FOOD COMPOSITION AND ADDITIVES

Development and Validation of a Surface Plasmon Resonance Biosensor for Specific Detection of Porcine Serum Albumin in Food

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Food allergies are a potential food safety and public health concern worldwide. To assure the safety of people who experience allergic reactions, it is necessary to establish effective and reliable methods for rapid detection of food allergens. This paper reports an innovative method for the rapid detection and analysis of porcine serum albumin (PSA), known as a major allergen in pork, based on a surface plasmon resonance (SPR) biosensor. The antibodies known to have a high bioactivity against PSA were verified by competitive indirect-ELISA and then immobilized on the SPR sensor surface, thus allowing them to capture PSA. The developed SPR demonstrated a linear range from 1.0 to 450 ng/mL for the measurement of PSA with a detection limit of 19.81 ng/mL. Within-day RSD (1.97–4.02%) and between-day RSD (1.88–4.15%) were no more than 5%. The SPR was evaluated for analysis of six commercial food samples and showed almost perfect agreement between the results obtained by ELISA test kits without significant differences (*P* > 0.05). Therefore, this assay permits accurate, specific, and sensitive detection of PSA in pork and pork products.

With the improvement of living standards, people have gained access to more types of food; however, the prevalence of food allergies is also increasing significantly in industrialized countries. Food allergies are considered to be an emergent public health problem, and it is estimated that they affect 4–6% of children and 1–3% of adults worldwide (1). It has been reported that about 90% of food allergies are caused by the eight major allergenic foods: soybean, peanuts, wheat, nuts, milk, eggs, fish, and crustaceans (2). Allergic reactions to meat products, featured in recent studies, are a newly recognized and important food safety issue. The incidence of meat allergies is about 0.5–8%, and the main symptoms include rash, measles, eczema, asthma, vomiting, diarrhea, pinkeye, and itching and swelling of the throat (3). Beef allergy has been reported in many studies and has drawn a great deal of attention; however, although pork is the most heavily consumed meat in the world, pork-related allergic reactions have not received adequate attention from researchers. Porcine serum albumin (PSA, 67 kDa) has now been determined to be a major allergen (4), and the pork-related allergies induced by PSA have an incidence around 3–10% (5). PSA may have cross-reactivity with other serum albumins (such as beef and sheep; 6).

To protect the health and safety of these allergic people, many countries and regions have established laws and regulations related to food allergen labeling and identification. This has become a new area for establishing technical barriers in international trade. In this aspect, quantitative analysis of food allergens plays a key role and so research in this area has received intensive attention. At present, analysis of food allergens depends mainly on ELISAs (7, 8), PCR (9, 10), and MS (11). Compared with these conventional methods, surface plasmon resonance (SPR) is a valuable analytical tool due to its obvious advantages of label-free, stable, high-throughput, online monitoring and analysis (12). SPR occurs at a certain angle and is observed as a minimum in reflected light intensity (13). When the refractive index of the thin gold film is altered, for example, when antigen binds to the immobilized capture antibody, the angle at which SPR occurs is changed. The changes are proportional to the amount of bound protein and are expressed as resonance units (RU). Thus far, SPR has been widely applied to numerous biomedical

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fields including molecular interaction (14, 15), clinical diagnosis (16, 17), proteomics (18, 19), environmental monitoring (20, 21), bacterium and virus identification (22–25), and cellular interaction (26, 27). However, not much research in the food allergen field has been published. Thus, in the current work, we presented an SPR-based immunoassay to accurately and rapidly detect trace levels of PSA in commercial food products. In general, this method exhibits excellent specificity and sensitivity, shortens the operating time, and remains competitive with the other technologies.

Materials and Methods

Reagents and Materials

Albumins from porcine, bovine, sheep, rabbit, and egg white, rabbit anti-goat immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate, *N*-hydroxysuccinimide (NHS, 0.1 M), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 M) were purchased from Sigma Chemical Co. (St. Louis, MO). Goat anti-PSA IgG was obtained from Bethyl Laboratories Inc. (Montgomery, TX).

Carboxymethylated dextran modified CM5 sensor chip, glycine-HCl (10 mM, pH 2.1), sodium acetate buffer (10 mM, pH 5.0), and ethanolamine hydrochloride (1 M, pH 8.5) were obtained from GE Healthcare (Pittsburgh, PA). Albumin (Pig) ELISA Kit was produced by Abnova (Walnut, CA). 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solutions was purchased from Promega Corp. (Madison, WI). Commercial food products including pork floss, fermented ham slices, Jing Hua ham, salami, air-dried sausage, and raw pork were acquired from a local supermarket in Nanjing, China. All the solutions were prepared in deionized water (18.2 MΩ·cm, an arium® pro ultrapure water purification system from Sartorius, Göttingen, Germany). All solutions used for analysis were filtered by passing through 0.22 µm filter membrane and degassed with vacuum or ultrasonic treatment.

Crude PSA Extraction

Crude PSA extracts obtained from different commercial food products were obtained by the extraction method described by Kim et al. (28), and only minor modifications to this method were taken. Five grams of commercial food products were cut and homogenized at 10 000 rpm for 1 min in 100 mL 10 mM phosphate-buffered saline (PBS, pH 7.3). Homogenates were centrifuged at 16 000 × *g* for 30 min. Finally, the supernatant was stored at −20°C for further use.

Competitive Indirect (Ci)-ELISA

The ELISA test was based on a competitive indirect configuration. A 96-well microtiter plate was covered with 100 µL of 10 µg/mL PSA solution in a 0.2 M bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were obstructed with 1% gelatin in 10 mM PBS at 37°C for 2 h. We added 50 µL PSA solution or other albumins and 50 µL diluted goat anti-PSA IgG (1:500) to each well, followed by incubation at 37°C for 2 h. We then added 100 µL diluted rabbit anti-goat IgG HRP conjugate (1:4 000) to the wells, followed by incubation at 37°C for 2 h. We added 150 µL TMB to the wells, followed by incubation at 37°C for 10 min. The reaction was halted with 50 µL 2 M H_2SO_4. The plates were washed three times with 10 mM PBS containing 0.1% (v/v) Tween 20 following every step of the incubation process. The optical density (OD) at 450 nm was then read on an iMark microplate reader (Bio-Rad, Hercules, CA).

Standard Curve of Ci-ELISA

The well was coated with 10 µg/mL PSA solution in a 0.2 M bicarbonate buffer (pH 9.6) at 4°C overnight. The plates were then blocked with 1% gelatin in 10 mM PBS at 37°C for 2 h. Ten levels of serial dilution containing 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL PSA were prepared in PBS. Then, 50 µL of 0.195–100 µg/mL PSA solution and 50 µL diluted goat anti-PSA IgG (1:500) in 10 mM PBS were added to the well. The following steps and conditions for the experiments were the same as the method of the developed Ci-ELISA described.

Analysis of Antibody Specificity

Specificity of the antibody was determined by the developed Ci-ELISA described. We added 50 µL of each primary antibody and 10 mM PBS into each well for 100% binding (C_{max}) among the covered PSA solution and goat anti-PSA IgG without competitive antigens. We used 100 µL of 10 mM PBS as the blank. Every sample was completed in triplicate. The IgG-binding capabilities were established with the equation:

$$\text{IgG-binding capabilities (\%)} = \frac{\text{OD of } C_{max} - \text{OD of Sample}}{\text{OD of } C_{max} - \text{OD of PSA}} \times 100\% \quad (1)$$

SPR-Based Immunoassay for PSA detection

All experiments were performed at 25°C using a Biacore™ T200 Biosensor (GE Healthcare, Uppsala, Sweden). Prior to use, goat anti-PSA IgG was immobilized using amine-coupling chemistry on the surface of a CM5 sensor chip. Briefly, surface was activated with a 1:1 (v/v) ratio of a mixture of EDC and NHS. Goat anti-PSA IgG (100 µg/mL in 10 mM sodium acetate buffer, pH 5.0) was then flowed for 7 min at a rate of 10 µL/min, and the remaining activated groups were blocked with a 50 µL injection of 1 M ethanolamine hydrochloride (pH 8.0). The chips were then rinsed with 10 mM PBS containing 0.05% (v/v) Tween 20 to remove excess antibodies adsorbed on the chip. After blocking, PSA standard or food samples were injected for 5 min, at a flow rate of 10 µL/min, over the sensing surface. For immobilizations, the running buffer was 10 mM PBS containing 0.05% (v/v) Tween 20. The background resulting from the injection of running buffer alone was subtracted from the data before plotting. After the antibody-antigen binding step, the sensor surface was regenerated by short pulses of 10 mM Glycine-HCl (pH 1.5; 30 s at a flow rate of 30 µL/min), to allow us to repeat analysis on the same chip.

Sensitivity and Standard Curve of SPR

To verify the sensitivity of the biosensor chips for detecting PSA, seven levels of serial dilution containing 1, 5, 25, 100, 150, 300, and 450 ng/mL PSA were prepared in 10mM PBS. The concentration gradient for PSA was analyzed using

the biosensor chips prepared as described above. These measurements were obtained with three replicates of gradient detection. The concentration gradient of PSA representing the X-axis and the response variables (acquired by SPR) representing the Y-axis were used to calculate the standard curve. The LOD was calculated using the following formula: LOD = 3.3 s/m, where "s" represents the standard deviation of the Y-axis intercept, and "m" represents the linear slope (29).

Reproducibility of SPR

To determine the reproducibility of SPR, four levels of serial dilution containing 60, 120, 180, and 240 ng/mL PSA were prepared in 10 mM PBS. These PSA solutions were measured using SPR 5 times per day at 2-h intervals to determine the within-day RSD, and for 5 consecutive days to determine the between-day RSD.

Detection of Commercial Samples

To ensure the accuracy of our analysis, six food samples of different product groups were used to detect PSA. PSA from commercial samples were extracted by previously described methods. The extracts were then diluted with PBS to the range of the standard curve for immediate analysis or stored at −20°C for further use. Albumin (Pig) ELISA Kit was used for the control method. The allergen extraction, detection process, and analysis were executed with three replicates according to the manufacturer's instructions in ELISA Kit.

Statistical Analysis

Data were statistically examined by one-way analysis of variance with Microcal Origin 8.0 (Microcal Software, Inc., Northampton, MA). Statistical differences with P values <0.05 were considered to be significant. Data were presented as mean ± SD of three independent experiments.

Results and Discussion

Construction of Ci-ELISA

The standard curve of Ci-ELISA was obtained using 10 µg/mL antigen and 2 µg/mL goat anti-PSA IgG. The results showed that the regression equation of Ci-ELISA standard curve was Y = 2.22342 − 0.7491X (R^2 = 0.99369). And the PSA solution to goat anti-PSA IgG was quantitatively determined in the range from 0.78 to 100 µg/mL (Figure 1).

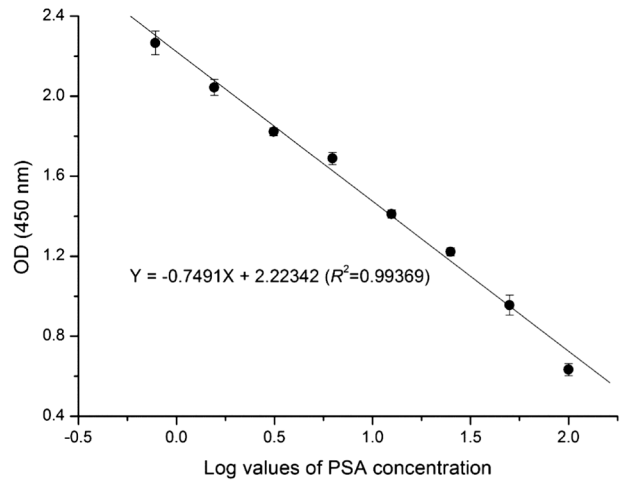

Figure 1. Standard curves of goat anti-PSA IgG to PSA by Ci-ELISA (eight levels of serial dilution containing 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL PSA were prepared in PBS). X-axis is Log values of PSA concentration; Y-axis is absorbance at 450 nm.

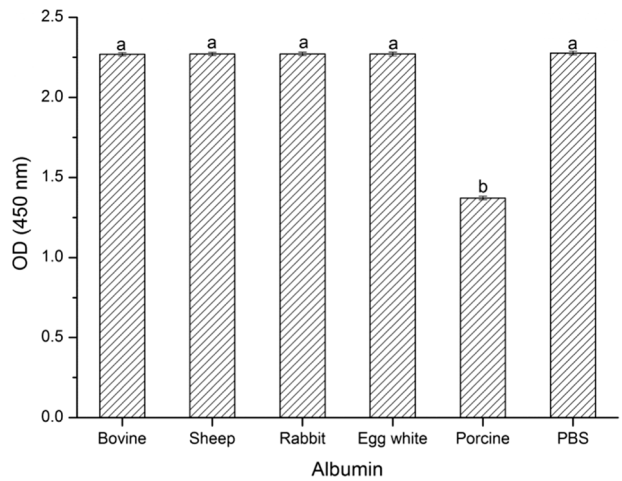

Figure 2. Ci-ELISA results from various albumins. Sample results expressed as absorbance at 450 nm. (a, b) Means in the bars bearing different letters are significantly different at P < 0.05.

Specificity Confirmation

Serum albumin from porcine, bovine, sheep, and rabbit and albumin in chicken egg sources (12 µg/mL in PBS) were tested using the developed Ci-ELISA for antibody specificity studies. As shown in Figure 2, the OD values of bovine, sheep, rabbit, and egg white sources of serum albumins were slightly lower than that of PBS (P > 0.05) and significantly higher than that of PSA (P < 0.05). According to the above results, the IgG-binding capabilities of bovine, sheep, rabbit, and egg white were measured at 0.86% ± 0.04, 0.70% ± 0.03, 0.66% ± 0.13, and 0.72% ± 0.21, respectively. These results indicated that the goat anti-PSA IgG exhibited the lowest cross-reactivity with other albumins except PSA in our study. We speculated that the differences found in amino acid sequences between the different serum albumins could be important for their susceptibility to the antibody recognition.

Preparation of SPR Biosensor

Preparation of the biosensor should fulfill the following conditions (Figure 3): (*1*) running the sensorgram and wait until the baseline becomes stable, (*2*) mixing the EDC and NHS and immediately injecting the mixture to activate the surface of the CM5 chip, (*3*) washing the chip with PBS with Tween 20 (PBST) buffer, (*4*) injecting the antibodies until the immobilization level reaches the target value, (*5*) washing the chip with PBST buffer, (*6*) injecting ethanolamine hydrochloride to block the excess reactive surface, and (*7*) washing the chip with PBST

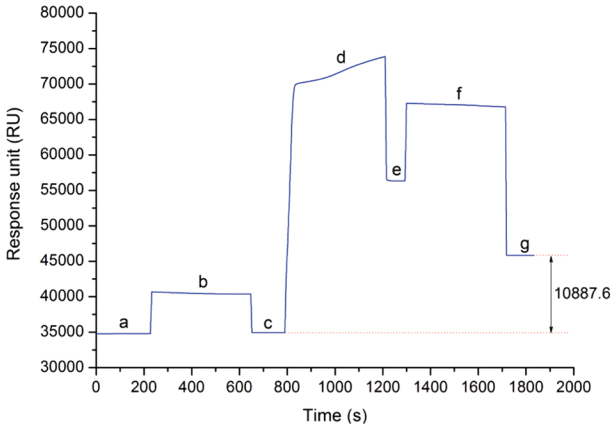

Figure 3. Description of the reactions taking place at the surface of the CM5 sensor chip. Data are expressed as RU against time.

buffer. Under the conditions above, goat anti-PSA IgG had been coupled at the high immobilization level of 10887.6 RU, where 1 RU corresponds to 1 pg protein accumulated per mm^2 (30). This indicated that SPR responses could exhibit good sensibility and accuracy ratio. Therefore, this technique should be sufficient to satisfy requirements for practical detection of PSA.

Sensitivity and Standard Curve

Seven concentrations of the PSA standard sample in serially diluted samples were used to confirm the standard curve and LOD of SPR. The results showed that as the concentration of PSA increased, the RU value was also found to increase (Figure 4). Under optimal experimental conditions, a linear range of 1.0–450.0 ng/mL (R^2 = 0.998; Figure 5) and LOD of 19.81 ng/mL was calculated for the detection of PSA in 10 mM PBS. Although the threshold for allergic reaction to food varies from person to person, trace amounts of allergens may cause serious reactions. Reactions with up to 14.1 g pork powder have been reported in a double-blind, placebo-controlled food challenge study (31). Therefore, the sensitivity of SPR in our study could be sufficient to satisfy requirements for practical application.

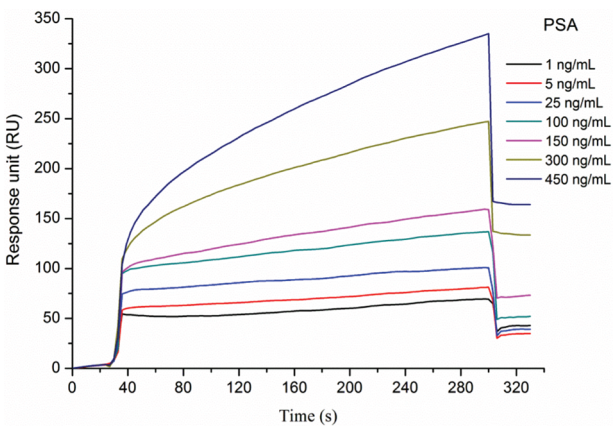

Figure 4. SPR-based immunoassay for PSA detection. Seven different concentrations of PSA (1.0–450.0 ng/mL) were injected for 5 min at a flow rate of 10 µL/min, over the immobilized goat anti-PSA IgG. The running buffer was PBST, pH 7.3. Data are expressed as RU against time.

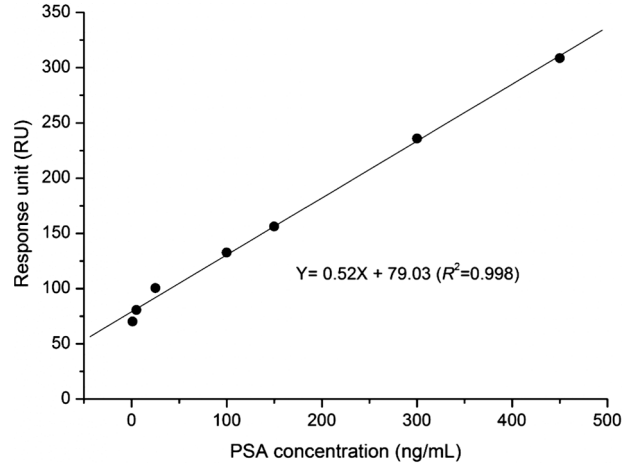

Figure 5. Standard curves obtained PSA concentrations against the SPR signal (seven levels of serial dilution containing 1, 5, 25, 100, 150, 300, and 450 ng/mL PSA were prepared in PBS). Data are expressed as RU against PSA concentration.

Reproducibility of SPR

Four concentration gradients of the PSA standard sample measured in serial diluted samples were applied to the assessment of the reproducibility (Tables 1 and 2). The results showed that both within-day RSD (1.97–4.02%) and between-day RSD (1.88–4.15%) were lower than 5%, which indicates that SPR method for the PSA detection has good reproducibility, reliability, and stability.

Detection of PSA in Commercial Samples

To examine the applicability and accuracy of the established SPR, we selected six different commercial samples as detected objects and compared the results obtained using SPR and ELISA methods (Table 3). The results of this analysis indicated that the ELISA test kits were used with the results shown to be almost consistent with those of SPR detection, and the results of the two methods had no significant difference ($P > 0.05$). As also described in Table 3, the contents of PSA in samples from different types of food processing had significant difference ($P < 0.05$). From the above results, we could infer that food processing treatments caused significant changes in the protein conformation of PSA, masking or destroying the antigenic epitopes. These changes may be important means of reducing the antigenicity of the allergen. Our studies indicate that the SPR assay described here showed good sensitivity and selectivity.

Table 1. Within-day reproducibility of the SPR-based method for PSA detection

PSA concentration, ng/mL	Times						
	1	2	3	4	5	Mean value	RSD, %
60	58.22	63.24	59.33	61.71	60.23	60.55	1.97
120	116.76	119.76	125.64	120.24	118.68	120.22	3.31
180	175.73	177.93	179.14	184.45	182.12	179.87	3.45
240	236.15	234.61	238.38	242.6	243.89	239.13	4.02

Table 2. Between-day reproducibility of the SPR-based method for PSA detection

PSA concentration, ng/mL	Time, day						
	1	2	3	4	5	Mean value	RSD, %
60	58.82	56.71	61.75	60.41	59.40	59.42	1.88
120	117.48	114.86	125.35	118.25	122.16	119.62	4.13
180	175.66	177.70	179.55	184.32	183.35	180.12	3.68
240	235.87	235.08	241.98	239.84	244.96	239.55	4.15

Table 3. Comparison of the results obtained by SPR and ELISA for the determination of PSA in commercial food products[a]

Product	SPR	ELISA
	PSA concentration, μg/mL	
Pork floss	8.43 ± 0.05^{Af}	8.53 ± 0.11^{Af}
Fermented ham slices	528.74 ± 2.64^{Ad}	530.15 ± 2.51^{Ad}
Jinhua ham	694.08 ± 2.02^{Ac}	697.38 ± 6.57^{Ac}
Salami	886.49 ± 2.72^{Ab}	881.73 ± 8.26^{Ab}
Air-dried sausage	987.51 ± 1.54^{Aa}	984.62 ± 5.03^{Aa}
Raw pork	449.39 ± 0.76^{Ae}	451.02 ± 5.33^{Ae}

[a] All data are shown as the means ± SD (n = 3). Different capital letters in the same row indicate significant differences between values (P < 0.05); different small letters in the same column indicate significant differences between values (P < 0.05).

It is, however, still dependent on the availability of the proper antibodies, but this is also the case for the commercially available ELISA assays. Nevertheless, in comparison with ELISA, SPR is a lot faster and less laborious.

Conclusions

The prevalence of food allergies has increased dramatically during the past few decades. Food allergies have been a growing food safety and public health concern. Therefore, food allergen detection has become an imperative task for helping allergic patients avoid contact with allergens. Nowadays, SPR is a good alternative method to measure food allergens. However, few publications have appeared in this field to date (32, 33). In the current study, a label-free biosensor based on SPR has been developed and validated to identify and measure levels of PSA present in pork and products. Further, this technique may be suitable for the qualitative and quantitative assessment of different ingredients in foods and for food allergen labeling.

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