal volume of proteinase K and incubation time were examined (Fig. 2). Figure 2 shows that DNA can be extracted efficiently from roasted soybean flour using more than 10 μl of proteinase K for a longer than 30 min-digestion, and from soy milk using more than 10 μl of proteinase K for a longer than 15 min-digestion. In Figure 2, the copy numbers at 0 min were increased depending on the amount of proteinase K. We speculated that the proteinase K reacted during a short time until the reaction stop by the acidic potassium acetate buffer. To make the developed method applicable for a variety of foods that differ in protein content, a 20 μl volume of proteinase K and 30 min digestion time were selected.

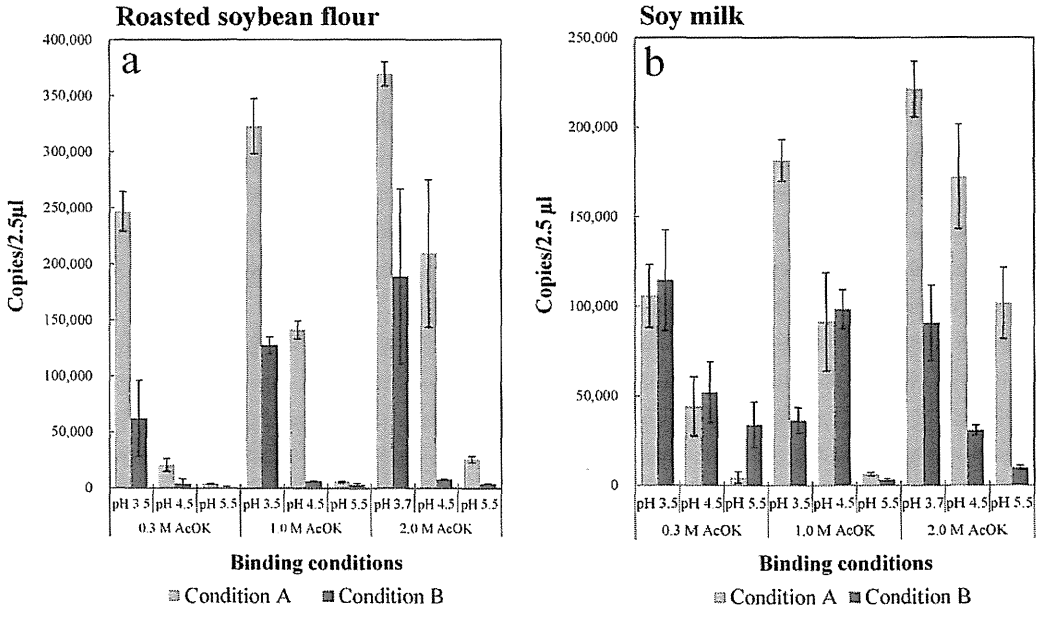

Fig. 1. Real-time PCR analysis of DNA extracts obtained under the various buffer conditions. A, roasted soybean; B, soy milk. Error bars indicate the standard deviation for three replicates. AcOK means potassium acetate.

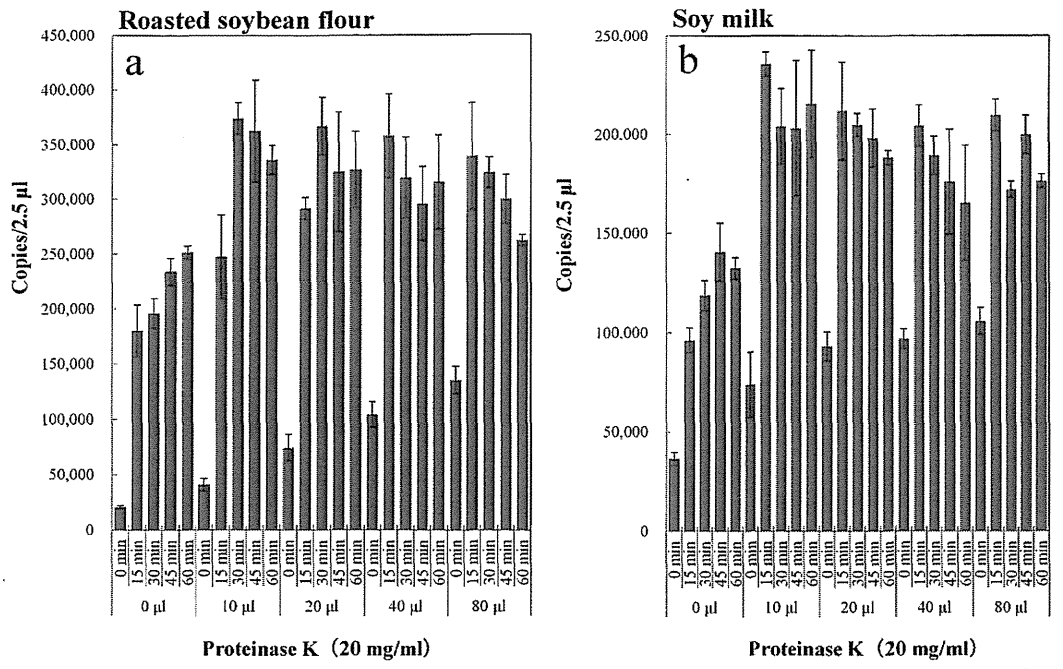

Fig. 2. Real-time PCR analysis of DNA extracts obtained under the various proteinase K digestion conditions. A, roasted soybean; B, soy milk. Error bars indicate the standard deviation for three replicates.

2. Evaluation of extracted DNA from processed foods by agarose-gel electrophoresis

Extracted DNA from roasted soybean flour and soy milk generated by the developed method were analyzed by agarose gel electrophoresis (Fig. 3). For DNA extracted from soy milk, smears of DNA fragments were observed in the range between 0.42 kb and 7.74 kb. On the other hand, only DNA fragments shorter than 0.42 kb were obtained from DNA extracted from roasted soybean flour. These results show that various length of DNA fragments, from short to long, can be recovered by the developed method.

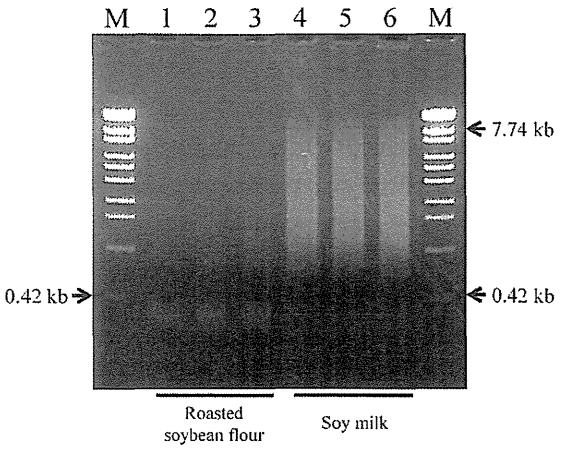

Fig. 3. Analysis of DNA fragmentation using ethidium bromide-stained agarose gel electrophoresis. Lane M, λ/*Sty* I digest (OneSTEP Marker 6, NIPPON GENE); Lane 1 to 3, DNA extracts from roasted soybean flour; Lanes 4 to 6, DNA extracts from soy milk.

3. Evaluation of extracted DNA from processed foods by absorbance measurements and PicoGreen® assay

UV absorbance at 260 nm is the most commonly used for DNA quantitation method; however, it is possible that the proteins or saccharides remaining in the DNA extracts may result in inaccurate readings. Meanwhile, although the PicoGreen® assay takes longer operation time and higher cost, it can detect double stranded DNA with high specificity. We measured DNA concentration using these two different methodologies. The DNA samples were extracted from processed foods (soy milk, roasted soybean flour, miso, canned whole kernel sweet corn, corn snack and dried soup mix) using the newly developed method and four other methods (Silica membrane-based, CTAB-based, CTAB/Silica membrane-based and anion exchange resin-based method). Then, the amounts of DNA were analyzed in triplicate (Table 1). In the official GMO testing methods[12], the use of DNA diluted to 10 ng/µl is recommended. The developed method and anion exchange resin-based method gave us above 10 ng/µl of DNA, while the other 3 methods showed insufficient DNA yield from several samples. Meanwhile, the anion exchange resin-based method showed large differences in the results of roasted soybean flour and dried soup mix, suggesting that impurities

Table 1. Comparison of DNA yields by five DNA extraction methods

Sample name	Extraction method	Absorbance measurements DNA Conc. (ng/μl)		PicoGreen® assay DNA Conc. (ng/μl)	
		Means	S.D.(i)	Means	S.D.(i)
Roasted soybean flour	New silica membrane-based method	105.62	7.05	119.86	4.98
	Silica membrane-based method	119.15	20.59	88.04	6.11
	CTAB-based method	240.08	56.00	46.01	9.54
	CTAB/Silica membrane-based method	42.30	1.86	25.39	0.84
	Anion exchange resin-based method	2218.50	43.49	535.92	11.68
Soy milk	New silica membrane-based method	80.18	6.46	49.90	4.86
	Silica membrane-based method	8.57	6.74	3.97	3.80
	CTAB-based method	18.50	1.37	6.79	0.42
	CTAB/Silica membrane-based method	35.88	1.10	14.04	1.19
	Anion exchange resin-based method	272.90	15.77	160.91	6.28
Miso	New silica membrane-based method	44.77	1.24	92.36	4.42
	Silica membrane-based method	7.62	2.14	9.17	3.32
	CTAB-based method	7.38	0.51	15.93	1.13
	CTAB/Silica membrane-based method	5.53	0.53	5.76	0.55
	Anion exchange resin-based method	171.28	7.67	337.07	1.50
Canned whole kernel sweet corn	New silica membrane-based method	71.42	7.46	89.19	13.32
	Silica membrane-based method	636.70	198.36	20.12	0.40
	CTAB-based method	127.23	13.72	17.17	2.01
	CTAB/Silica membrane-based method	6.02	0.53	4.65	0.70
	Anion exchange resin-based method	604.83	21.88	401.44	10.61
Corn snack	New silica membrane-based method	50.25	2.81	19.88	3.50
	Silica membrane-based method	121.07	13.70	14.31	1.36
	CTAB-based method	22.23	5.06	6.73	0.88
	CTAB/Silica membrane-based method	6.43	0.32	1.71	0.04
	Anion exchange resin-based method	238.68	20.21	113.37	8.99
Dried soup mix	New silica membrane-based method	224.80	29.20	165.36	6.76
	Silica membrane-based method	173.97	47.15	133.09	36.55
	CTAB-based method	64.10	4.45	38.17	4.31
	CTAB/Silica membrane-based method	27.08	3.35	18.02	1.86
	Anion exchange resin-based method	1661.33	119.05	405.99	6.86

(i) S.D. means standard deviation. n = 3.

remained in the DNA extracts. These results support that the developed method is a better choice in terms of stable DNA yield and purity.

4. Evaluation of extracted DNA from processed foods by real-time PCR analysis

DNA extracted from processed soy and maize foods were analyzed by real-time PCR without adjustment of DNA concentration (Tables 2 and 3). Although the anion exchange resin-based method showed high DNA concentration (Table 1), the DNA sample from roasted soybean flour did not show DNA amplification in the real-time PCR analysis. We speculated that the failed DNA amplification was attributed to PCR inhibition. To evaluated PCR inhibition in real-time PCR analyses, therefore, the DNA samples which were diluted 10-fold with TE buffer were also analyzed by real-time PCR and then, the obtained copy numbers were multiplied by 10 for comparison with the measurement results of the undiluted samples (Table 2 and 3). In case of the DNA samples from canned whole kernel sweet corn and dried soup mix, the calculated copy numbers from diluted samples were more than 100-fold higher, suggesting that undiluted samples caused PCR inhibition. Meanwhile, all the DNA extracts obtained by the developed method were not subject to PCR inhibition even in the undiluted state.

To compare the DNA extraction methods in terms of DNA extraction efficiency, total DNA copies obtained from 1 gram of the respective initial samples were calculated (Tables 2 and 3). Regarding the DNA samples which showed PCR inhibition i.e., DNA samples from roasted soybean flour, canned whole kernel sweet corn and dried soup mix by the anion exchange resin-based method, the copy numbers obtained from diluted samples were adopted for the calculation. The measured copy number in 2.5 μl of DNA sample was multiplied by the elution volume and divided by the weight of the initial sample. The calculated total copy numbers by the developed method was the highest for miso, the second highest for the canned whole kernel sweet corn and corn snack and the third highest for the other three processed food samples. We concluded that the developed method was comparatively good in terms of the DNA yield from a certain amount of starting material.

Table 2. Real-time PCR analysis of DNA extracts from processed soy foods

Sample name	Extraction method	Le1 copy number/2.5 μl Means	S.D.(i)	Sample weight (g)	Elution volume (μl)	Total × 10^3 copies /1 g sample
Roasted soybean flour	New silica membrane-based method	49325.63 50006.84 (ii)	1951.63 3323.63 (ii)	1.0	50	986.51
	Silica membrane-based method	11821.04 21518.22 (ii)	1951.04 2967.09 (ii)	1.0	100	472.84
	CTAB-based method	9109.98 8082.47 (ii)	2042.37 1921.79 (ii)	0.2	100	1822.00
	CTAB/Silica membrane-based method	6674.50 3707.23 (ii)	577.10 170.70 (ii)	2.0	100	133.49
	Anion exchange resin-based method	N.D. (iii) 301189.00 (ii)	18924.38 (ii)	2.0	50	3011.89 (ii)
Soy milk	New silica membrane-based method	135828.44 138207.22 (ii)	16608.37 4649.92 (ii)	1.0	50	2716.57
	Silica membrane-based method	13472.48 8586.11 (ii)	10837.53 10052.22 (ii)	1.0	100	538.90
	CTAB-based method	15667.50 10901.33 (ii)	1156.56 813.29 (ii)	0.2	100	3133.50
	CTAB/Silica membrane-based method	10106.03 4618.56 (ii)	846.67 332.49 (ii)	2.0	100	202.12
	Anion exchange resin-based method	418532.00 347826.67 (ii)	21997.07 54309.70 (ii)	2.0	50	4185.32
Miso	New silica membrane-based method	2792.68 2432.71 (ii)	230.39 109.09 (ii)	1.0	50	55.85
	Silica membrane-based method	228.12 202.85 (ii)	71.28 117.24 (ii)	1.0	100	9.12
	CTAB-based method	262.10 172.20 (ii)	16.58 42.04 (ii)	0.2	100	52.42
	CTAB/Silica membrane-based method	149.88 79.30 (ii)	3.15 22.21 (ii)	2.0	100	3.00
	Anion exchange resin-based method	5361.06 5217.98 (ii)	907.34 238.50 (ii)	2.0	50	53.61

(i) S.D. means standard deviation.
(ii) DNA samples were diluted 10-fold with TE buffer, and the obtained quantitative value was multiplied by 10.
(iii) N.D. means not detected.

Table 3. Real-time PCR analysis of DNA extracts from processed maize foods

Sample name	Extraction method	SSIIb copy number/2.5 μl Means	S.D.(i)	Sample weight (g)	Elution volume (μl)	Total × 10^3 copies /1 g sample
Canned whole kernel sweet corn	New silica membrane-based method	8526.71 5108.83 (ii)	508.66 992.13 (ii)	1.0	50	170.53
	Silica membrane-based method	1536.81 1257.50 (ii)	118.15 87.62 (ii)	1.0	100	61.47
	CTAB-based method	628.06 519.58 (ii)	15.31 29.78 (ii)	0.2	100	125.61
	CTAB/Silica membrane-based method	318.56 117.32 (ii)	72.60 58.00 (ii)	2.0	100	6.37
	Anion exchange resin-based method	4.17 19887.52 (ii)	4.53 1642.11 (ii)	2.0	50	198.88 (ii)
Corn snack	New silica membrane-based method	749.86 603.87 (ii)	82.81 82.00 (ii)	1.0	50	15.00
	Silica membrane-based method	209.30 200.51 (ii)	102.54 64.59 (ii)	1.0	100	8.37
	CTAB-based method	69.95 89.82 (ii)	9.47 18.95 (ii)	0.2	100	13.99
	CTAB/Silica membrane-based method	71.32 49.21 (ii)	10.87 13.37 (ii)	2.0	100	1.43
	Anion exchange resin-based method	2046.13 1786.51 (ii)	122.63 259.51 (ii)	2.0	50	20.46
Dried soup mix	New silica membrane-based method	205311.00 194405.44 (ii)	12155.67 24353.90 (ii)	1.0	50	4106.22
	Silica membrane-based method	129172.68 119840.50 (ii)	30538.63 34993.75 (ii)	1.0	100	5166.91
	CTAB-based method	16415.04 13302.74 (ii)	1886.49 721.43 (ii)	0.2	100	3283.01
	CTAB/Silica membrane-based method	21875.12 11439.08 (ii)	2858.30 1149.47 (ii)	2.0	100	437.50
	Anion exchange resin-based method	3232.16 1121762.22 (ii)	1493.46 45616.94 (ii)	2.0	50	11217.62 (ii)

(i) S.D. means standard deviation.
(ii) DNA samples were diluted 10-fold with TE buffer, and the obtained quantitative value was multiplied by 10.

5. Comparison of DNA extraction methods in terms of handling

To compare the usability of the DNA extraction methods, handling time, the number of pipette operations and use of toxic organic solvent were assessed and are summarized in Table 4. This survey revealed that the newly developed silica membrane-based method permitted the shortest handling time and the smallest number of pipette operations. Additionally, the method did not require toxic organic solvents. It is clear that our new silica membrane-based method is superior in terms of user-friendliness.

Ⅳ Conclusion

We developed a novel silica membrane-based DNA extraction method suitable for processed foods. The developed method was evaluated against four conventional methods using six kinds of processed food as analytical samples. The developed method showed wide applicability to various process foods and it gave sufficient amounts of DNA with high purity. Also, the user-friendliness of the method was extremely high because of the short handling time, the small number of pipette operations and non-use of toxic organic solvents. It is expected that our novel method will be practically used in food testing to detect GMOs, allergens and pathogenic microorganisms.

Ⅴ Acknowledgments

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Table 4. Summary of operation procedures of the DNA extraction methods

DNA extraction method		Handling time[(i)]	The number of pipette operations[(iii)]	Toxic organic solvent[(iv)]
Method name	Commercially available kit			
New silica membrane-based method	GM quicker 2 kit	60 min	11	–
Silica membrane-based method (protocol A, for soybean)	DNeasy® Plant Maxi kit	190 min (+12-24 hours)[(ii)]	26	–
Silica membrane-based method (protocol B, for maize)	DNeasy® Plant Maxi kit	160 min (+12-24 hours)[(ii)]	19	–
CTAB-based method	–	130 min	19	Phenol and chloroform
CTAB/Silica membrane-based method	DNeasy® *mericon*™ Food kit	70 min	11	Chloroform
Anion exchange resin-based method	Genomic-tip 20/G	130 min (+12-24 hours)[(ii)]	40	–

(i) Approximate time needed for DNA extraction from one sample.
(ii) DNA precipitant was suspended for 12-24 hours.
(iii) When using micropipettes up to 5 ml.
(iv) Toxic organic solvents as designated by the Poisonous and Deleterious Substances Control Act of Japan.

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論　　文

加工食品に適した新規 DNA 抽出法の開発と評価

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キーワード：加工食品、DNA 抽出法、グアニジン塩酸塩、シリカ膜、遺伝子組換え農産物

概　要

加工食品から簡便迅速に DNA を抽出することを目的としてシリカ膜を用いた新規 DNA 抽出法を開発した。本法は穀物を対象とする DNA 抽出キット GM quicker 2 を基盤とし、加工食品からの DNA 抽出に最適化を図ったものである。試料の前処理にはプロテイナーゼ K（20 mg/ml）を 20 µl 添加し、65℃で 30 分間処理する条件を採用した。また、より多くの DNA を回収する条件として、2M の酢酸カリウム溶液（pH3.7）を（液状試料には）200 µl、（乾燥試料には）400 µl、及び 8M のグアニジン塩酸塩溶液を 600 µl 抽出に用いることとした。次に、6 種類の加工食品（きな粉、豆乳、味噌、トウモロコシ缶詰、スナック菓子、粉末コーンスープ）を用い、既存の 4 種類の DNA 抽出法と新たに開発した方法の比較を行った。その結果、DNA の収量、純度及び操作性の各指標において、新たに開発した方法は従来法と同等かそれ以上の性能を有することが確認された。

本研究で開発した DNA 抽出法は、遺伝子組換え農産物や、アレルゲン、食中毒菌などを対象とする加工食品の遺伝子検査に広く活用されることが期待される。

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FOOD COMPOSITION AND ADDITIVES

Interlaboratory Validation Study of an Event-Specific Real-time Polymerase Chain Reaction Detection Method for Genetically Modified 55-1 Papaya

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Genetically modified (GM) papaya line 55-1 (55-1) is resistant to papaya ringspot virus infection, and is commercially available in several countries. A specific detection method for 55-1 is required for mandatory labeling regulations. An event-specific real-time PCR method was developed by our laboratory. To validate the method, interlaboratory validation of event-specific qualitative real-time PCR analysis for 55-1 was performed in collaboration with 12 laboratories. DNA extraction and real-time PCR reaction methods were evaluated using 12 blind samples: six non-GM papayas and six GM papayas in each laboratory. Genomic DNA was highly purified from all papayas using an ion-exchange column, and the resulting DNA sample was analyzed using real-time PCR. Papaya endogenous reference gene chymopapain (*CHY*) and the event-specific 55-1 targeted sequence were detected in GM papayas whereas *CHY* alone was detected in non-GM papayas in all laboratories. The cycle threshold values of *CHY* and the 55-1 targeted sequence showed high repeatability (RSD_r 0.6–0.8%) and reproducibility (RSD_R 2.2–3.6%). This study demonstrates that the 55-1 real-time PCR detection method is a useful and reliable method to monitor 55-1 papaya in foods.

In recent years, an increasing number of genetically modified (GM) crops have been developed in many countries and regions using recombinant DNA technology. Over the past 15 years, a number of these crops have been approved for commercialization following safety evaluations (1). However, the use of GM crops for foods remains controversial. Labeling of GM foods is required to allow consumers the freedom of choice with respect to foods. Therefore, many countries have mandated the labeling of foods containing a specified threshold level of GM organisms: 0.9% in the European Union (EU); 3% in Korea; and 5% in Japan (2).

Papaya (*Carica papaya* L.) is an economically important fruit in tropical and subtropical areas (3). The infection of papaya with papaya ringspot virus (PRSV) is the most serious problem for papaya cultivation worldwide (4). GM papayas resistant to PRSV have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, the Philippines, and Vietnam (4). GM papaya line 55-1 (55-1), released in Hawaii in 1998, was the first commercialized PRSV-resistant GM papaya (5), and is authorized in the United States, Canada, and Japan (6), indicating its popularity as a GM fruit. GM papaya Huanong No. 1 was developed in China and commercialized there in 2006 (7). However, to date, GM papayas developed elsewhere have not yet been authorized by any countries.

Because 55-1 is commercially available, development of a specific, as well as quantitative, detection method was necessary to enable identification of 55-1 use and monitoring for validity of labeling in papaya fresh fruit and processed papaya products. We previously established a histochemical assay (8) and a conventional PCR method (9, 10) for 55-1 detection. The former is a simple and less costly assay for β-glucuronidase activity expressed in 55-1. However, the assay requires the manual excising of 12 seed embryos per sample, a labor-intensive and time-consuming task in proportion to the sample number. Moreover, this method is not applicable to processed papaya products because of enzyme denaturation and the disposal of papaya seeds during processing. On the other hand, the latter is a simple assay for 55-1 detection. However, the assay is not applicable to fragmented DNA in processed papaya products because of long PCR amplicon length (>150 bp) from the targeted sequence (11–13).

We recently developed an event-specific real-time PCR method for sensitive detection of 55-1 contamination in papaya fruit and processed products (14). This method was based on the detection of a 55-1 targeted sequence located across a junction region between genomic papaya DNA and the recombinant 55-1-derived DNA sequence in combination with papaya endogenous reference chymopapain (*CHY*). This assay uses shorter amplicones (approximately 70 bp) than the previous

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Table 1. Oligonucleotide primers and probes used in this study

Name		Oligonucleotide sequence (5′-3′)
For event-specific detection of 55-1 papaya		
Primer	55-1 primer1	CAG CCT TAG ATG CTT CAA GAA AAG A
	55-1 primer2	TCC GCC TCC ATC CAG TCT ATT
Probe	55-1 probe	FAM-TCT TCT AGC TTC CCG GCA ACA AT-TAMRA
For the detection of papaya endogenous reference gene (*CHY*)		
Primer	Q-CHY-1F2	CCA TGC GAT CCT CCC A
	Q-CHY-2R	CAT CGT AGC CAT TGT AAC ACT AGC TAA
Probe	Q-CHY-P	FAM-TTC CCT TCA T(BHQ1)CC ATT CCC ACT CTT GAG A

conventional PCR assay, and is therefore better applicable to fragmented DNA in processed papaya products.

Despite the fact that the 55-1 real-time PCR detection method is required for labeling regulation in Japan and the monitoring of 55-1 contamination in countries where 55-1 is not authorized, interlaboratory validation of this method had not yet been demonstrated. Therefore, we conducted an interlaboratory validation study of this methodology. Twelve laboratories participated in this study, each following the same DNA extraction and real-time PCR methods. The results show that the 55-1 real-time PCR detection method is specific and accurate for fresh papaya fruit.

Experimental

Samples

Non-GM papaya (Sunset) and GM papaya (SunUp, a 55-1 line homozygous for PRSV coat protein gene; and Rainbow, an F_1 hybrid of SunUp × non-GM papaya Kapoho) fruits were purchased from a Japanese trade agency via the Hawaii Papaya Industry Association through the Consumer Affairs Agency of Japan. The fruits were stored at –30°C until used.

Preparation of the Test Samples

The frozen fruits were thawed and cut in half lengthwise, and then the seeds and pericarp were removed. The flesh was ground using a mixing mill, and 10 g each of the resulting samples was placed in 50 mL centrifuge tubes. Samples were stored at –30°C until further use.

DNA Extraction

DNA was extracted from samples (10 g) using Genomic-tip 100/G (QIAGEN, Hilden, Germany) columns according to the manufacturer's instructions, with the following modifications: A 30 mL amount of Buffer G2 (QIAGEN), 20 µL of 100 mg/mL RNase (QIAGEN), and 500 µL of cellulase (Sigma-Aldrich Co., St. Louis, MO) were added to the sample, vortexed thoroughly, then incubated at 50°C for 1 h. The mixture was incubated at 50°C for another 1 h after the addition of 200 µL Proteinase K (QIAGEN). During incubation, the samples were

Table 2. Homogeneity test results of the samples

Sample	$\Delta Ct_{55\text{-}1\ -\ CHY}$	RSD%[a]	*n*	*F*-ratio	*F* crit[b]
Rainbow	2.760	2.7	10	1.45	3.02
SunUp	1.682	6.2	10	1.73	3.02

[a] RSD calculated from sampling and analysis SD.

[b] *F* crit, 5% critical value of the *F*-test statistic for duplicates.

mixed several times by inverting the tubes. The samples were then centrifuged at 3000 × *g* at 4°C for 20 min. The supernatant was applied to a Genomic-tip 100/G column, which was pre-equilibrated with 4 mL of Buffer QBT (QIAGEN). The column was washed three times with 7.5 mL of Buffer QC (QIAGEN) and once with 1 mL pre-warmed (50°C) Buffer QF (QIAGEN), then transferred to a fresh centrifuge tube. The DNA was eluted by addition of 2 mL pre-warmed (50°C), Buffer QF (QIAGEN). Isopropyl alcohol (2 mL) was added, and the mixture mixed thoroughly. An aliquot of the mixture (1 mL) was transferred to a 1.5 mL centrifuge tube, then centrifuged at 10 000 × *g* at 4°C for 15 min. The pellet was rinsed with 1 mL 70% (v/v) ethanol and centrifuged at 10 000 × *g* at 4°C for 5 min. The supernatant was discarded and the precipitate dried. The DNA was dissolved in 50 µL distilled water per sample for use in analyses. The DNA concentration was determined by measuring the UV absorption at 260 nm using a spectrophotometer. The extracted DNA was diluted with an appropriate volume of distilled water to a final concentration of 10 ng/µL. In cases where the concentration of the extracted DNA did not reach 10 ng/µL, the extracted DNA was used in real-time PCR assay without dilution.

Real-time PCR Procedure

Real-time PCR was performed using the ABI PRISM™ 7900 Sequence Detection System (Life Technologies Co., Carlsbad, CA). The 55-1 primer 1/55-1 primer 2 pair amplifies a DNA sequence across a junction between genomic papaya DNA and the recombinant DNA sequence present in 55-1, and the 55-1 probe, which is labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine at the 5′ and 3′ ends, respectively, were used for real-time PCR, as described previously (Table 1; 14). For detecting the papaya endogenous reference gene, *CHY*, Q-Chy-1F2/Q-Chy-2R primer pair and FAM-labeled Q-Chy-P containing black-hole quencher 1 at the internal thymidine were used as described previously (Table 1; 7). The reaction mixture (25 µL) consisted of 5 µL of sample DNA solution, 12.5 µL TaqMan® Gene Expression Master Mix (Life Technologies Co.), 0.8 µM forward and reverse primers, and 0.1 µM probe. The PCR conditions were as follows: 2 min at 50°C, 95°C for 10 min, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

All primers and 55-1 probe, and Q-Chy-P were synthesized by Food Assessment and Management Center Co., Ltd (Atsugi, Japan) and Life Technologies Co., respectively. All primers and probes were diluted with an appropriate volume of distilled water and stored at –20°C until further use.

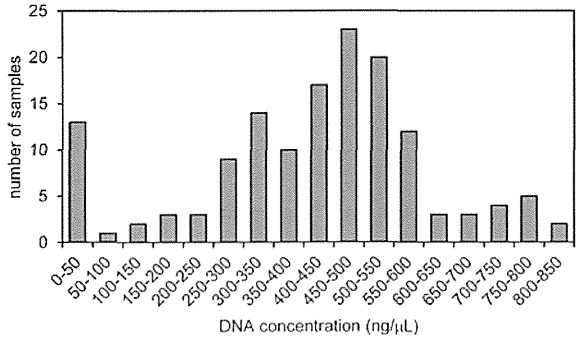

Figure 1. Histogram of the DNA concentration of blind samples determined by UV absorption measurements at 260 nm.

Data Analysis

Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reaction with Ct values of <48 and exponential amplification, as judged by visual inspection of the respective ΔRn plots and multicomponent plots, were scored as positive. If the Ct value could not be obtained, the reaction was scored as negative. Reactions with Ct values of <48, but without exponential amplification, as judged by visual inspection of respective ΔRn plots and multicomponent plots, were scored as negative.

Homogeneity Tests of the Test Materials

Before dispatch to the 12 laboratories, the homogeneity of the test samples was verified by the National Institute of Health Sciences (NIHS) according to the procedure described in the International Harmonized Protocol for Proficiency Testing of Analytical Laboratories (15), except that the number of test samples was 10. As duplicate reactions for each GM sample were tested, 20 reactions in total were analyzed using the ABI PRISM 7900 Sequence Detection System. The differences between Ct for *CHY* detection and Ct for 55-1 detection ($\Delta Ct_{55\text{-}1\ -\ CHY}$) were calculated and subjected to one-way analysis of variance (ANOVA).

Interlaboratory Validation

We referred to the guidelines for collaborative study to determine the general procedure for the interlaboratory validation (16, 17). The interlaboratory validation of the 55-1 real-time PCR detection method, conducted with the participation of 12 laboratories, was organized by the NIHS. Each laboratory received 12 blind samples, including six tubes with 10 g ground samples of non-GM papaya (Sunset) and three tubes with 10 g ground samples of each GM papaya (Rainbow and SunUp), Genomic-tip 100/G columns, other reagents for DNA extraction, the primer pairs, probes, and TaqMan Gene Expression Master Mix used for real-time PCR as described above, as well as the experimental protocol from the NIHS. The participants extracted and purified DNA from 12 blind samples, then duplicate reactions for each DNA sample were conducted using real-time PCR.

Results and Discussion

Homogeneity of the Test Materials

Ten samples from each GM papaya (Rainbow and SunUp) were randomly selected to confirm homogeneity. The samples were analyzed using the 55-1 real-time PCR detection method to obtain Ct values, $Ct_{55\text{-}1}$ and Ct_{CHY}. One-way ANOVA for

Table 3. Summary of the results of all blind samples

	CHY detection												55-1 detection											
Lab	Sunset			Rainbow			SunUp			Sunset			Rainbow			SunUp								
A	+/+[a,c]	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–[b,c]	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
B	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
C	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
D	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
E	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
F	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
G	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
H	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
I	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
J	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
K	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+
L	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+

[a] + = Positive reaction.

[b] – = Negative reaction.

[c] +/+ and –/– = the results in duplicate reactions for each sample.

Table 4. Results of the interlaboratory study

Detection sequence	*CHY*			55-1		
Primer pair and probe	Q-Chy-1F2/Q-Chy-2R/Q-Chy-P			55-1 primer 1/55-1 primer 2/55-1 probe		
No. of laboratories	12			12		
No. of laboratories that evaluated	12			12		
No. of samples/laboratory	12			12		
No. of total samples	144			144		
No. of total reactions	288			288		
No. of accepted results	288			288		
No. of samples containing target detection material	144			72		
Cultivar	Sunset	Rainbow	SunUp	Sunset	Rainbow	SunUp
No. of reactions	144	72	72	144	72	72
No. of positive reactions	144	72	72	0	72	72
No. of negative reactions	0	0	0	144	0	0
Positive reaction ratio, %	100	100	100	0	100	100
False-negative reaction ratio, %	0	0	0	0	0	0

$\Delta Ct_{55\text{-}1\ -\ CHY}$ showed that the RSD values calculated from both sampling and analysis errors were low (2.7% in Rainbow and 6.2% in SunUp), and *F*-ratios for Rainbow (1.45) and SunUp (1.73) were less than 5% critical value (3.02) of the *F*-test statistic for duplicates (*F* crit, Table 2; 18). These results indicate that samples were homogeneous, i.e., all of the same quality.

Interlaboratory Study: DNA Extraction

Twelve blind samples, comprising six replications of Sunset, three of Rainbow, and three of SunUp, were analyzed by each laboratory. DNA was extracted as described, and the concentration and purity were determined. Data from a total of 144 blind samples from the 12 laboratories were collected and statistically analyzed. The concentration of DNA extracted from all laboratories ranged from 2 to 816 ng/μL (Figure 1); most (72.9%) were in the range of 250–600 ng/μL. The purity of DNA was quite appropriate for PCR analysis (>1.7 in A_{260}/A_{280} and in A_{260}/A_{230}), although the quality and yield of DNA extracted by Laboratory A was low (<30 ng/μL) and impure (1.0–1.5 in A_{260}/A_{280} and 0.7–1.5 in A_{260}/A_{230}). This may have been caused by inappropriate operation in the extraction steps.

Interlaboratory Study: Real-time PCR

GM 55-1 papaya was detected using the 55-1 real-time PCR detection method. A summary of the results is shown in Table 3. Samples were determined to be either negative ($Ct \geq 48$) or positive ($Ct < 48$) in each real-time PCR reaction (*CHY* and 55-1). Namely, samples carrying both *CHY* and the 55-1 targeted sequence were judged as a 55-1 sample. For *CHY* detection, the positive reaction rates for Sunset, Rainbow, and SunUp samples were all 100% (Table 4). For 55-1 detection, the rates for Sunset, Rainbow, and SunUp samples were 0, 100, and 100%, respectively (Table 4). There were no misjudged samples, despite the low quality DNA in Laboratory A. These results suggest that the 55-1 real-time PCR detection method, including the DNA extraction step, produces accurate results for 55-1 detection, similar to validation studies previously reported (19, 20).

To evaluate the repeatability and reproducibility of the 55-1 real-time PCR detection method, we statistically analyzed the Ct values obtained from all laboratories after eliminating outliers using Cochran's ($P < 0.025$) and Grubbs' tests ($P < 0.0125$). The identified outlier data appeared to come from a laboratory with lower relative DNA yields (Laboratory A, <30 ng/μL) and samples that were relatively lower with respect to DNA yield (two samples from Laboratories B and C, 144 and 180 ng/μL, respectively). According to internationally accepted guidelines, which stipulate that the data from more than two out of nine laboratories cannot be removed (16), only two were removed as outliers. Subsequently, statistical analysis was performed to determine mean, RSD, reproducibility RSD (RSD_R) and repeatability RSD (RSD_r) of the Ct value for each papaya targeted sequence (Table 5). The RSD, RSD_R, and RSD_r ranged from 1.2 to 1.7%, 2.2 to 3.4%, and 0.7 to 0.8%, respectively, indicating the high reproducibility and repeatability of the 55-1 real-time PCR detection method in this interlaboratory validation study.

Conclusions

An interlaboratory validation study of the 55-1 real-time PCR detection method, including DNA extraction, was conducted according to internationally accepted guidelines (16, 17). The quality of DNA samples extracted by the various participating

Table 5. Variance analysis of Ct values

	CHY detection			55-1 detection	
	Sunset	Rainbow	SunUp	Rainbow	SunUp
Mean	22.34	22.38	22.31	25.09	24.01
RSD, %	1.4	1.7	1.3	1.6	1.2
RSD_R, %	3.4	3.3	2.5	3.0	2.2
RSD_r, %	0.8	0.8	0.7	0.7	0.7

laboratories was acceptable for the subsequent PCRs. The 55-1 specific real-time PCR reaction gave accurate results. Statistical analysis of Ct values indicated the high reproducibility and repeatability of this method. This study demonstrated that the 55-1 real-time PCR detection method is a useful and reliable method for monitoring GM 55-1 papaya.

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A DNA extraction and purification method using an ion-exchange resin type kit for the detection of genetically modified papaya in processed papaya products

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ABSTRACT

A method for the extraction and purification of genomic DNA from processed papaya products is essential for the detection of approved genetically modified (GM) papaya, according to GM labeling regulations, and unapproved GM papaya, to restrict the import or sale of products containing it. Here, we investigated methods for the extraction of DNA from processed papaya products, including dried papaya, canned papaya and papaya jam. The extraction of DNA from dried papaya and canned papaya required a pre-digestion step, using RNase, cellulase and proteinase K. In the case of papaya jam, α-amylase was found to be indispensable to obtain DNA with high yield and purity. The DNA yield was considerably higher when an ion-exchange resin type kit (IER-100G) was used, compared with other five methods (IER-20G, QIAamp DNA Stool Mini Kit, DNeasy Plant Maxi Kit, GM Quicker 3 Kit and Wizard Cleanup Resin System). We developed a suitable method for the extraction and purification of DNA from processed papaya products, which could be used to detect GM papaya.

1. Introduction

Papaya (*Carica papaya* LINNAEUS) is cultivated worldwide. The fruit is affected by the papaya ringspot virus (Gonsalves, Tripathi, Carr, & Suzuki, 2010). Genetically modified (GM) papaya line 55-1, having resistance to the papaya ringspot virus, was developed by Cornell University, the University of Hawaii and the Upjohn Company (Fitch, Manshardt, Gonsalves, Slightom, & Sanford, 1992), and has been cultivated in Hawaii since 1998. As of 2010, line 55-1, which includes cultivars such as Rainbow and SunUp (the dominant cultivar grown in Hawaii), accounts for more than 70% of Hawaii's papaya acreage (Gonsalves et al., 2010). Line 55-1 is commercially grown and consumed in Hawaii and the rest of the United States. The Canadian government approved the import of line 55-1 in 2003, and the Food Safety Commission of Japan approved its import in 2010. Furthermore, GM papaya line 63-1 was developed by Cornell University and Hawaii University, GM papaya line X17-2 was developed by University of Florida, GM papaya Huanong No. 1 was developed by South China Agricultural University. The molecular characterization and the method for the detection of Huanong No. 1 papaya were reported by Guo et al. (2009). Three lines of GM papaya were approved for growing and commercialization in each country where GM papaya was developed. However those GM papayas were unauthorized in Japan. The European Union, Japan and Korea have enforced mandatory GM labeling regulations for approved GM foods, and the import of any unauthorized GM food has been prohibited. Another GM papaya having resistance to the papaya ringspot virus YK strain (PRSV-YK) was recently detected in commercially processed food in Japan, as well as in papaya seeds imported from Taiwan (Nakamura et al., 2011). To date, the cultivation of unauthorized GM papaya resistant to PRSV-YK has not been allowed by the Cartagena Protocol, an international agreement regulating the international movement of GM organisms. Thus, the cultivation and use of GM papaya resistant to PRSV-YK as food has been limited, and it cannot be imported into Japan (MAFF, 2011; MHLW, 2011).

Therefore, detection methods for the authorized line 55-1, unauthorized line 63-1, line X17-2, Huanong No. 1 papaya and PRSV-YK resistant papaya are required to ensure the reliability of food labeling, and to monitor the presence of the unauthorized GM

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