

Expression of the soybean allergenic protein P34 in *Escherichia coli* and its indirect ELISA detection method

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Abstract To detect the soybean allergen P34 (*Gly m* Bd 30K) from soybean products, the full-length cDNA sequence of P34 was synthesized and inserted into the prokaryotic expression vector pET-28a. The P34 protein was expressed in *Escherichia coli* BL21 (DE3) as an inclusion body under the induction of 0.8 mmol/L isopropyl β -D-1-thiogalactopyranoside. After purification with His-Bind affinity chromatography, the purity quotient of the recombinant protein was over 92 %, and its molecular weight (approximately 33 kDa) was very close to that of the native soybean P34. The polyclonal antibody (pAB) against P34 was prepared with the purified recombinant P34. The generated pAB, named as pAB-P34, exhibited high specificity to the P34 protein of the soybean meal. The indirect enzyme-linked immunosorbent assay (iELISA) based on pAB-P34 was established to determine the P34 content of soybean products. The CVs of the recovery tests of P34 were less than 7.77 %, which indicated that iELISA had high reproducibility and accuracy. Therefore, the recombinant P34 produced in the *E. coli* expression system, the prepared pAB-P34, and the developed iELISA could provide a valuable tool for sensitive detection of P34 in

various soybean products and for future studies on allergies related to soybean P34.

Keywords Soybean allergen · P34/*Gly m* Bd 30K · Prokaryotic expression · pAB · Indirect ELISA

Introduction

Soybean (*Glycine max*) is widely utilized in diets fed to humans and animals because of its high levels of protein and well-balanced amino acid profile (Friedman and Brandon 2001). However, soybean is also one of the “the big eight” foods, identified as the most common sources of human food allergens, accounting for over 90 % of the documented food allergies worldwide (van Bostel et al. 2008; Batista et al. 2007; Hoffmann-Sommergruber et al. 2008; Yaklich et al. 1999). Classic immunoglobulin-E (IgE)-mediated food allergies are classified as type-I immediate hypersensitivity reaction and around 1–2 % of adults and up to 5–7 % of children suffer from some type of food allergy with foods (Mills and Breiteneder 2005). Furthermore, food hypersensitivity seems to be on the rise and has become a major public health concern around the world in recent years (Bu et al. 2010; Sathe and Sharma 2009).

So far, 38 soybean allergens have been characterized in the AllergenOnline database (<http://www.allergenonline.org/>) (Batista et al. 2007; L'Hocine and Boye 2007). Among these allergenic proteins, the P34 also known as *Gly m* Bd 30K was recognized as a major allergenic protein in soybean seeds (Batista et al. 2007). IgE binding assays using immunoglobulin from soybean-sensitive individuals indicated that 65 % of the total allergenic response was induced by P34 (Helm et al. 2000; Herman et al. 2003). P34 is a monomeric, insoluble glycoprotein consisting of 258 amino acid residues attached

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by disulfide linkages in the 7S globulin fraction and may play a role in protein folding (Helm et al. 2000). As a thiol protease, P34 is a member of the papain superfamily (Sewekow et al. 2008) and has a tendency to bind to lipid and oil, which is likely significant in its role as an allergen (Mills et al. 2004). P34 exhibits post-translational production derived from a 46- to 47-kDa precursor protein by the partial removal of 122 N-terminal amino acid residues, and its tertiary conformation was similar to the papain family (Ogawa et al. 2000).

To date, the studies on P34 have focused on their structural and functional properties including their HPLC assay and immunological detection by means of monoclonal antibody based on epitope predication, and in the meanwhile, the change and diversity of P34 structure frequently occurring in processing was seldomly considered and tracked when these assays were tried to develop (You et al. 2008; Wilson et al. 2008; Amnuaycheewa and de Mejia 2010; Egounlety and Aworh 2003; Hotz and Gibson 2007; van Boekel et al. 2010). The detection of allergens in food products is very difficult as allergens are often present in trace amounts and can be masked by the food matrix (Poms et al. 2004). Although the quantification of soybean allergens can be carried out by high-performance liquid chromatography and immunohistochemical techniques, these methods require time-consuming cleanup steps and expensive equipment (Mujoo et al. 2003; Pedersen et al. 2008; L'Hocine and Boye 2007). Immunoassay methods have been widely applied in many fields including detection for food allergen because they involve the use of relatively inexpensive equipment and are suitable for high-throughput screening (Asensio et al. 2008). The indirect enzyme-linked immunosorbent assay (iELISA) had been developed to quantify P34 in various commercial soy ingredients and products based on the existing monoclonal α -P34 antibody (Tsuji et al. 1995; Wilson et al. 2008). But some problems exist in the above typical immunoassay approach based on the monoclonal antibody: on the one hand, the preparation of pure P34 from raw soybean and highly sensitive monoclonal antibody against P34 from animal immunization are critical and crucial; in fact, besides clinical diagnosis in hospital, the detection assay of soy allergens including P34 with merits such as more simple, quick, efficient, or high-throughput screening is more urgently required in many fields such as food and feed processing and some regions where people are often insensitive to soy allergens such as Asia. Moreover, on the other hand, the immunoassay assay based on monoclonal antibody is limited on a single epitope and shows probably no effect when this epitope was destroyed during the process. On the contrary, the assay based on polyclonal antibody could cover multiple epitopes and showed a lower detection limit (Pedersen et al. 2008). So far, there is no iELISA method to detect P34 using polyclonal antibody pAB against recombinant P34 fusion protein. In this study, the P34 protein was expressed in *E. coli*, pAB was

prepared, and then an iELISA for the detection of P34 using pAB was established and evaluated.

Materials and methods

Preparation of the substances

Soybean seed (SBS), the soybean meal (SBM), and fermentation soybean meal (FSBM) were ground into powder with an FW100 Series of High-Speed Universal Grinder (Beijing Zhongxingweiye Instrument Co., Ltd.) and passed through a 60-mesh sieve. Each gram of powder above was added to 15 mL of 0.01 mol/L PBS (8.0 g NaCl, 0.2 g KH_2PO_4 , 2.13 g Na_2HPO_4 , and 0.2 g KCl per liter, pH 7.4) and kept overnight at 4 °C. After centrifugation at 13,500×g for 15 min at 4 °C, the supernatants were stored at −20 °C until analysis.

Construction of the pET28a-30K plasmid

The 771-bp cDNA sequence of P34 mature peptide (GenBank: EU883600.1) was optimized for expression in *E. coli* and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The full length of the P34 cDNA was amplified by PCR with forward primer 30K-F, 5'-GGAATTCATATG AAGAAAATGAAGAAGGAA-3' (*Nde* I site underlined) and reverse primer 30K-R, 5'-CGGAATTC AAGAGGAGAGTGATCAAC-3' (*EcoR* I site underlined). The PCR products were digested with *Nde* I and *EcoR* I and inserted into similarly digested pET-28a to generate plasmid pET28a-30K.

Expression of P34 in *E. coli*

The plasmid pET28a-30K was transformed into *E. coli* BL21(DE3) and was identified by restriction enzymes *Nde* I and *EcoR* I. The transformant cells were cultured in 5 mL of Luria–Bertani medium containing 50 $\mu\text{g/mL}$ kanamycin (Kan), with shaking at 37 °C overnight. The preculture (5 mL) was then transferred into 500 mL fresh Terrific Broth medium containing 50 $\mu\text{g/mL}$ Kan and grown with shaking at 37 °C until OD_{600} was 0.5–0.8, at which time protein expression was induced by adding 0.8 mM isopropyl β -D-1-thiogalactopyranoside (IPTG).

Purification of recombinant P34 protein

After 6 h of induction, the cultured cells were harvested by centrifugation at 5,000 rpm for 30 min at 4 °C and resuspended in 50 mL of lysis buffer (0.5 mol/L NaCl, 20 mmol/L Tris–HCl, 8 mol/L urea, 1 mmol/L PMSF, pH 7.9). The suspended cells were sonicated for 20 min with an Ultrasonic Crasher Noise Isolating Chamber (SCIENTZ,

Ningbo Science Biotechnology Co. Ltd, China) on ice. The suspension was centrifuged at 12,000 rpm for 10 min to separate the unlysed cells from the lysate, and the supernatant fraction was analyzed by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R₂₅₀.

The supernatant of the lysate was loaded onto a His-Bind Resin column (Novagen, USA), pre-equilibrated with the lysis buffer. The loaded column was extensively washed with washing buffer (50 mmol/L Na₂HPO₄, pH 8.0, 300 mmol/L NaCl, and 6 mol/L urea) containing 10, 20, 40, 60, 80, 120, and 200 mmol/L imidazole, respectively. The washing buffers were tested by 12 % SDS-PAGE. The purified recombinant P34 proteins were lyophilized at a freeze dryer (ALPHA 1-2 LD plus, Christ, Germany).

Preparation of the polyclonal antibody

The recombinant P34 lyophilized powder was dissolved in 0.01 mol/L PBS (pH 7.4) to 1 mg/mL. Two New Zealand white rabbits (named A and B), weighing 2 kg (Beijing CoWin Biotech Co., Ltd.), were immunized with the recombinant P34 solution emulsified with complete or incomplete Freund's adjuvant of the same volume by three subcutaneous injections at multiple subcutaneous sites. The first injection consisted of 600 µg (0.6 mL) of recombinant P34 emulsified with complete Freund's adjuvant (0.6 mL) (PIERCE Biotechnology, USA). Subsequent two injections consisted of 400 µg (0.4 mL) of recombinant P34 emulsified with incomplete Freund's adjuvant (0.4 mL; PIERCE Biotechnology, USA) were given every 2 weeks. The rabbits were then bled by cardiac puncture 10 days after the final injection, and the blood was clotted and retracted on ice overnight. Then, serum was isolated by centrifugation at 3,000 rpm for 30 min using refrigerated centrifuge (HC-3018R, ANHUI USTC ZONKIA SCIENTIFIC INSTRUMENTS CO., LTD, China). The serum antibody titers were determined using iELISA and the recombinant P34 protein. A higher titer serum was named pAB-P34.

SDS-PAGE and Western blotting analysis

In order to assess the specificity of the pAB-P34 to P34, the Western blot technique, as described below, was performed. The recombinant P34 and total proteins of SBM were dissolved in 0.01 M PBS, respectively; the protein concentration of the samples was measured using Bradford Protein Assay kit (TIANGEN BIOTECH Co., Ltd, Beijing, China) and bovine serum albumin (BSA) as the standard. Then, samples (15 µg/well) were subjected to SDS-PAGE using a minigel apparatus (BioRad Laboratories, Hertfordshire, UK) with 12 % acrylamide separating gel and 5 % acrylamide stacking gel according to the method of You et al. (2008).

The separated protein was transferred electrophoretically from gel to a 0.45-µm polyvinylidene fluoride microporous membrane (Millipore Corporation, USA) in a semi-dry electrophoretic transfer cell (BioRad, USA). Semi-dry blotting was performed at 12 V for 40 min. The membrane was blocked in Tris-buffered saline (TBS-T) (10 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) containing Tween-20 (0.1 %) and 5 % BSA overnight at 4 °C. The membrane was rinsed again four times for 10 min each in TBS-T and incubated with pAB-P34 diluted 8,000-fold with TBS-T and 1 % BSA for 2 h at room temperature (RT). After washing with TBS-T, the membrane was probed with the secondary antibody (goat anti-rabbit IgG (H+L)-AP, Beijing CoWin Biotech Co., Ltd.) diluted 5,000-fold for 30 min at RT. After washing with TBS-T four times, bound antibodies were detected with the BCIP/NBT Chromogenic Substrate Kit (TIANGEN BIOTECH Co., Ltd.).

iELISA protocol

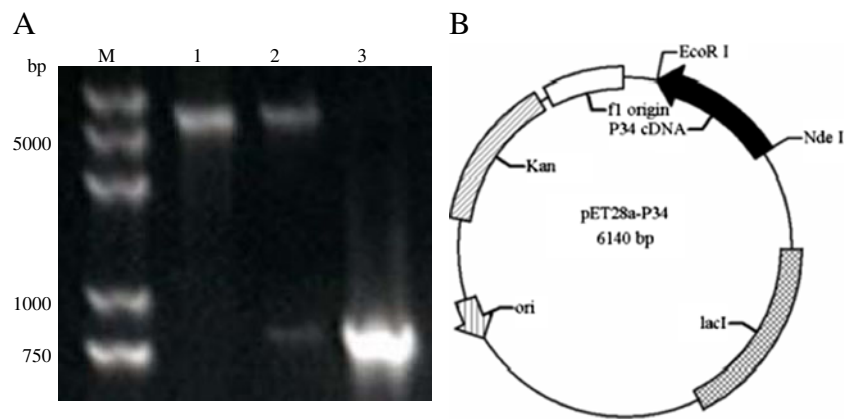
All of the assay parameters were optimized and standardized. The coating antigen was serially diluted from 5 to 80 ng/mL, and the pAB-P34 was diluted threefold from 1/1,000 to 729,000.

One hundred microliters of recombinant P34 or extracts of soybean samples in 0.05 M carbonate buffer (pH 9.6) were incubated overnight at 4 °C in 96-well microtiter plates (Nunc-Immuno Plate MaxiSorp, Denmark). After washing three times with 300 µL/well of 0.01 M PBST (8.0 g NaCl, 0.2 g KH₂PO₄, 2.13 g Na₂HPO₄, 0.2 g KCl/L, 0.05 % Tween-20, pH 7.4), the plate was blocked for 2 h at 37 °C by adding 200 µL per well of 0.01 M PBST containing 2 % BSA. The plate was washed three times again. Next, 100 µL of pAB of soybean P34 (diluted with 1 % BSA in 0.01 M PBST) was added to the wells and incubated for 1.5 h at 37 °C. After washing the plate three times, 100 µL goat anti-rabbit-HRP IgG (H+L)-HRP was diluted 1:2,000 in 0.01 M PBST containing 1 % BSA and added to each well. The plates were incubated for 1.5 h at 37 °C and then washed three times. Finally, 100 µL of TMB diluted in substrate buffer was added to each well and incubated in the dark at RT for 20 min. The color reaction was stopped by adding 50 µL of 2 M H₂SO₄ to each well. The absorbance of each well at 450 nm was determined by an automatic ELISA plate reader. Positive and negative serum controls were included in each plate (Beijing PuLang New Technology Ltd., Beijing, China).

Establishment of iELISA standard curve

A standard curve is constructed by plotting the absorbance obtained from each reference standard against its concentration in nanograms per milliliter.

Fig. 1 Schematic diagram of the pET28a-P34 plasmid and digestion of the restriction enzymes. **a** Digestion of the restriction enzymes. **b** Schematic diagram of the pET28a-P34 plasmid. 1 pET-28a plasmid; 2 pET28a-P34 plasmid/*Nde* I, *EcoR* I; 3 P34 gene; M DNA marker



Validation and repeatability of the assay

The repeatability of the experiment was assessed by assaying eight replicates of the P34 samples at 20, 40, 60, and 100 ng/mL, respectively. The recovery and coefficient of variation (CV) were calculated by measuring eight replicates each of four P34 samples. Eight replicates were determined for each sample.

Detection of the contents of P34 in soybean products

Protein solutions from SS, SM, and FSM were diluted in assay buffer to their proper concentrations before assessment. The P34 content from these soybean products was analyzed by the developed iELISA based on recombinant P34 and pAB-P34.

Results

Construction of the recombinant plasmid pET28a-30K

As described in the “Materials and methods,” the recombinant plasmid pET28a-P34 was successfully constructed and identified by PCR method and restriction enzyme digestion (Fig. 1). Nucleotide sequencing confirmed that the construct was in the correct reading frame.

Expression and purification of P34

The expression of recombinant P34 protein in *E. coli* BL21 (DE3) was induced for 6 h by adding 0.8 mM IPTG at 37 °C. In order to analyze the expression and the solubility of the recombinant proteins, lysate and soluble and insoluble fractions were examined by SDS-PAGE. As shown in Fig. 2, most of the expressed P34 was present in the insoluble bodies (lane 4) leaving only a minute fraction in the soluble form (lane 3). The inclusion bodies could be soluble in 6 M urea. The

molecular weight of the recombinant P34 was approximately 33 kDa, which is the expected size of the fusion protein.

The purification of this recombinant protein P34 was achieved by a His-Bind Resin column (lane 1; Fig. 3a). The purified P34 was of more than 92 % without other bands and met the requirement for the next preparation of polyclonal antibody pAB-P34.

Preparation of pAB-P34 and Western blotting analysis

The sera titers of two rabbits were detected by iELISA using P34 as a coating antigen. When the sera titer of rabbit A was

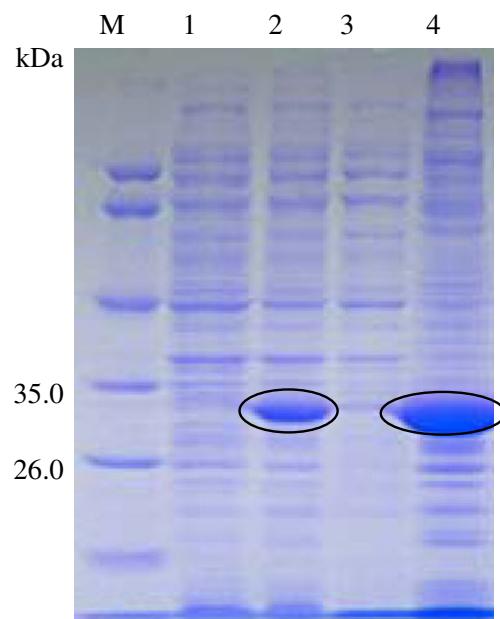


Fig. 2 SDS-PAGE analysis of the expression pattern of the recombinant P34 protein. M standard protein marker; 1 the lysates of pET28a-P34/BL21(DE3) (non-induced); 2 the lysates of pET28a-P34/BL21 (DE3) (induced); 3 the soluble fractions of pET28a-P34/BL21(DE3) (induced); 4 the insoluble fractions of pET28a-P34/BL21(DE3) (induced)

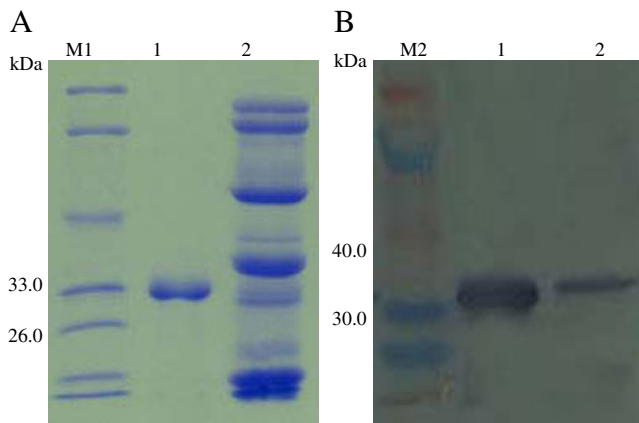
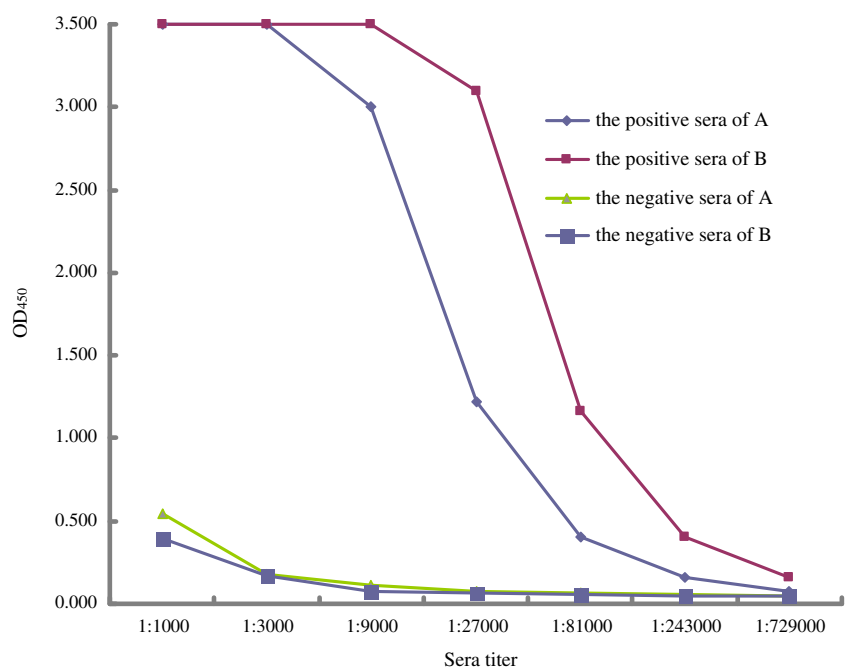


Fig. 3 SDS-PAGE and Western blotting analysis of the recombinant P34 protein powder and extruded protein of SBM. **a** SDS-PAGE; **b** Western blotting. *M1* standard protein marker, *M2* prestained protein molecular weight marker, *1* purified recombinant P34 protein, *2* extruded proteins of SBM

lower than 81,000, its ratio of P/N was higher than 2.1 and the *P* value was larger than 0.1. When the sera titer of rabbit B was lower than 729,000, its ratio of P/N was higher than 2.1 and the *P* value was larger than 0.1 (Fig. 4). Thus, the sera of rabbit B was chosen and named as pAB-P34.

Western blot analysis indicated that pAB-P34 could specifically recognize the purified recombinant P34 and extracted proteins from SBM with a specific signal at 33 kDa (Fig. 3) but could not recognize any other SBM proteins, indicating that the recombinant P34 protein induced a specific immunological response, and thus, the prepared pAB-P34 antibody had a high level of specificity.

Fig. 4 Sera titers of the rabbits



iELISA

When pAB-P34 samples were serially diluted 9,000, 27,000, 81,000, and 729,000 times, the OD₄₅₀ values correspondingly increased with the content of recombinant P34. A linear relationship between the P34 content and OD₄₅₀ values was observed at 9,000 dilution times of pAB-P34 (Fig. 5). The iELISA was optimized on the basis of 40 ng/mL of antigen and 9,000 dilution times of pAB-P34.

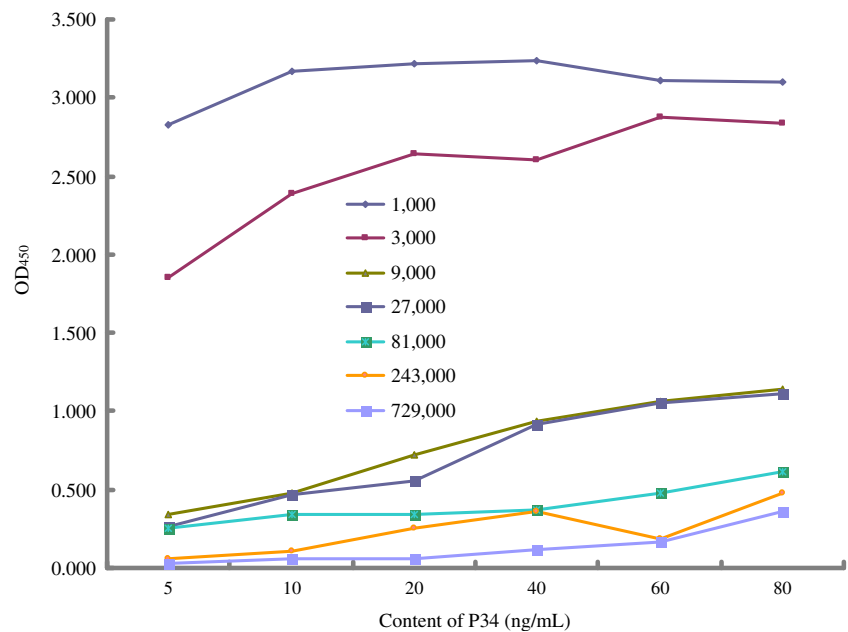
A calibration curve for the iELISA was established based on the optimal concentrations of P34 and pAB-P34. The linear formula of the method was $y=0.034x+0.0838$ ($R^2=0.9831$; Fig. 6).

In order to evaluate the validation and reliability of the recombinant P34 and pAB-P34 for iELISA, recovery tests were performed using 20, 40, 60, and 100 ng/mL recombinant P34. As shown in Table 1, the recoveries at the four spiked levels were from 100.44 to 112.42 %, and the CVs were in the range from 5.95 to 7.77 %. Obviously, both recoveries and CVs of the iELISA method were within acceptable ranges, indicating that the iELISA based on P34 and pAB-P34 was sensitive, reliable, and reproducible.

Quantification detection of P34 in soybean products

The developed iELISA assay was used to detect the P34 content in SBS, SBM, and FSBM. As shown in Table 2, the immunoreactive P34 in unprocessed SBS had a higher content at 4.62 µg/mg when compared with 3.91 µg/mg of the immunoreactive P34 content in SBM.

Fig. 5 Relationship of absorption values with the sera of rabbit B titer and the content of P34



The contents of immunoreactive P34 in fermented soybean meal were significantly reduced with fermentation time. The P34 desensitization efficiency from *Candida utilis* and *Saccharomyces cerevisiae* was higher than that of other microbial strains, and then followed by *Endomycopsis fibuligera*, *Geotrichum candidum*, and *Bacillus subtilis*. The immunoreactive P34 could not be detected when the soybean meals were fermented by *C. utilis*, *S. cerevisiae*, and *E. fibuligera*, respectively, for 72 h, which indicated that the main allergen P34 of SBM could be reduced by fermentation with these microbes.

Discussion

In previous studies, P34 was extracted from soybean oil bodies using hydrophobic interaction chromatography (Sewekow et al. 2008). A subsequent purification, however, is in any case necessary. Moreover, it is incredibly time consuming and depends on expensive equipment. In comparison, prokaryotic recombinant protein expression systems (*E. coli*) have several advantages, including ease of culture, rapid cell growth, and relatively simple purification procedures (Bohle and Vieths 2004; Clavijo et al. 2004; Guo et al. 2008; Lorenz et al. 2001; Utsumi et al. 2002). In addition, immunoblot analysis showed that both soybean and recombinant P34 proteins cross-reacted not only with polyclonal and monoclonal antibodies produced against P34 and crude soybean protein but also with the sera from 361 patients with atopic dermatitis; it means that the recombinant P34 is immunologically reactive, indicating that the recombinant P34 expressed by *E. coli* has similar epitope structures to natural soybean P34 protein and thus can be used as the standard

allergen of P34 (Babiker et al. 2000). In this study, the recombinant P34 (approximately 33 kDa) was expressed in the *E. coli* to replace the extraction of natural P34 used for the detection of allergic protein in P34-ELISA within scope of molecular mass as 30–34 kDa of the native P34 (Wilson et al. 2005; Ogawa et al. 1991; Batista et al. 2007); some minor MW differences between native and recombinant forms might result from the fact that the native P34 in soybean is glycosylated at position Asn₁₇₀, whereas the recombinant protein generated in *E. coli* lacks this post-translational modification (Babiker et al. 2000). In addition, this purified recombinant P34 by nickel affinity chromatography is able to insure the unique component of target protein and the decrease of non-specific binding during immunization (Kleine et al. 1993).

The objective of this work was to develop a rapid and simple assay method for detecting P34 protein in soy based

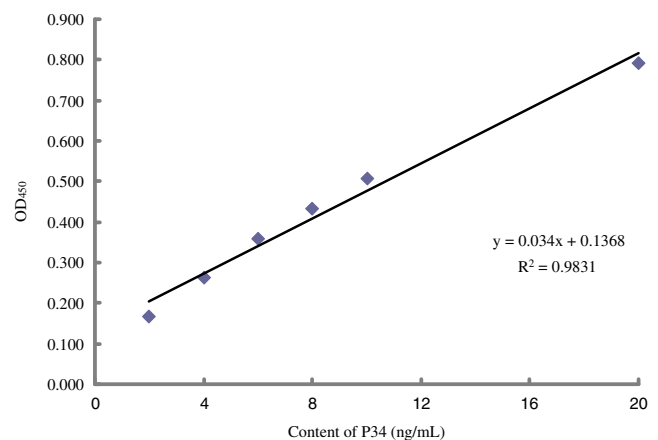


Fig. 6 Standard curve of the iELISA based on pAB-P34 and the recombinant P34 protein for the detection P34 of soybean and soybean products

Table 1 Accuracy analysis of iELISA for P34 detection

P34 (ng/mL)	Mean±SD (OD ₄₅₀) ^a	P34 determined (ng/mL)	Recovery (%)	CV (%)
20	0.736±0.045	20.49	102.45	6.11
40	0.958±0.057	41.57	103.93	5.95
60	1.110±0.072	67.45	112.42	6.49
100	1.235±0.096	100.44	100.44	7.77

^aData represent the means of eight replicate subsamples

on pAB. In previous studies, the sandwich ELISA and cELISA methods aimed at native soy proteins (such as P34, Gly m Bd 28K, β -conglycinin, and glycinin) had been developed (Tsuji et al. 1993; Bando et al. 1998; You et al. 2008; Ma et al. 2010). However, these methods based on monoclonal antibodies need a longer time for the preparation of monoclonal antibody and the higher manufacture cost compared with those assays depending on polyclonal antibody (Pedersen et al. 2008). By comparison, a rabbit pAB against the recombinant P34 protein was easily produced in terms of time and cost special for immunoassays of P34. Western blot analysis revealed that the prepared pAB-P34 antibody reacted strongly with the purified recombinant P34 protein corresponding to the size of the P34 protein in soy (Fig. 3). The result indicated that pAB-P34 was able to specifically identify P34 protein similar to a previous report (Kleine et al. 1993) and thus could be used to develop immunoassay methods for detecting P34.

In the previous studies, using the sandwich ELISA based on two different kinds of monoclonal antibodies, the P34 protein content in SBS was determined as 7.29 $\mu\text{g}/\text{mg}$ (Tsuji et al. 1995). By ELISA, Wilson et al. (2008) determined the P34 protein content in 138 soybean cultivars from a diverse germplasm collection from ten different countries, and the results indicated that P34 protein was present in all strains tested, ranging from 2.3 to 12 $\mu\text{g}/\text{mg}$. Our result showed that the P34 protein content in SBS was 4.62 $\mu\text{g}/\text{mg}$ detected by the developed iELISA based on the pAB-P34 (Table 2), which was compatible with the previous value, and the advantage over pAB-P34 is low cost and shorter time consumed by comparison with the assay based on monoclonal antibody. In previous reports, the contents of P34 in total soybean proteins, which account for 40.0 % of the total weight of the seed (You et al. 2008), were near 1 % (Herman et al. 2003; Wang et al. 2003). These data indicate that the P34 content of the total SBS was about 4.0 $\mu\text{g}/\text{mg}$. Obviously, the assay for P34 detection showed high sensitivity and low cost of equipment from our results, as can be seen from Table 2; the content of the P34 protein in SBS (4.62 $\mu\text{g}/\text{mg}$) was higher than that of SBM (3.91 $\mu\text{g}/\text{mg}$).

As can be seen from Table 2, the immunoreactive P34 contents in soybean products especially in FSBM (i.e., soybean meal and fermented soybean meal by *B. subtilis*, *G. candidum*, *E. fibuligera*, *C. utilis*, and *S. cerevisiae*) were

Table 2 Detection of the P34 in soybean seed and fermented soybean meal by iELISA based on pAB-P34

Samples	Duplicates (n)	P34 content ($\mu\text{g}/\text{mg}$)	SD	CV (%)
Soybean seed ^a	8	4.62	0.371	8.0
Soybean meal ^b	8	3.91	0.501	12.8
Fermented soybean meal by <i>Bacillus subtilis</i> ^c				
24 h	8	1.85	0.212	11.5
48 h	8	0.93	0.111	11.9
72 h	8	0.10	0.006	5.7
Fermented soybean meal by <i>Geotrichum candidum</i> ^d				
24 h	8	1.02	0.125	12.3
48 h	8	0.67	0.077	11.5
72 h	8	0.02	0.002	10.0
Fermented soybean meal by <i>Endomycopsis fibuligera</i> ^e				
24 h	8	0.92	0.115	12.5
48 h	8	0.55	0.065	11.8
72 h	8	—	—	—
Fermented soybean meal by <i>Candida utilis</i> ^f				
24 h	8	0.88	0.079	9.0
48 h	8	0.49	0.052	10.6
72 h	8	—	—	—
Fermented soybean meal by <i>Saccharomyces cerevisiae</i> ^g				
24 h	8	0.83	0.069	8.3
48 h	8	0.52	0.045	8.7
72 h	8	—	—	—

Data represent the means of eight replicate subsamples

— Not detected

^a Zhonghuang 13 soybean seeds, obtained from the Crop Research Institute, Chinese Academy of Agricultural Sciences

^b Soybean meal, obtained from the Sanhe Huifu Foodstuff and Oil, Ltd.

^c Samples of fermented soybean meal prepared with *B. subtilis* in the Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences

^d Samples of fermented soybean meal prepared with *G. candidum* in the Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences

^e Samples of fermented soybean meal prepared with *E. fibuligera* in the Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences

^f Samples of fermented soybean meal prepared with *C. utilis* in the Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences

^g Samples of fermented soybean prepared with *S. cerevisiae* in the Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences

much lower than that in soybean seeds, which was consistent with previous results (Song et al. 2008a, b; Ma et al. 2010; Liu et al. 2012), indicating that the main allergen P34 of SBM could be greatly reduced by fermentation. The fermentation could not only improve the nutritional value of SBM by enhancing the bioavailability of nutrients and reducing contents of the anti-nutritional factors (Teng et al. 2012), but also degrade antigen protein (Sealey et al. 2009; Song et al. 2010; Liu et al. 2012).

What is more important is that the immunoassay assay based on monoclonal antibody can only react with the substance containing the given single epitope and thus shows very likely no response when the structure of this epitope or individual allergen was destroyed or changed during the process (You et al. 2008). In fact, many factors in processing as heating, expanding, and aqueous alcohol extracting could result in the change of the special structure and immunoreactivity of P34 in soybean products (Wilson et al. 2008; Amnuaycheewa and de Mejia 2010; Egounlety and Aworh 2003; Hotz and Gibson 2007; van Boekel et al. 2010), but the assay based on polyclonal antibody could cover multiple epitopes and showed a lower detection limit (Pedersen et al. 2008). Therefore, the iELISA assay from polyclonal antibody pAB-P34 special for P34 detection established in this work is expected to effectively hold the panorama of different allergenic structures of P34 in soybean products that resulted from various processes instead of an individual special structure and thus should be encouraged to be used in quality evaluation and control for food and feed products from soybean.

In conclusion, the recombinant pET28a-P34 plasmid was constructed and transformed to *E. coli* BL21 (DE3). The recombinant P34 protein was expressed in the form of inclusion bodies under the induction of IPTG and was highly purified by means of His-Bind affinity chromatography. The pAB-P34 against soybean allergen P34 was prepared through rabbit immunization by the purified recombinant P34 protein, and it showed high specificity for the P34 protein in SBM and no cross-reactivity with other proteins from SBM. Based on the pAB-P34 and the recombinant P34 protein, the developed iELISA exhibited high reproducibility and accuracy. Therefore, the recombinant P34 and the developed iELISA could provide a valuable tool for the sensitive detection of P34 protein in various soybean products and for future studies on allergies related to soybean P34 protein from soybean products.

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