



Analytical Methods

A two-site monoclonal antibody immunochromatography assay for rapid detection of peanut allergen Ara h1 in Chinese imported and exported foods

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ABSTRACT

Food allergen labeling has not yet been implemented in China. Therefore, a gold immunochromatography assay (GICA) was developed using two monoclonal antibodies (mAb) against the peanut allergen Ara h1. The GICA was specific for standard peanut samples with a sensitivity of 10 ng/ml. Peanut protein traces extracted from 124 food products imported and exported by China Customs were easily and rapidly detected by GICA. 68 food samples originally labeled as containing peanuts were positive for Ara h1 and 54 food samples labeled as not containing peanuts were negative for Ara h1, indicating that the labels from the manufacturers were accurate. However, 2 food samples labeled as not containing peanuts tested positive for Ara h1. The present GICA provides a fast, simple, semi-quantitative method for the determination of peanut allergens in foods. This detection system can be used to ensure the safety of food imported and exported by China Customs.

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1. Introduction

Food containing peanut ingredients can be allergenic and may even cause life-threatening allergic reactions (Bock, Munoz-Furlong, & Sampson, 2001, 2007). Death resulting from peanut allergies has been reported both in China and the USA (Ji et al., 2009). Threshold doses for allergic reactions can be as low as 100 µg of peanut protein (Hourihane et al., 1997). As there is currently no effective causative therapy for peanut allergies, labeling of food containing peanut ingredients has proven an effective and simple method for avoiding allergic reactions (Vierk, Koehler, Fein, & Street, 2007). The Food and Drug Administration (FDA) Bureau of the USA implemented a regulation named Food Allergen Labeling and Consumer Protection Act (FALCPA) in Jan 2006, requiring food allergen labeling of all foods sold in the USA (FDA, 2004). Similar regulations have been passed in other developed regions, such as Japan and the European Union (Li et al., 2009). However, no such regulation has been implemented in China, although food import and export between these developed countries and China is increasing (McKay, 2007). Therefore, it would be beneficial for Chinese Customs to be able to perform rapid detection of peanut allergens in order to establish a food safety surveillance system and avoid international trade disputes.

Ara h1 is an ideal biomarker for peanut allergen detection for a number of reasons (Pomés et al., 2003). Firstly, Ara h1 is the major

component of peanut, comprising 12–16% of the total peanut protein (Koppelman et al., 2001). Secondly, it has a stable structure so that it does not degrade during food processing procedures, such as heating or roasting (Beyer et al., 2001). Thirdly, Ara h1 is the most important major allergen from peanuts.

Food detection systems used by Customs should be fast, sensitive and specific. GICA has been commonly used previously by Customs because it is quick and easy to use (Stephan, Möller, Lehmann, Holzhauser, & Vieths, 2002). Lateral flow devices based on polyclonal antibodies for the detection of peanut allergens have previously been shown to be simple, sensitive and rapid (Wen, Borejsza-Wysocki, DeCory, & Durst, 2005). However, false positive results can be obtained with this approach due to cross-reactivity of the polyclonal antibodies (Schubert-Ullrich et al., 2009). The high specificity of mAbs can be used to address the problem of false positives. In this study, we developed a new, two-site Ara h1 specific, mAb-based sandwich GICA method which allows the rapid, highly selective and simple semi-quantitative determination of Ara h1 from food products imported and exported by China Customs.

2. Materials and methods

2.1. Purification and identification of Ara h1

Fresh peanuts were supplied by a local vendor. Ara h1 was purified from peanut extract by ammonium sulfate precipitation and ion exchange chromatography as previously reported (Maleki et al., 2000). The purified native Ara h1 (nAra h1) was separated

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and detected by SDS–PAGE. Identification of the allergen was confirmed by MS/MS. Isolated Ara h1 was used as the antigen for mAb production and as a standard for ELISA.

2.2. Production of anti-Ara h1 mAbs

Two anti-Ara h1 mAbs (clones 2G9 and 5G4) were produced and purified according to a standard protocol (Chang, Sutherland, & Parsons, 1995). Their isotypes and titres were also determined according to standard protocols. Indirect competitive ELISA was performed to investigate whether there was cross reactivity between these two mAbs (Sano et al., 1998).

2.3. Primary epitope analysis of mAbs

Using DNASTAR software (<http://www.dnastar.com/products/lasergene.php>), five potential antigenic peptide fragments of Ara h1 (1–614 aa) were selected: Ara h1 A (residues 25–80), B (residues 74–204), C (residues 197–345), D (residues 335–425) and E (residues 462–604). Their corresponding coding cDNA sequences were cloned into prokaryotic expression vectors (pTrc-CKS) constructed by our laboratory and then expressed as CKS fused proteins in *E. coli* (Zhan et al., 2010). Immunoblotting and indirect ELISAs were performed with each purified CKS-fusion polypeptides against mAbs 2G9 and 5G4 respectively, using CKS as the negative control.

2.4. Two-site mAb ELISA for Ara h1

A two-site mAb ELISA was established using mAb 5G4 as the coating antibody and biotin-conjugated mAb 2G9 as the capture antibody. A standard curve was prepared with serially diluted nAra h1 protein and the detection limit was determined. All experiments were performed according to classic immunological methods (Luczynska et al., 1989).

2.5. Two-site mAb-based GICA for Ara h1

A two-site mAb-based GICA assay was developed according to the method of (Lee et al., 2006). Purified mAb 2G9 was conjugated with colloidal gold as the mobilising antibody for capturing Ara h1 and mAb 5G4 was absorbed onto nitrocellulose membranes to serve as the detection line. These two antibodies constituted a sandwich immunochromatography detection strip. The sensitivity of these assembled GICA strips was tested against serially diluted peanut protein crude extracts. Specificity was tested with a series of food sample protein extracts such as soybean, broad bean, pea, black bean, green bean, red bean, sunflower seeds, wheat, buckwheat, rye, sesame, black sesame, pistachio, American almond, Chinese almond, nut, walnut, hazel, egg, milk, shrimp or fish.

2.6. Detection of Ara h1 in foods using GICA at China Customs

124 exported and imported food product samples were obtained from the Shenzhen Entry-Exit Inspection and Quarantine Bureau of China. The food samples were ground to a fine powder. 1 g of powder was dissolved in 20 ml extraction buffer (200 mM PBS, 1 M NaCl, 2% Tween, pH 8.0) at 60 °C for 30 min, with rigorous shaking every 10 min. The solution was centrifuged at 12 000 rpm for 10 min. The supernatant was then tested using the GICA. Strips with visible detection lines were defined as positive results; strips without any visible lines were defined as negative results.

3. Results

3.1. Anti-Ara h1 mAbs

Western blotting was performed to identify the mAbs. Both mAbs bound purified nAra h1 (about 63 kDa) (Fig. 1). Identification of purified nAra h1 in this experiment was confirmed by MS/MS analysis (see supplemental data). Isotyping assays demonstrated that mAb 2G9 and 5G4 are both IgG2a subtype. The titers of both mAbs exceeded 1:4000,000. Indirect ELISA inhibition assays showed that mutual inhibition of mAb 2G9 and 5G4 was less than 5% (Table 1). This demonstrated that these two mAbs recognised two different epitopes, which allowed the development of a two-site mAb sandwich immunoassay. These results show that the two mAbs generated had high titers and specificity without cross-reactivity.

3.2. Primary epitope analysis

The epitopes of the two mAbs were analysed by their reactivity with the five Ara h1 fragments through Western blot and indirect ELISA assays. As shown in Fig. 2, both mAbs, 2G9 and 5G4, showed ELISA reactivity against polypeptide A (25–80 aa) (Fig. 2a). Reactivities were confirmed by immunoblot analysis (Fig. 2b). The corresponding amino acid sequence of Ara h1 25–80 aa is shown in Fig. 2c.

3.3. Two-site mAb ELISA for Ara h1

A two-site mAb ELISA was established using mAb 5G4 as the coating antibody and biotin-conjugated mAb 2G9 as the capture antibody. The optimum dilution folds for coating antibody (mAb 5G4) and capture antibody (biotin-conjugated mAb 2G9) were determined to be 1:4000 and 1:3000, respectively. Using serially diluted nAra h1, the ELISA detection limit was determined to be 3 ng/ml (Fig. 3). The response was linear between 6 and 100 >ng/ml ($R^2 = 0.9899$) (Fig. 3).

3.4. Sensitivity and specificity of the two-site mAb-based GICA for Ara h1

GICA strips were generated by coating mAb 5G4 onto nitrocellulose membranes and labeling mAb 2G9 with Au³⁺ as the mobilising antibody. The sensitivity of GICA strip was determined to be

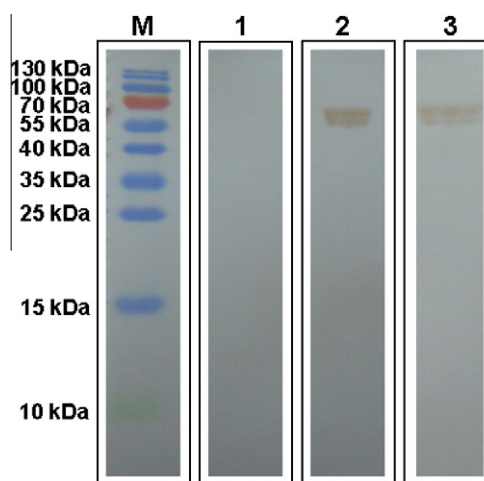


Fig. 1. Immunoblot analysis of mAb 2G9 and 5G4 specificity. M: protein marker; 1: negative control; 2: mAb 2G9 + purified nAra h1; 3: mAb 5G4 + nAra h1.

Table 1

Cross reactivity of mAb 2G9 and 5G4 as determined by indirect competitive ELISA.

Competitive biotinylated mAb dilutions	1:8000	1:4000	1:2000
Inhibition ratio, mAb 2G9:mAb 5G4	3.13%	3.45%	4.12%
Inhibition ratio, mAb 5G4:mAb 2G9	3.56%	3.15%	4.25%

10 ng/ml (Fig. 4). GICA strips did not react with any food sample protein extracts from soybean, broad bean, pea, black bean, green bean, red bean, sunflower seeds, wheat, buckwheat, rye, sesame, black sesame, pistachio, American almond, Chinese almond, nut, walnut, hazel, egg, milk, shrimp or fish (data not shown). This revealed that this method had good sensitivity and specificity.

3.5. Detection of Ara h1 in foods with GICA by China Customs

GICA was used to detect peanut allergens in 124 exported and imported food product samples from the Shenzhen Entry-Exit Inspection and Quarantine Bureau of China (Table 2). In our GICA tests, 68 food samples labeled as containing peanuts tested positive for Ara h1 and 54 samples labeled as not containing peanuts tested negative for Ara h1. These results indicate that the original food allergen labels were accurate. This was not the case for all samples tested. 2 food samples labeled as not containing peanuts

tested positive for peanut allergen contamination. These results demonstrate that GICA is effective for food allergen detection in food inspection and quarantine in Customs.

4. Discussion

Currently, most commercially available kits for the detection of peanut allergens are based on polyclonal antibodies against total peanut protein or Ara h1 (Schubert-Ullrich et al., 2009). Ara h1 is a good target for peanut allergen detection (Pomés et al., 2003). It is a member of the vicilin family of seed storage proteins, showing 30% to 45% amino acid identity with other vicilin storage proteins from legumes such as soybean, garden pea, and beans according to our blast results (blast data not shown). This homology could result in cross-reaction of polyclonal antibodies against Ara h1 with other allergens from botanically related families, leading to false positive results (Schubert-Ullrich et al., 2009). The possibility of generating false positive results makes these assays unsuitable for daily use in a custom setting. In order to prevent false positives, we developed a mAb based detection method. The two mAbs used in our method recognize epitopes in the 25–80 aa region of Ara h1. This region has the lowest degree of homology with other members of the vicilin family (blast data not shown). This could also improve the specificity of our method. This is

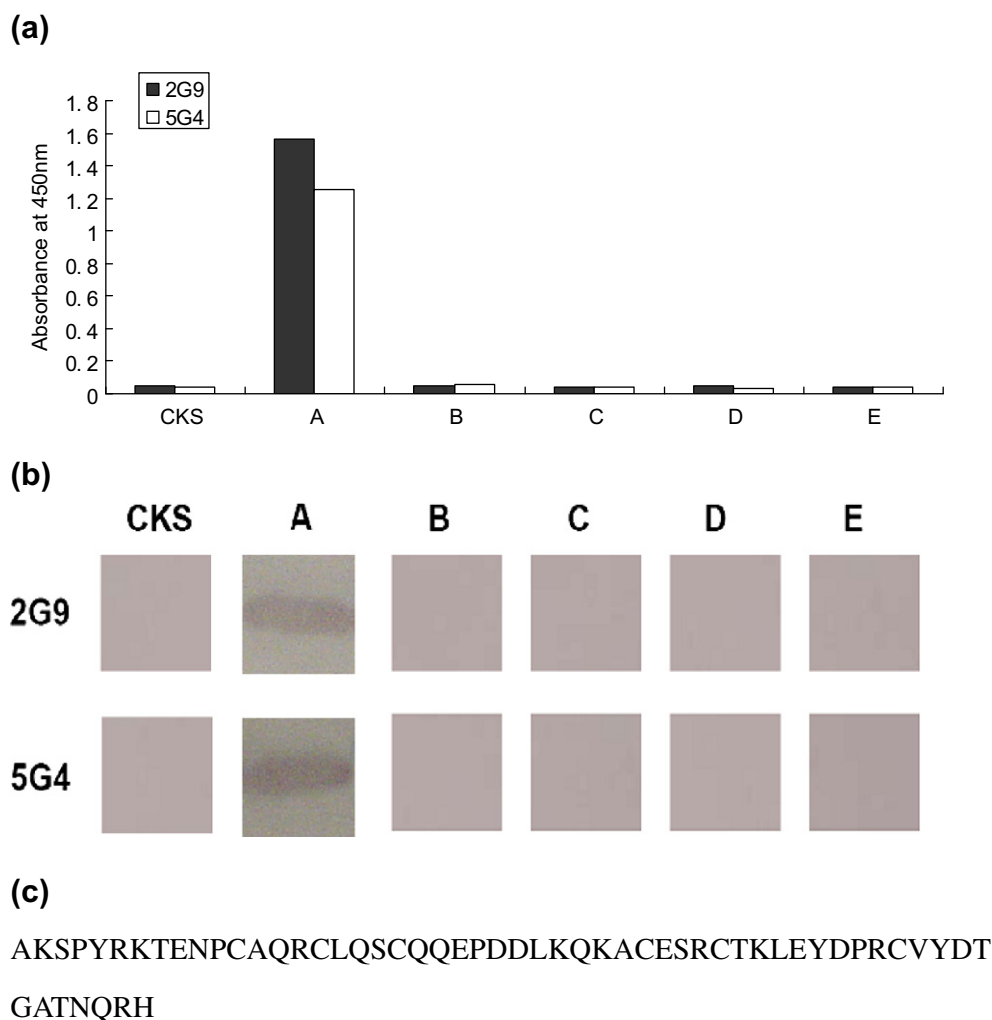


Fig. 2. Primary epitope analysis of mAbs. mAb reactivity to five Ara h1 fragments (A: 25–80 aa; B: 74–204 aa; C: 197–345 aa; D: 335–425 aa; E: 462–604 aa) and CKS fusion protein (control) was examined by ELISA (a). Each peptide was separated on 15% SDS–polyacrylamide gel and immunoblotting was performed with monoclonal antibodies 2G9 and 5G4 (b). The amino acid sequence of Ara h1 25–80 aa (c).

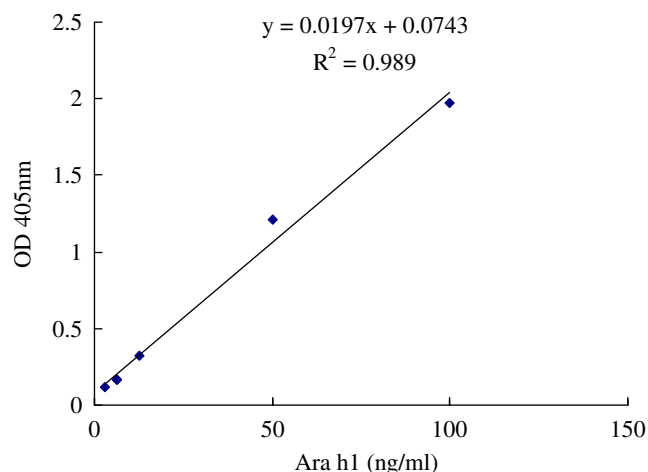


Fig. 3. Sandwich ELISA with biotinylated mAb 2G9 as the capture antibody and mAb 5G4 as the coating antibody. The standard curve is linear for nAra h1 from 6 to 100 ng/ml and the detection limit was 3 ng/ml ($R^2 = 0.989$).

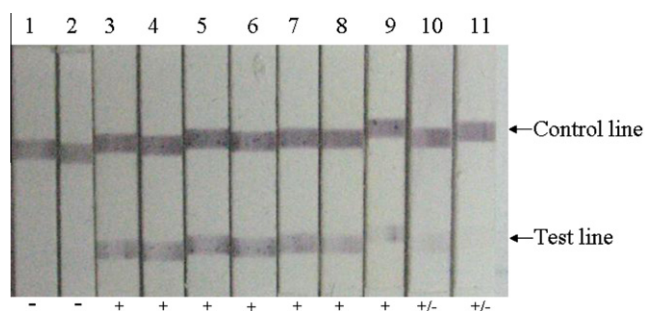


Fig. 4. Detection of peanut crude protein extracts with GICA strips; 1: Extract buffer; 2: PBS; 3: 1000; 4: 500; 5: 250; 6: 100; 7: 50; 8: 25; 9: 10; 10: 5; 11: 2.5 ng/ml. The concentrations indicated are the quantity of total protein per ml of crude peanut extract. +: positive; -: negative; +/-: not definitive.

consistent with our GICA experimental results where these two mAbs demonstrated high specificity.

The sensitivity of the GICA strips was determined as 10 ng/ml total peanut protein extract, which is comparable to the sensitivity of the sandwich ELISA system (30 ng/ml) developed by Pomés for peanut allergen detection (Pomés et al., 2003). However, the GICA method described here is much simpler and faster than an ELISA assay, making it more suitable for large volume food sample testing in the Customs environment.

Threshold doses for allergic reactions can be as low as 100 µg of peanut proteins (Hourihane et al., 1997). Since every individual food sample varies in peanut ingredients, our method could not fully guarantee detection of peanut allergen at the threshold levels. However, our GICA method is still an effective tool for the detection of peanut allergens in imported and exported food samples by China Customs.

The key to a successful two-site monoclonal antibody immunoassay is two mAbs with high titers, high specificity and no cross reactivity (Sander, Neuhaus-Schröder, Borowitzki, Baur, & Raulf-Heimsoth, 1997). Although Ara h1 protein is a stable antigen, traditional food processing practice such as roasting or boiling may still fragment the protein into smaller peptides (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). Therefore, for a two-site monoclonal antibody immunoassay, the two non-cross reactive mAbs should recognize different epitopes in an adjacent region of the antigen. If the two epitopes are far apart on the allergen, fragmentation from food processing procedures may prevent allergen

Table 2

Representative ($n = 20$) GICA analysis of peanut allergen Ara h1 in food samples from Chinese Customs.

Food Description	Imported (I) /Exported (E)	Labeling for peanut presence	GICA results ($n = 3$)
Cookies, almond	E	+	Positive
Cookies, walnut	E	–	Negative
Cookies, peanut	E	+	Positive
Cookies, corn	E	–	Negative
Cookies, chocolate	I	–	Negative
Cake, nuts	E	–	Negative
Cake, lotus seed	E	–	Negative
Chocolate I	I	–	Negative
Chocolate II	I	–	Negative
Chocolate III	I	–	Negative
Chocolate IV	I	–	Positive
Chocolate, Milk	I	–	Positive
Chocolate, strawberry	E	–	Negative
Chocolate, almond	E	–	Negative
Chocolate, coffee	I	–	Negative
Chocolate, liqueur	I	–	Negative
Hazelnuts, milk	I	–	Negative
Corn bar	I	–	Negative
Legumes and nuts I	I	+	Positive
Legumes and nuts II	I	+	Positive

Note: Theoretically, in our experiments, GICA positive results indicated that the concentrations of the foods were greater than 200 g peanut total protein/kg food sample whereas negative results represented non-existence of any peanut ingredients or less than 200 g peanut total protein/kg food sample.

detection. Fortunately, in the method presented here, the two mAbs recognised two adjacent, distinct epitopes located in a small region (25–80 aa of Ara h1) but without cross reactivity.

A number of Western countries have issued a series of food labeling regulations to protect allergic consumers from accidental consumption of food allergens (Food and Drug Administration, 2004; Ji et al., 2009; Vierk, Koehler, Fein, & Street, 2007). Currently, China does not have such regulations, although it exports a large amount of food globally. One of the most common food allergens is peanut. Therefore, it would be beneficial for Chinese customs to be able to detect peanut allergens in imported and exported foods. As the volume of food passing through customs is so large, it is essential that any detection method for peanut allergens is fast and simple with high sensitivity and specificity. The GICA method presented here meets these requirements for use in Chinese Customs. In addition to fulfilling the demands of China's import and export food trade, the GICA method could also form the technological basis for China's food labeling management.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.foodchem.2011.04.055](https://doi.org/10.1016/j.foodchem.2011.04.055).

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Corrigendum

Corrigendum to ‘A two-site monoclonal antibody immunochromatography assay for rapid detection of peanut allergen Ara h1 in Chinese imported and exported foods’ [Food Chemistry 129 (2011) 541–545]Kun-Mei Ji, Jia-Jie Chen, Chen Gao, Xiao-yu Liu, Li-Xin Xia^{*}, Zhi-Gang Liu^{*}, Liang Li, Suhan Yang*State Key Laboratory of Respiratory Disease for Allergy at Shengzhen University, School of Medicine, Shenzhen University, Nanhai Ave 3688, Shenzhen Guangdong 518060, PR China*

The authors regret that an error appeared in the ‘Note’ of Table 2 of this article. The corrected note follows here:

Note: Theoretically, in our experiments, GICA positive results indicated that the concentrations of the foods were greater than 200 µg peanut total protein/kg food sample whereas negative

results represented non-existence of any peanut ingredients or less than 200 µg peanut total protein/kg food sample.

The authors would like to apologise for any inconvenience caused.

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