

学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目 : Study on the remediation of Thai food safety with respect to food hypersensitivities
(タイの食品への過敏症に対する安全性の改善策に関する研究)

Title of Dissertation

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Introduction

Food hypersensitivities are the abnormal reaction of the body's immune system to a particular food; commonly occur in a sensitive population of individuals. It can be divided into two major categories based on mechanism: (1) immunoglobulin E (IgE)-mediated hypersensitivity also known as food allergies (allergy to protein in milk, egg, peanut etc.) is the most severe and immediately life-threatening adverse reactions to foods, especially food containing offending proteins, and (2) non-IgE-mediated hypersensitivity (gluten hypersensitivity or celiac disease, CD), allergic symptom is provoked by ingestion of gluten from gluten containing cereal namely wheat, rye and barley in their diets ((Jackson, 2003; Sampson, 2004). In CD patients, gluten causes damage to the lining of the small intestine which consequently leads to a severe malabsorption syndrome. Food hypersensitivity is now increasing globally and becomes serious problematic health issues in many countries. IgE-mediated is frequently high prevalence in industrialized countries, affecting nearly 1% of world's adults' population and around 6-8% of children (Ajala *et al.*, 2010). The increasing of food allergy in rapidly developing countries was also reported (Prescott *et al.*, 2013). While, CD originally is high prevalence in white Europeans, affect around 1% of population in Europe and the United States and now distributed worldwide (Ludvigsson and Montgomery, 2005) followed the mankind wheat consumption and the migratory flows (Gujral *et al.*, 2012). In Thailand, prevalence of food allergy and CD is uncommon among general Thai populations. However, there are many reports those confirmed the existing of food allergy among Thai people (Piromrat *et al.*, 2008, Lao-arrya and Trakultivakom, 2012). While, CD prevalence is quite rare this might be due to an underestimate and the adhering with Thai staple diets commonly based on rice, which are gluten free diets naturally. A large number of foods could provoke allergic reactions which are varying by region. Among the foods; eight types namely peanuts, nuts, wheat, soy, milk, egg, fish and shellfish; those widely used in food manufacturing, are reportedly responsible for causing more than 90% of allergic reaction (Bush and Hefle, 1996). However, there is a significant differences in the types of food known to cause allergies in Asian populations compared to Western populations such as peanut allergies are very common in the West, they are relatively rare in Asia. Generally, food component those caused allergic symptom called "food allergens" are commonly protein or glycoprotein, characterized by their ability to induce a pathogenic IgE response in susceptible individuals. The allergenic protein usually has high structural stability, highly soluble in aqueous solution, stable against enzymatic and chemical degradation as well as processing procedures (Besler *et al.*, 2001). Commonly, food allergens comprise divalent or multivalent molecules with two or more antibody-binding sites calls epitopes, which responsible for interacting with immune effector molecules including interaction with IgE antibodies. Among the allergenic food sources, tree nuts are big group generally include almond, cashew nut, chestnut, hazelnut, macadamia, pecan, pine nut, pistachio, walnut

and coconut, which are often used by the food industries owing to their taste. Obviously, many reports mentioned that tree nut allergens, particularly storage proteins which are abundant in nut, are always led to severe allergic reactions. In Thailand coconut are popularly use in various Thai food products as main ingredients, surprisingly the United States classified coconut as nut and has to declare as allergic ingredients on the label. Though allergic reactions to coconut are relatively rare, severe reactions as anaphylaxis shock to coconut fruit has occasionally been reported (Rosado *et al.*, 2002). However, there are many potentially cross reactive allergen among the tree nuts, thus it was recommended patients hypersensitivity to nut avoid coconut in the diets.

Currently, there is no medication for food allergy and celiac disease; strict avoidance of the allergy-causing food is the most secure methodology for preventing a reaction. In order to protect the allergic sufferers, food allergen labeling and gluten free labeling regulation are enforced both international and national level (Sicherer and Sampson, 2010), and the food manufacturers are requested to provide reliable product labels regarding to allergen issue thus allergic individuals can avoid the consumption of such allergic ingredients. However, still a wide range of products is suspected to contain undeclared allergens due to oversight by food manufacturers. Presently, undeclared allergens in food staffs are being recognized as a growing concern that cause incredible health hazard in highly sensitive persons. Additionally, these might be resulted in big loss of economic and brand reliability due to food allergen- related recalled. Therefore, to ensure that the food products have appropriate labelling and safe for allergic consumers, food allergen management programs should be applied through the food supply chain in order to reduce chance of cross-contract or cross-contamination of those allergens in place. In the same time, there is a need for quick and accurate methods to detect allergenic residue in food. Though there are various methods for allergen detection in food products, protein based immunoassays and DNA based polymerase chain reaction (PCR) are the two main methods popularly used for determination of residues from allergens (López-Calleja *et al.*, 2015). Commercially available test kits are primarily based upon immunochemical procedures as enzyme-linked immunosorbent assay (ELISA), especially in a sandwich format (Poms *et al.*, 2004). ELISAs can provide the most reliable and routinely used technique by the food industry and regulatory agencies for analyzing foods and for monitoring the safety of food products because ELISA is ease to use, fast, highly specificity, high sensitivity, simple handling and can perform for either qualitative or quantitative or both, through the specific binding of antigen and antibodies (Asensio *et al.*, 2008). A multitude of ELISA methods have been developed for the detection of several different food allergens. However, commercially available allergen kit might different in its performance which depended upon the manufacturer's technology such as choosing a format, selection of the appropriate antigen to raise good antisera (highly specific, no cross-reactivity), preparation antigen standard for the assay, gathering the needed components, and constructing a working protocol, systematically adjusting and testing of many components and variables to help ensure the accurate results (Yeung, 2006).

Food allergen issue is particularly important for Thailand, as a food producing and exporting country, and their ambition of becoming kitchen of the world. Thus the effective management for ensuring safety food that meet the global food safety standard level is crucial and challenge for Thai food industries as well as others stakeholders. At the periods of studying, we ensured that there is no food allergen and gluten free labeling regulation enforcing in Thailand, thus practically labeling of allergen and gluten free food is totally depended on the voluntary participation of food manufacturers, especially for domestic products. Presently, Thailand has limited data on current situation in term of scientific-base of allergen-related information. Therefore recognizing the need for a collective approach in addressing the safety issue with respect to food hypersensitivity is urgent required. The purposes of the present study are: (a) Investigation of undeclared food allergen in commercial Thai food products; (b) Survey of commercial Thai food products that have been reported to contain no wheat, rye barley or gluten according to their labels whether it is safe for CD patients; (c) Investigation on the understanding and implementation of food allergen management among Thai food manufacturers; (d) Establishment of reliable

Enzyme-Linked Immunosorbent Assay for the determination of coconut milk proteins in processed foods.

1. Investigation of undeclared food allergens in commercial Thai food products

Most of the food products examined by ELISA were determined to have an allergen content less than the LOQ (0.3 ppm), shown as “ND”. The result revealed that the labeling was properly done in most of the examined products (figure 1.1).

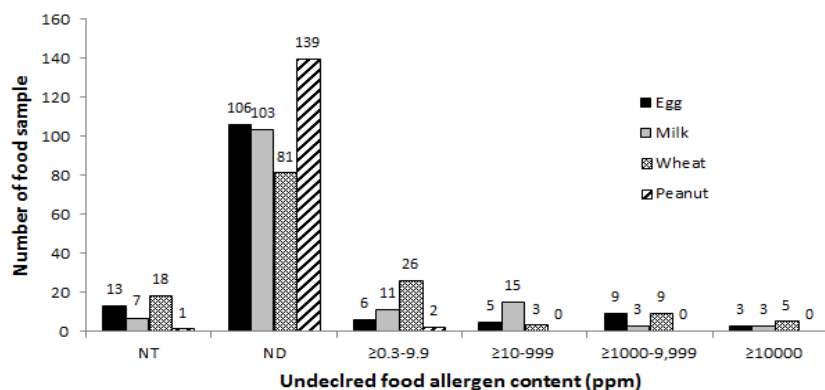


Figure 1.1 Distributions of undeclared food allergen content in commercial Thai food products examined by ELISA. ND = not detected (detected food allergen is less than LOQ (0.3 ppm); NT = not tested (food allergen in question is labeled).

Out of 142 commercial food products, a total of 55 positive cases of undeclared food allergens were observed. The frequency of undeclared milk-positive cases was 21, followed by wheat- and egg-positive cases; both with 17 and no peanut-positive cases were found. In our survey, milk was the most common positive allergen found in various kinds of processed food *i.e.* instant noodles, curry, salad dressing, dessert mix and snack. It was noticed that the labels of milk-positive food products often contained the ingredients “cream,” “coconut cream powder” or “non-dairy cream.” Commonly, “cream” is defined as the milk part rich in fat, so “cream” is considered to principally contain milk protein. While egg-positive frequently found in fishery products as fish balls, fish tofu, these products were commonly used egg white to improve the texture (Wang and Wang, 2009). Wheat –positive cases found in fish balls, crab stick and imitation crab stick. The inclusion of wheat in these products is due to wheat protein which is widely used to improve the texture of fishery products; and wheat-derived seasoning or wheat hydrolysate, which is added to enrich the taste. Another speculated cause is “starch,” which is also a popular ingredient in a wide variety of food products. Among the positive cases, there were a significant number of cases containing undeclared food allergen greater than 1%, namely wheat (5 cases), egg (3 cases) and milk (3 cases). In this study confirmation test for the present of undeclared milk (casein) or egg (ovalbumin) were showed positive result by Western blot analysis. Our results should help to increase the awareness among Thai food manufacturers of the need for more careful management of allergenic food ingredients, and to encourage the labeling of allergen information for allergic consumers, thus reducing the health hazard from food allergy.

2. Survey of commercial Thai food products those have been reported to contain no wheat, rye barley or gluten according to their labels whether it is safe for CD patients

The 129 commercially available Thai food products with neither wheat, rye, barley, nor gluten on the

product labels were included snacks, noodle, Thai dessert, fish and meat products, pre-cooked rice, sauce, instant drink powder, spread, flour, rice grain, cassava (tapioca) pellets, rice paper, and gelatin powder. Those products had different main ingredients and 68 samples of 129 (52.7%) declared rice as a major ingredient. The ELISA examinations presented that 97 out of 129 products had the gluten level below the LOQ (Table 2.1).

Table 2.1 Gluten content of examined Thai food products by wheat ELISA kit

Gluten content (ppm)	Number of food products
Less than LOQ (<0.255ppm gluten)	97
>0.255-5 ppm	22
>5 ppm-20ppm	0
>20ppm-1000 ppm	2
>1000 ppm	8
Total	129

LOQ was 0.255 ppm gluten according to the kit instruction

Overall, the vast majority of the products, *i.e.* 119 of 129 products (92.2%), contained less than 20 ppm gluten, and satisfied the safe gluten level for the CD individuals. On the other hand, a significant number of products (10 products, 7.8%) showed gluten exceeding 20 ppm. The product group most frequently found gluten exceeding 20 ppm was “Snacks” (4 products), followed by “Instant drink powder” (2 products), and then “Fish and meat products”, “Pre-cooked rice”, “Noodles”, and “Rice paper” (1 product each). Among those 4 samples contain gluten level >1 %. ELISA is known to give a false-positive result sometimes due to the food matrix effect and antibody cross-reaction, therefore all products observed exceeding 20 ppm gluten by ELISA were further examined by PCR analysis. The PCR results showed clearly presented positive bands verifying the presence of wheat DNA in those ten products. The incidence of gluten presence was potentially found in the products that were manufactured with complex formulation and/or with complicated process *i.e.* “Instant drink powder” and “Snack”. Our survey suggests that CD individuals can consume most of the examined Thai food products. Although Thai food has the advantage to provide gluten-free food option for CD patients, this study also indicated the effective gluten management in the supply chain and manufacturing process including finished product testing are essential to keep the safety, quality and compliance with gluten-free regulations.

3. Investigation on the understanding and implementation of food allergen management among Thai food manufacturers

Selection of objective companies

The information presented in this report was collected via a field survey through questionnaires and direct interviews of Thai food manufacturers during the years 2008–2009. A total of 121 HACCP certified food manufacturers representing 14 primary food categories, were selected for the investigation.

Data collection and data analysis

The questionnaire was composed of four parts: (1) general manufacturer profile; (2) production line characteristics; (3) food allergen control implementation; and (4) knowledge, understanding and concern regarding food allergen management. Questionnaires as well as further interviews were directed to the most knowledgeable person(s) responsible for quality control (QC) in order to assess the level of awareness, understanding and practices addressing food allergen management on-site. Percentage responses are calculated based on the number of respondents answering the question. Depending on the questions, results are presented using descriptive statistics together with correlation analysis among parameters of company profiles, production

line characteristics, understanding of food allergens, food allergen control procedures, and food allergen concerns.

Food manufacturers profile information

From the total of 121 HACCP-certified food manufacturers, 72 (59.5%) returned completed questionnaires, which included large-size companies (51; >100 employees), followed by medium (17; 10–100 employees) and small (4; <10 employees) companies. The 72 surveyed companies comprised 14 food categories. Five categories *i.e.* baby formula, snacks, frozen and ready-to-eat meals, meat, and hydrogenated and refined oils were represented almost entirely by large-size companies, bakery products, on the other hand, were produced only by medium-size companies. Four food categories – beverages and soy milk, milk and dairy products, sauces and condiments, and premixed flour- were manufactured by companies of all sizes. Among the food categories, convenience food products were gaining the popularity among consumers because of the changing Thai lifestyle, and additionally are exported extensively with the encouragement of the government’s “Thai Kitchen to the World” policy. Forty-eight food manufacturers (66.7%) primarily distributed their products in the domestic market, and 24 (33.3%) overseas. The top three export markets are the European Union, North America and Japan/Korea, represent 61.3% of the total export market, where food allergen labeling regulations are already enforced.

Table 3.1 Overseas customers of Thai food manufacturers.

Country	Percentage (%)
European Union	23.8
North America	19.6
Japan / Korea	17.9
China	14.3
Australia / New Zealand	13.1
Other countries in Asia	11.3
Total	100

Production line characteristics

Production line characteristics can be categorized as dedicated line, shared line and outsourcing. Among those dedicated lines were the most common practice; this probably reflects a preference for managing primary production under their own production line, especially for frozen and ready-to-eat meal product category. According to the interviews, outsourcing was commonly employed to prepare the intermediate mixed ingredients, *i.e.* processed fruits and vegetables, premixed flour products, for manufacturing the final product. Forty-two of 72 (58.3%) companies had a reworking process mainly for identical products. Managing the production line, 20 companies used a scheduled cleaning strategy to prevent food allergen cross-contamination and evaluated for the risk of contamination in place. None of the QC managers felt that their products had a high risk of allergen contamination when produced by their own manufacturing lines. However, 12/72 (16.7%) companies recognized a medium to somewhat high risk level of allergen cross-contamination in their products, which could possibly occur by the unintentional inclusion of a food allergen through the food ingredient supply chain. This implied that nationwide food allergen management is needed in Thailand. Among the examined companies, major allergic ingredients widely used were wheat, rice, soybeans, eggs, milk. Although wheat, soybeans and their derivatives are imported, they are still widely used in various kinds of food products due to their nutritional value and functional properties.

Implementation of food allergen control in the production line

The increasing prevalence of food allergies in recent years has led food manufacturers to be more proactive in their responsibility for preventing any contamination by implementing effective allergen control programs (Chang et al., 2013). Forty manufacturers (55.6%) reported implementing food allergen control as part of the quality management platform of HACCP, Good Manufacturing Practices (GMP) or British Retail Consortium (BRC) programs. Their practical control activities related to allergen such as purchasing, inspection of raw materials, receiving and storage, formulation, cleaning etc. Among those, ACPs were most frequently applied in the purchasing process, food formulating and cleaning program. Process validation and finished product inspection were conducted less frequently because they requiring special resources, i.e. knowledgeable experts, special reagents/equipment. Inspections for food allergen control in cleaning program were occasionally performed by protein detection test, Adenosine triphosphate (ATP) test, and allergen-specific enzyme-linked immunosorbent assay (ELISA). The correlation between export regions and the adoption of a food allergen control program by Thai food manufacturers showed that 64.3% of food exporters conducted ACPs, but the percentage levels were different upon the exporting market. Results revealed that companies provided food allergen information more frequently for export products (45.9%) than for domestic products (6.9%) because the lack of food allergen labeling laws in Thailand. Allergen-related labeling practices used *i.e.* “contain [allergen]”, “may contain [allergen]”, and “Produced in facility/equipment processing allergen”. In regard to domestic product labeling, food allergen labeling of their products in response to requests from Thai consumers.

Table 3.2 Export regions and the application of food allergen control programs.

Export region	Food allergen control program			
	Applied*		None	
	Number	Percentage (%)	Number	Percentage (%)
European Union	26	63.4	15	36.6
Japan/Korea	25	75.8	8	24.2
North America	19	63.3	11	36.7
China	13	56.5	10	43.5
Australia/New Zealand	12	57.1	9	42.9
Other county in Asia	13	65.0	7	35.0
Subtotal	108	64.3	60	35.7

*Applied ACP following the HACCP, BRC or GMP system.

Understanding of food allergen management and labeling

The majority of QC managers (70.8 %) had medium to good understanding of food allergen management. The level of understanding was mostly related to their experience in food safety, more specifically in their engagement with food allergen management. Additionally, QC managers of companies with a higher proportion of exports had significantly higher scores of understanding. Food manufacturers believed that allergen labeling will provide benefits, especially those whose major product is for export. The greatest benefit was to increase the product’s reliability in foreign markets where food allergen labeling regulations are enforced. On the other hand, the responses of companies whose major market is domestic indicated comparatively fewer benefits of labeling. Earlier implementation of labeling regulations has been driven by adherence to WTO (Codex) rules.

The results of this survey indicated the substantial effectiveness of the present allergen control programs of Thai food manufacturers, and also their potential capability of food allergen management for domestic products at a level equal to that for export products.

4. Reliable Enzyme-Linked Immunosorbent Assay for the determination of coconut milk proteins in processed foods

Purification of Coconut Milk Protein (P40-60).

SDS-PAGE of defatted coconut milk showed 4 major bands with a molecular mass between 15 and 25 kDa. One main single band with molecular weight around 25 kDa and some minor bands were observed in purified P40-60. The protein fractions of interest in a highly processed coconut product; canned coconut milk; are still retained on SDS-PAGE. While, there was no corresponding band around the 25 kDa of both defatted hazelnut and walnut. Therefore, P40-60 containing main protein was used as an antigen to provided coconut-specific polyclonal antibodies against P40-60. Western blotting also confirmed the ability of the anti-P40-60 antibody to recognize P40-60 coconut milk protein. The 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. Accordingly, the P40-60 protein was considered to be suitable as a coconut marker protein for the determination of coconut milk in processed foods.

Construction of Sandwich ELISA

The standard curve was evaluated for optimal concentration range for the detection of coconut proteins, using anti-P40-60 rabbit serum.

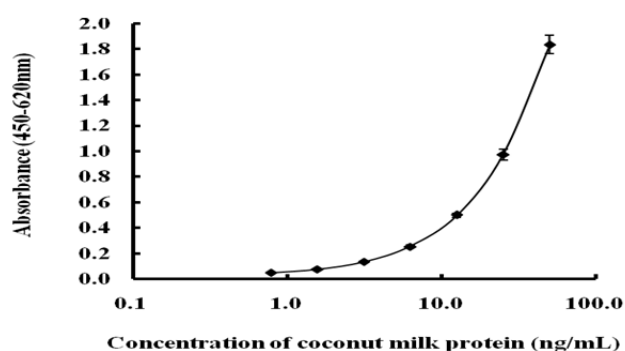


Figure 4.1. Representative calibration curve of the developed ELISA.

The calibration curve was prepared with concentrations of standard solution (0.78-50 ng/ml). 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 $\mu\text{g/g}$ in foods, respectively, indicating the ability of the assay to determine a small amount of coconut milk in a given food sample. The LOD and LOQ were calculated as three times and 10 times the SD of the buffer blank mean values after three experiments. The developed P40-60 ELISA showed no cross-reactivity with 42 of 43 foods (except oat), reflecting a very high specific detection system. Detection of coconut allergens present in various processed coconut milk showed very high reactivity. Tetra pack coconut milk showed high reactivity (100-110% recovery) compared to the highly process as canned coconut milk (52-54% recovery). The severe processing might lead to great conformational epitope changes, leading to loss of the ability of antibodies to bind directly to specific epitopes. Application of establish ELISA to commercial food products with coconut meat or coconut milk listed as ingredients (biscuit, curry, crispy roll shredded pork, dessert mix), were clearly detected and showed very high reactivity. These results suggest that the established ELISA could be applicable for the determination of coconut milk proteins in processed foods.

Conclusion

Our work is the first evidence-based safety report to assess the risk of Thai commercial food products

with respect to food allergens. Analysis result for the presence of allergenic proteins (egg, milk, wheat and peanut) and gluten using ELISA assays in various Thai commercial food products demonstrated that most of examined samples were properly labeled for the presence of allergenic protein / gluten. The results of gluten survey in processed Thai foods indicated the potential of the examined Thai products as new diets for CD patients. However, some products contained high undeclared allergen level. Those results reflect appropriate allergen and gluten management strategies need to be implemented by Thai food manufacturers to ensure accurate labelling and to protect the safety of food allergic individuals. In addition this could be an alarm for all food safety stakeholders in Thailand to consider improve allergen management, particularly from the standpoint of product liability. However, survey on the understanding and practices of HACCP certified Thai food manufacturers by questionnaires and direct interviews of quality control managers concerning food allergens revealed a substantial number of Thai food manufacturers have already implemented food allergen management practices at an international food safety level by extending the existing food safety system. This demonstrated a very high awareness of food allergy, the management and labeling of food allergens, indicated the potential capability of Thai food manufacturers to adopt the upcoming food allergen regulations. Since food allergy are varies depending on eating habit, reliable testing for Thai specific allergen as coconut milk ELISA was developed and could be used to detect coconut milk proteins from various coconut milk products with acceptable accuracy results.

Our researches are the first scientific based evident related to health risk on food hypersensitivity. The results obtained demonstrated the valuable information for the present food safety situation. In the absence of food allergen labeling and gluten free labeling regulation, these scientific information will be used as guideline which enable better decisions for future food safety policies dealing with food hypersensitivity in Thailand (*i.e.* food allergen labeling, food allergen control) in order to protect the public's health and ultimately reduce the burden of illness cause by allergen hazard. In addition, the knowledge on ELISA establishment for coconut milk protein detection will enable to be used for ELISA establishment of other allergens in the future. Since food allergen is considered as global emerging issue, Thai food labeling regulation was then be revised wherein allergen information was required for the first time by December, 2014. The list of priority allergens in this new regulation is simply follow the Codex. With this regulation, accurate and informative labelling of allergenic ingredients need to be provided thus help to prevent potentially serious reactions in food hypersensitive individuals.

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