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Reliable Enzyme-Linked Immunosorbent Assay for the Determination of Coconut Milk Proteins in Processed Foods

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ABSTRACT: This study was designed to develop a novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of coconut milk proteins in processed foods. The developed sandwich ELISA was able to detect coconut milk proteins from various coconut milk products and did not show any cross-reactivity with 41 of 42 kinds of popularly used food ingredients, thus reflecting great specificity for coconut milk proteins. In addition, the established ELISA is highly sensitive and allowed the detection of 0.31 μg/g of coconut milk protein in complex food matrices. This proposed assay could serve as a useful tool for the detection of the presence of hidden coconut milk proteins in processed foods.

KEYWORDS: coconut milk, food allergy, sandwich ELISA

INTRODUCTION

Reactions triggered by food allergens are increasing and becoming a major health concern worldwide, especially in industrialized countries. It is estimated that up to 8% of children and 2% of adults are affected by food allergies,[1−3] with symptoms ranging from relatively mild to severe anaphylactic reactions with fatal consequences.[4] Many foods are known to trigger food allergies; among these, eight types (peanuts, nuts, wheat, soy, milk, eggs, fish, and shellfish) are responsible for causing the majority (>90%) of allergic reactions.[5]

Tree nuts are one of the most potent of all known food allergens and are often attributed as the cause of food anaphylaxis and anaphylactic death.[6−8] Among tree nuts, those most commonly responsible for allergic reactions are hazelnut, walnut, cashew, and almond, whereas those less frequently associated with allergies are pecan, chestnut, Brazil nut, pine nut, macadamia nut, pistachio, and coconut.[8] Although technically not a tree nut, coconut has been categorized as such by the U.S. Food and Drug Administration (FDA) since 2006. Allergy to coconut is very infrequent.[9,10] However, coconut can elicit allergic reactions, including immediate reaction and anaphylaxis, especially in patients who have been previously diagnosed with a severe sensitivity to nuts.[9,11−13] Cross-reactivity between coconut and other tree nuts, such as hazelnuts and walnuts, has been demonstrated.[12,13] This probably increases the risk of coconut allergy in consumers who are allergic to tree nuts or peanuts, although there is one study that showed no evidence.[14] Since the FDA updated their tree nut allergen list to include coconut, foods that contain coconut ingredients have required mandatory labeling. Coconut (*Cocos nucifera*) is one of the most economically important palm species. Its endosperm, particularly in the form of coconut milk, is widely used as an ingredient in many Asian cuisines, including Thailand.[15] Food products containing coconut include curry, biscuits, dessert mix, and ice cream. In most factories, many different food products are produced, with various food ingredient compositions. A problem of hidden allergens might result from cross-contamination in the food production line, through inadequate cleaning of shared equipment or by reworking of allergen-containing products.[16,17] Thus, strict control of coconut allergens in food factories is required to minimize the risk to coconut-allergic consumers and to comply with product labeling regulations. To practically and effectively manage food and food ingredients, especially traces of coconut, a simple and reliable detection method for detecting hidden coconut allergens is necessary. Presently, numerous ELISA kits suitable for specific nut allergen detection (hazelnuts, almonds, walnuts, etc.) are available,[7,18,19] whereas only one commercial product offers a lateral flow test for the qualitative detection of coconut allergen. To date, no quantitative enzyme-linked immunosorbent assay (ELISA) test suitable for coconut milk allergen determination has been developed.

Coconut allergens are identified to be seed storage proteins such as 11S (legumin-like) globulin, known as cocosin, and 7S (vicilin-like) globulin.[11] Globulins comprise 75% of the total protein in coconut; of this, 11S is estimated to constitute 86% and 7S, 14%.[20] Some researchers have already shown that cocosin, a major coconut allergen, is a hexamer with a molecular mass of approximately 300 kDa, with each heterogeneous 54 kDa subunit comprising an acidic and basic polypeptide chain linked by disulfide bonds.[11] Under reducing conditions, these proteins migrate on gel electrophoresis as bands of about 35 and 32 kDa and a complex of 22 kDa. The 7S globulin, on the other hand, is a trimeric protein with a native

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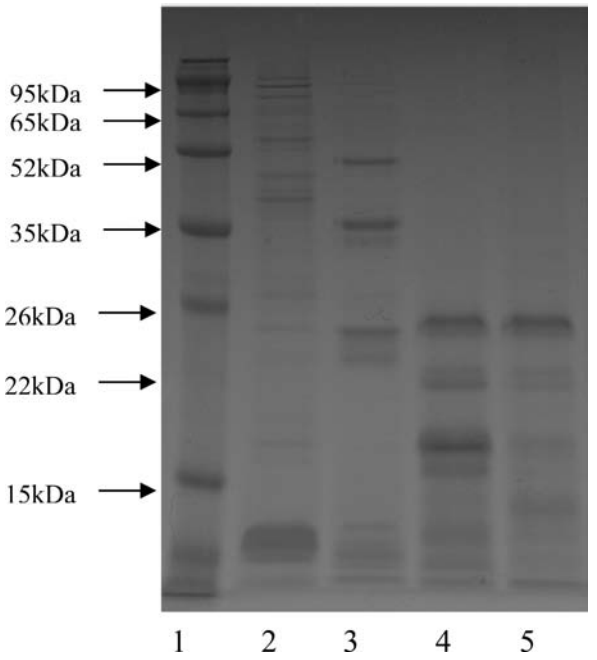

Figure 1. SDS-PAGE of defatted coconut milk IV, P40−60, defatted hazelnut, and defatted walnut. Lanes: (1) molecular weight marker; (2) defatted walnut; (3) defatted hazelnut; (4) defatted coconut milk IV; (5) P40−60. Each protein sample (2 μg) was separately applied to lanes 2, 3, 4, and 5. Arrows indicate molecular weight markers.

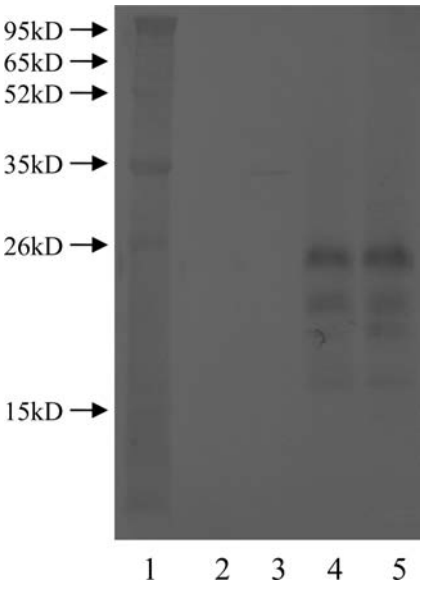

Figure 2. Western blotting analysis of defatted coconut milk, P40−60, defatted walnut, and defatted hazelnut. Lanes: (1) molecular weight marker; (2) defatted walnut; (3) defatted hazelnut; (4) defatted coconut milk IV; (5) P40−60. Each protein sample (0.1 μg) was separately applied to lanes 2, 3, 4, and 5. Arrows indicate molecular weight markers.

molecular mass of ~150 kDa. It shows no disulfide bond[20] and is quickly digested by pepsin.[11] However, during the processing of coconut milk proteins, the reactivity of the allergenic proteins might be altered due to structural changes, affecting their ability to be detected in the food matrix. Thus, it is very important to obtain the specific antibodies that can recognize denatured coconut milk proteins for ELISA in certain foods after processing. Preparation of these antibodies presents a challenge. Moreover, a major problem with most ELISAs developed for allergen detection from nuts and seeds is that many nuts and seeds are closely related phylogenetically.[21] Therefore, a highly sensitive and specific ELISA without cross-reactivity is crucial, to avoid incorrect judgments. The aim of this study was to develop the first highly coconut specific and sensitive sandwich ELISA that can detect and quantify trace amounts coconut milk proteins in processed foods. This assay is developed to be used by the food industry and regulatory agencies to protect consumers and to avoid loss of sales due to allergen-related recalls.

MATERIALS AND METHODS

Food Materials. Three kinds of coconut milk and dried coconut were purchased at local supermarkets (Bangkok, Thailand) in 2009. Coconut milk I is 100% coconut milk, Chaokoh brand, made in Thailand (Ampol Food Processing Co., Ltd.). Coconut milk II is 100% coconut milk, Aroy-D brand, made in Thailand (Thai Agri Foods Public Co., Ltd.). Coconut milk III is 100% coconut milk scented candles, Chaokoh brand, made in Thailand (Ampol Food Processing Co., Ltd.). Dried coconut meat was prepared from fully mature coconut endosperm and cut into small pieces before drying at 80 °C in a hot-air oven for 2 h. Two other kinds of coconut milk were purchased at local supermarkets in Yokohama, Japan, in 2009. Coconut milk IV was canned 100% coconut milk, Ayam brand, made in Malaysia. Coconut milk V was a canned product made of coconut milk (53%), water, citric acid, and sulfite (for bleaching), Chaokoh brand, made in Thailand (Thep Padung Porn Coconut Co., Ltd.).

Chemicals and Reagents. 2-Mercaptoethanol (2-ME), sodium sulfite, sodium dodecyl sulfate (SDS), polyoxyethylene sorbitan monolaurate (Tween 20), ammonium sulfate, and hexane were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). Albumin from bovine serum (BSA), horseradish peroxidase (HRP), and 3,3′5,5′-tetramethylbenzidine (TMB) were provided by Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan), Toyobo Co., Ltd. (Osaka, Japan), and SurModics, Inc. (Eden Prairie, MN), respectively. In addition, the following buffers were prepared for the experiment: 150 mM Tris-HCl (pH 7.4) containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20 (buffer A); and 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.05% (v/v) Tween 20 (buffer B).

Preparation of Coconut Milk Protein (P40−60). Coconut milk IV was defatted with hexane (1:2.5 v/v) by stirring for 2 h; then the hexane layer was removed after centrifugation at room temperature (5000*g*, 5 min). The procedure of defatting was repeated two times. Two hundred and twenty milliliters of defatted coconut milk was obtained from 400 g of coconut milk. Defatted coconut milk was added to solid ammonium sulfate at 40% saturation with stirring for 30 min at 4 °C followed by centrifugation (15000*g*, 30 min, 4 °C). The supernatant was then added to solid ammonium sulfate at 60% saturation under the same conditions. The precipitated proteins were centrifuged (15000*g*, 30 min, 4 °C) and dissolved in 20 mM Tris-HCl (pH 7.4) buffer. The precipitated proteins (P40−60) were used as antigens.

Preparation of the Antibody to P40−60. The rabbit antiserum against P40−60 was produced by immunization of Japanese white rabbits using P40−60 solution (1 mg for P40−60, PBS containing 0.6% SDS (w/v), 0.1 M sodium sulfite) in Freund's complete adjuvant. Injections were repeated five times at the appropriate intervals (14 days). Whole blood was collected, and the serum was separated. Specific polyclonal antibodies against denatured P40−60 were purified from the serum using a 1 mL HiTrap NHS-activated column (GE Healthcare, U.K.) in which 10 mg of the denatured coconut protein from dried coconut was fixed. The obtained antibodies were examined for the construction of the ELISA for detection of the coconut milk protein.

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were diluted 1:1 with sample buffer (4% w/v SDS, 28% v/v glycerol, 0.24 mg/mL bromophenol blue, 20% v/v 2-ME). Under reducing conditions, the samples were boiled for 5 min. Polyacrylamide gels and molecular weight standards used were 12.5% Q-PAGE mini (TEFCO, Tokyo, Japan) and SeeBlue Plus2 Prestained Standard (Invitrogen Life Technologies Corp., Carlsbad, CA). Electrophoresis was run at constant voltage (200 V) for 36 min using a STC-808 (TEFCO). The gels were stained with Rapid CBB KANTO (Kanto Chemical Co., Inc., Tokyo, Japan).

Western Blotting. Samples were subjected to SDS-PAGE and transferred onto a PVDF membrane (Amersham Hybond-P, GE Healthcare, U.K.) by a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). The PVDF membrane was incubated for 1 h in a blocking solution (buffer B containing 0.1% BSA). Coconut protein was detected on blot using subsequently anti-P40−60 antibodies (20 ng/mL, 60 min), biotinylated anti-rabbit IgG antibodies (1 μg/mL, 30 min) from a Vectastain ABC-AP Kit (Vector Laboratories, Inc., Burlingame, CA), and alkaline phosphatase conjugated streptavidin (20 min) also from a Vectastain ABC-AP Kit, and it was used according to the manufacturer's description. Signals were detected by a BCIP/NBT Alkaline Phosphatase Substrate Kit IV (Vector Laboratories, Inc.).

Sample Extraction. A Millser IFN-700G homogenizer (Iwatani International Corp., Osaka, Japan) was used to homogenize the food samples. Samples were treated with the Millser a few times for 30 s until homogenized. Nineteen milliliters of buffer A, containing additional 0.6% (w/v) SDS and 0.1 M sodium sulfite, was added to 1 g of a homogenized sample. The sample was then shaken at 90−110 rpm over 12 h at 25 °C for extraction. After confirmation of sample pH at around 6.0−8.0 with pH paper, the sample was centrifuged at 3000*g* for 20 min; the supernatant was then filtered through 5A filter paper (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) to obtain the extract. As needed, the obtained extracts were analyzed with a 2-D Quant protein assay kit (GE Healthcare, U.K.). The sample extract, diluted 20-fold with buffer A, was used for ELISA. In the case of additional dilution, the 20-fold diluted sample extract was diluted with buffer A containing 0.03% (w/v) SDS and 0.005 M sodium sulfite.

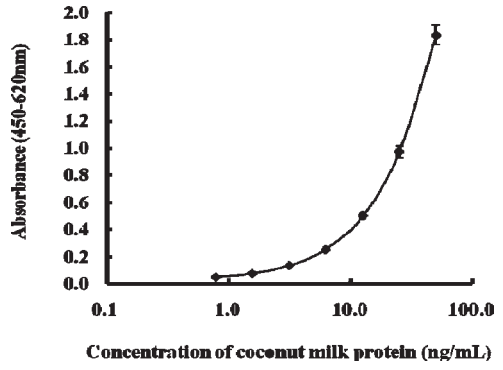

Figure 3. Representative calibration curve of the developed ELISA. The calibration curve shows the mean value of 10 experiments and the standard deviation in each point of the curve. Concentrations of standard solution: 0.78 ng/mL (0.31 μg/g), 1.56 ng/mL (0.62 μg/g), 3.13 ng/mL (1.25 μg/g), 6.25 ng/mL (2.5 μg/g), 12.5 ng/mL (5 μg/g), 25 ng/mL (10 μg/g), and 50 ng/mL (20 μg/g).

Preparation of Calibration Standard Solution. For the determination of coconut milk protein using ELISA, the preparation of a calibration standard solution is necessary. Coconut milk I powder, after freeze-drying, was defatted with hexane (1:2 w/v) by stirring for 1 h; this was followed by paper filtration. The procedure of defatting was repeated three times. Twenty milliliters of buffer A containing 0.6% (w/v) SDS and 0.1 M sodium sulfite was added to 0.2 g of the defatted coconut milk powder. The mixture was then shaken at 90−110 rpm for 16 h at 25 °C for extraction. The extract was centrifuged at 10000*g* for 30 min, and the supernatant was filtered through a 0.8 μm microfilter (DISMIC-25CS, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The protein content of the filtrate was assayed using a 2D Quant protein assay kit (GE Healthcare, U.K.). The original filtrate was diluted to 10 times its volume with phosphate buffered saline (PBS, pH 7.4). Next it was further diluted to twice its volume by PBS (pH 7.4) containing 0.2% BSA and then stored at −80 °C for preservation. Finally, it was diluted to 50 ng/mL using buffer A containing 0.03% (w/v) SDS and 0.005 M sodium sulfite. The prepared calibration standard solution for the ELISA was stored at 4 °C.

ELISA. A microtiter plate (F8Maxisorp Nunc-Immuno module, Thermo Fisher Scientific Inc., Waltham, MA) was coated at 25 °C for 2 h with the prepared specific polyclonal antibody to anti-P40−60 (100 μL of 2.5 μg/mL antibody protein solution in 50 mM sodium carbonate, pH 9.6). The plate was blocked at 25 °C for 2 h with buffer B containing 0.1% w/v BSA, as described in a previous study.[7] The blocking buffer was removed, followed by drying of the plate. The diluted food sample and the coconut milk standard solution were added to the plate (100 μL/well) and incubated for 1 h at 25 °C. After six washings with buffer B, the anti-P40−60 antibody labeled with HRP using the method described in previous literature[22] was diluted with buffer B containing 1% BSA. This was added to the ELISA plate at 100 μL/well and allowed to stand for 30 min. After six washings with buffer B, TMB (100 μL/well) was added, and the enzyme reaction was allowed to run at 25 °C for exactly 10 min. The reaction was stopped by the addition of 100 μL/well of 0.5 M H_2SO_4. The absorbance was measured at 450 nm, the dominant wavelength, and at 620 nm, the subdominant wavelength. The amount of coconut milk protein was calculated using the calibration standard curve of coconut milk protein based on P40−60 assay as a coconut marker protein. All experiments were performed in duplicate. Each sample determination was expected to have a mean CV of <10%.

RESULTS

Purification of Coconut Milk Protein (P40−60). Figure 1 shows the analysis of defatted walnut, defatted hazelnut, defatted

Table 1. Reactivity of Five Coconut Milk Varieties Using the Developed ELISA[a]

sample	type of package	protein assay (μg/g)	ELISA (μg/g)	recovery (%)
coconut milk I	Tetra pak	16800 ± 1100	18000 ± 700	107
coconut milk II	Tetra pak	16000 ± 500	16000 ± 300	100
coconut milk III	Tetra pak	20000 ± 300	22000 ± 500	110
coconut milk IV	can	24000 ± 700	13000 ± 300	54
coconut milk V	can	18000 ± 500	9300 ± 300	52

[a] Coconut milk extracts were prepared according to the procedure given under Materials and Methods. The protein assay for determination of protein concentration was analyzed by a 2D Quant protein assay kit (GE Healthcare, U.K.). The ELISA mean shows the average value (coconut milk protein weight/coconut milk weight) of the coconut milk proteins (n = 3).

coconut milk IV, and purified P40−60 using the SDS-PAGE method. Three major bands with molecular masses between 15 and 25 kDa were observed in lane 4 (defatted coconut milk IV). One main single band with molecular mass around 25 kDa and some minor bands were observed in lane 5 (purified P40−60). Defatted hazelnut exhibited four major bands with molecular masses between 15 and 52 kDa (lane 3). Defatted walnut, on the other hand, showed some weaker bands and one major band with a molecular mass of <15 kDa (lane 2). However, there was no corresponding band around the 25 kDa of both hazelnut and walnut. Therefore, P40−60 containing main protein was used as an antigen, obtaining anti-P40−60 antibody as discussed under Materials and Methods. Figure 2 shows the results of Western blotting to confirm the ability of the anti-P40−60 antibody to recognize P40−60 coconut milk protein. Two major bands with molecular masses around 25 and 22 kDa were observed in lane 4 (defatted coconut milk IV) and lane 5 (purified P40−60). Obviously, no band was observed in the other two nuts (lanes 2 and 3). These results indicated that the obtained antibodies were specifically recognized P40−60 in coconut milk, and these antibodies were not immunologically cross-reactive with walnut and hazelnut. In the experiment we observed that in a highly processed coconut product such as canned coconut milk (coconut milk IV), the protein fractions of interest are retained on SDS-PAGE. Accordingly, we considered the P40−60 protein to be suitable as a coconut marker protein for the determination of coconut milk in processed foods.

Construction of Sandwich ELISA. We constructed a sandwich ELISA for the detection of coconut proteins, using anti-P40−60 rabbit serum. Figure 3 shows a representative calibration curve of the developed sandwich ELISA for the determination of the coconut standard calibrators. All calibrators were measured using the mean values of 10 experiments. The dose response curves were obtained in the assay. The model that best describes the relationship between the absorbance and concentration of the antigen is a four-parameter logistic curve. The limits of detection (LOD) and quantification (LOQ) were found to be 0.39 and 0.78 ng/mL whole coconut milk proteins, equivalent to 0.15 and 0.31 μg/g in foods, respectively. The LOD and LOQ were calculated as 3 times and 10 times the SD of the buffer blank mean values after three experiments.

Reactivity of Five Coconut Milk Varieties. As shown in Table 1, we examined the protein content in all five coconut milk products using the developed ELISA, and the average values were obtained from three experiments. The activity of three coconut milk product varieties (I, II, and III) ranged from 100 to 110% (ratio to protein assay). On the other hand, the other coconut milk product varieties (IV and V) ranged from 52 to 54% (ratio to protein assay). Canned products such as coconut milks IV and V were produced under high temperature and high pressure. Therefore, we considered that the reactivity of coconut milks IV and V might be decreased by the manufacturing process. However, our coconut milk ELISA could detect all five kinds of coconut milk with adequate sensitivity. We tried to improve the reactivity of coconut milks IV and V using antibodies, which were purified from the serum using a 1 mL HiTrap NHS-activated column (GE Healthcare, U.K.), in which 10 mg of the denatured P40−60 was fixed. However, it was ineffective in increasing the reactivity to coconut milks IV and V (data not shown).

Cross-Reactivity to Various Food Ingredients. As shown in Table 2, 43 foods such as grains, wheat, eggs, soybeans, milk, and sesames were evaluated using the ELISA method developed in this study. The P40−60 ELISA showed cross-reactivity to oat (0.59 μg/g), but no cross-reactivity to the other 42 foods. These results indicated that the P40−60 ELISA developed in this study had high specificity, although the established ELISA rarely has cross-reactivity to oat.

Table 2. Cross-Reactivity to Various Foods Using the Developed ELISA[a]

food	ELISA mean (μg/g)
rice powder	<0.31
buckwheat	<0.31
wheat	<0.31
soybean	<0.31
peanut	<0.31
shrimp (boiled black tiger)	<0.31
crab (boiled snow crab)	<0.31
egg	<0.31
boiled egg	<0.31
freeze-dried whole egg	<0.31
milk	<0.31
skim milk	<0.31
corn flour	<0.31
polished rice	<0.31
millet (hie)	<0.31
millet (awa)	<0.31
millet (kibi)	<0.31
rye	<0.31
barley	<0.31
malt	<0.31
oat	0.59 ± 0.06
adlay	<0.31
quinoa	<0.31
amaranthus	<0.31
sago palm	<0.31
roasted almond	<0.31
roasted cashew	<0.31
roasted macadamia nut	<0.31
salmon	<0.31
squid	<0.31
salmon roe	<0.31
beef	<0.31
chicken	<0.31
pork	<0.31
cumin	<0.31
poppy seed	<0.31
coriander	<0.31
roasted white sesame	<0.31
roasted black sesame	<0.31
potato flake	<0.31
roasted peanut	<0.31
roasted hazelnut	<0.31
roasted walnut	<0.31

[a] Cross-reactivity of the developed ELISA for 43 kinds of foods. Sample extracts were prepared according to the procedure described under Materials and Methods. Values are the calculated mean concentrations (coconut milk protein weight/sample weight) of coconut milk (n = 3).

Table 3. Analysis of Coconut Milk Content in Commercial Foods Using Sandwich ELISA[a]

sample	declaration on label	calculated coconut milk protein (μg/g)	ELISA mean R (μg/g)	recovery (%)
biscuit I	coconut meat (11%)	1800	1400 ± 60	78
biscuit II	desiccated coconut (5.26%)	880	1700 ± 60	193
curry I	coconut milk (17%)	2900	890 ± 20	31
curry II	coconut milk (52%)	8700	9900 ± 360	114
curry III	coconut milk (21%)	3500	2300 ± 80	66
crispy roll shredded pork	coconut milk (45%)	7600	3200 ± 60	42
dessert mix (custard agar)	coconut milk powder (10.3%)	1700	2000 ± 40	118
dessert mix (custard mix)	coconut milk powder (8.4%)	1400	1800 ± 100	129

[a] Coconut milk was assayed in eight kinds of commercial foods. Sample extracts were prepared according to the procedure described under Materials and Methods. Listed means are the calculated mean concentrations (coconut milk protein weight/sample weight) of coconut milk (n = 3). The amount of coconut milk is calculated from ELISA means (coconut milk protein weight μg/sample weight g). Coconut milk protein is calculated on the basis of protein assay of coconut milk I (16800 μg/g) in Table 1. The recovery (%) is the amount of coconut milk determined by ELISA compared to the coconut milk amount declared on the label.

Application to Commercial Food Products. Eight various commercial food samples were analyzed by the established ELISA. Each commercial food was homogenized, and the extracts were prepared according to the extraction procedure described under Materials and Methods. As shown in Table 3, proteins in eight commercial foods with coconut meat or coconut milk listed as ingredients were clearly detected and showed very high reactivity (890−9900 μg/g). There were eight positives and no false negatives from the declarations on the ingredients lists of the samples analyzed in this study.

These results suggest that the established ELISA could be applicable for the determination of coconut milk proteins in processed foods.

DISCUSSION

Allergen detection is of increasing interest for food labeling purposes, because the misidentification of an allergen in food could be fatal to an allergic consumer and have devastating consequences for the food business.[23] Coconut, classified as a tree nut, is reported to cause allergy, and its presence needs to be declared on the label. The unintentional presence of coconut protein in processed food might cause a problem, due to the lack of a suitable detection assay. In this study we established a sandwich ELISA for the detection of coconut milk proteins in foods, using coconut-specific polyclonal antibodies against P40−60 from rabbit as the source of the detector antibody. This is the first report that a highly specific ELISA for quantitative determination of coconut milk protein has been established. To ensure the reliability of analytical techniques, validation of the performance of the assay is essential. This developed ELISA was characterized for its specificity to coconut milk proteins and for its selectivity in challenging with other food ingredients and coconut allergens present in various processed food products. In Table 1 we have shown that the developed ELISA could detect all five coconut milk products with acceptably high sensitivity. In actuality, the qualities of coconut milk products are different, depending on the manufacturing process (milk extraction procedure, temperature, and pressure, etc.) as well as other variables such as coconut varieties and coconut maturity. This results in the diversified characteristics of such products, which might affect ELISA testing. As the results demonstrate, Tetra pack (ultrahigh temperature, UHT) coconut milk provided higher reactivity (100−110% recovery) than canned coconut milk (52−54% recovery). Canned products such as coconut milks IV and V are produced under high temperature and high pressure (121 °C, 15 lb), with a longer time (∼15 min) under these conditions. This might lead to great conformational epitope changes, leading to loss of the ability of antibodies to bind directly to specific epitopes. In comparison, coconut milks I, II, and III (Tetra packed) were manufactured under industrial-like conditions at 135 °C in a very short time 2−5 s, therefore resulting in only a slight change in protein structure. Specificity is one of the inherent factors that affect the ELISA assay. Usually the assay specificity needs to be high enough that other related food components will not cause false positives, especially at the limit of detection, but still able to detect allergenic protein regardless of the processing method.[24,25] As presented in Table 2, the developed P40−60 ELISA showed no cross-reactivity with 42 of 43 foods (except oat), reflecting a very high specific detection system. This means only coconut milk protein can bind to the P40−60 antibody on the coated plate exclusively, whereas the other components are found to be negligible. Furthermore, the LOQ of our established ELISA for the sample solution was 0.78 ng/mL, equivalent to 0.31 μg of coconut milk protein/g of food sample weight, indicating the ability of the assay to determine a small amount of coconut milk in a given food sample. Although little is known about the minimum dose of coconut milk that can elicit an allergic reaction in sensitive individuals, the threshold guidance for allergen content at levels of 10 μg/g (allergenic ingredient protein weight/food sample weight) was used, on the basis of Japanese food allergen labeling regulations.[7,26] Therefore, it is necessary for the detection method to be able to accurately detect the allergenic coconut milk protein ingredient at this level.

Because coconut and coconut milk are common ingredients in biscuits, curry, and flour mix products, successful detection of coconut milk in these types of food is crucial. The eight commercially processed food products labeled as containing coconut meat or coconut milk were examined for coconut milk protein content using the developed ELISA. As shown in Table 3, the proposed ELISA could detect coconut milk proteins with a positive result for all products and showed very high reactivity (890−9900 μg/g) in accordance with the labeling of coconut on the ingredients lists. Among the eight food products, two samples (curry I and crispy roll shredded pork) possessed low recoveries of 31 and 43%, respectively. This was probably because the coconut protein had degraded, aggregated, or bound to other components and, therefore, was not accessible for detection with the antibody, especially in the case of crispy roll shredded pork, which is produced under direct heat at high temperature.

With this study, we have demonstrated that the established ELISA could be applicable for the determination of coconut milk proteins in various processed foods matrices, to ensure the validity of the labeling of coconut. We are considering future studies to assess this ELISA kit for universal application to processed foods containing coconut.

In conclusion, we have developed a rapid, specific, and sensitive immunoassay system to detect coconut milk proteins in processed foods, with acceptable accuracy results and few false positives. This method has been demonstrated to be suitable for the quantitative measurement of specific coconut milk proteins in processed foods.

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