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Detection of the Soybean Allergenic Protein *Gly m* Bd 28K by an Indirect Enzyme-Linked Immunosorbent Assay

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ABSTRACT: The full-length cDNA sequence of *Gly m* Bd 28K was chemically synthesized and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) as an inclusion body under the induction of 0.2 mmol/L of isopropyl *β*-D-1-thiogalactopyranoside (IPTG). The purity of the recombinant protein was over 90% following Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography, and its molecular weight was 29.71 kDa. The polyclonal antibody (pAB) against *Gly m* Bd 28K was prepared and referred to as pAB-28K, and it exhibited high specificity for the protein in soybean meal. We established an indirect enzyme-linked immunosorbent assay (iELISA) using the pAB-28K and the recombinant *Gly m* Bd 28K protein to determine the *Gly m* Bd 28K content in soybean products. The R^2 value of the standard curve was 0.9910, the average relative standard deviation (RSD) was 16.93%, and the average recovery was 95.50%, which indicated that the iELISA was highly reproducible and accurate. Therefore, the pAB-28K and the iELISA provide valuable tools for the rapid and sensitive detection of *Gly m* Bd 28K in food and feed products from soybeans. This protocol meets the technical requirements for quality control and food safety as related to soybean.

KEYWORDS: *soybean allergen, Gly m Bd 28K, prokaryotic expression, pAB, indirect ELISA*

INTRODUCTION

Although soybeans (*Glycine max*) and soybean meal have been widely consumed by humans and animals as a popular protein source for many years,[1,2] at least 38 soybean allergens have shown the capacity to bind IgE antibodies in the sera of soybean-sensitive patients.[3,4] *Gly m* Bd 28K is one of the predominant soybean allergens and can be recognized by serum antibodies in approximately 25% of soybean-sensitive patients.[5,6] *Gly m* Bd 28K is isolated as a glycoprotein with the glycan moieties from the 7S globulin fraction of soybean proteins, and it represents the $Man_3GlcNAc_2$ backbone containing the $\beta1\rightarrow2$ xylose and $\alpha1\rightarrow3$ fucose branches.[1] The open reading frame of the *Gly m* Bd 28K cDNA is 1419 bp and encodes a pro-protein of 473 amino acids. This protein is highly homologous to the MP27/MP32 proteins, which are found in the protein storage vacuoles of pumpkin seeds, and a globulin-like protein of carrot (50.4% and 45.9% identity, respectively). Its precursor protein contains a 21 amino acid signal peptide that can be converted into two mature proteins, *Gly m* Bd 28K (240 amino acids) and a 23 kDa protein (212 amino acids), during the development of the soybean cotyledons.[6,7]

Approximately 1−2% of adults and up to 5−7% of children suffer from a food allergy.[8] The soybean ranks among the eight most significant food allergens and is the largest source of protein in the world.[9,10] The rapid detection of food allergens is an important topic as food hypersensitivity has become a major public health concern around the world in recent years.[8,11,12] The two common assays used for the detection of soybean allergens include high pressure liquid chromatography (HPLC) and the monoclonal antibody-based assay. The HPLC depends on expensive equipment and highly pure standards,[4,9,13] while the latter, known as the enzyme-linked immunosorbent assay (ELISA), has been applied in the detection of food allergens for years due to its inexpensive equipment and its capacity for high-throughput screening.[14] The sandwich ELISA has now been developed to quantify *Gly m* Bd 28K in various soybean products.[15] However, there are some limitations to the ELISA method. First, it is dependent on the preparation of a highly pure antigen from raw soybeans, and the development of a monoclonal antibody against the soybean antigen by animal immunization is a very tedious and critical process.[16] Second, an alteration of the native soybean structure during processing could disrupt the immunological detection that relies on particular epitopes.[15,17−19] These challenges, particularly the latter, are often ignored, and this could influence the detection results by unintentionally targeting an altered antigen structure. This situation can be explained as follows: not all of the "*n*" types of processing-induced structure impairments can be tested by "1" monoclonal antibody based on epitope predication, as the antibody can only detect "1" epitope. At the same time, it is impossible, and also unnecessary, to prepare "$n - 1$" monoclonal antibodies to screen the subsequent "$n - 1$" derivates of the soybean antigen.[3] The above uncertainty could be further complicated by the facts that the allergen concentrations in nature are constantly in flux, and the surrounding components in soybean products can interfere with the results.[20]

Therefore, an ideal immunoassay should incorporate multiple existing epitopes that are derived from the same antigen. One obvious solution is to use a polyclonal antibody-based

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assay, as this covers multiple epitopes with a lower detection limit.[13,16] In fact, this assay provides more power than one that relies on a monoclonal antibody for a single epitope. The methods that can simply, quickly, and efficiently detect multiple derivatives of the soybean allergen *Gly m* Bd 28K are attractive to clinicians. They are emphasized in the food and feed industries and regions where people are often insensitive to soybean allergens, such as Asia. In addition, an immunoassay based on a monoclonal antibody that is limited to a single epitope is likely to register a pseudonegative result if the epitope is destroyed during processing.[13,16]

Methods for detecting other soy allergens, such as P34, the α subunit of β-conglycinin, β-conglycinin, and glycinin, have been developed on the basis of polyclonal or monoclonal antibodies.[16,21−25] However, there is currently no iELISA method to detect *Gly m* Bd 28K using a polyclonal antibody against the recombinant *Gly m* Bd 28K protein rather than the native protein. In this study, the recombinant *Gly m* Bd 28K protein was expressed in *E. coli*, a pAB was prepared in rabbits, and an iELISA was established and evaluated for the detection of *Gly m* Bd 28K.

■ MATERIALS AND METHODS

Preparation of Substances. Different samples of soybean seed, soybean meal, and fermentation soybean meal were ground into powder with a High-speed Universal Grinder (model FW-100, Yongguangming Medical Treatment Instrument Factory, Beijing, China) and passed through a 60-mesh sieve. One gram of each sample was added to 15 mL of 0.01 M 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. Following centrifugation at 13 500 rpm for 15 min at 4 °C, the protein concentration of the samples was measured using the Bradford Protein Assay kit (Tiangen Biotech Co., Ltd., China) with bovine serum albumin (BSA) as the standard.

100 μL of recombinant *Gly m* Bd 28K or soybean extract samples in 0.05 mM bicarbonate buffer (pH 9.6) was added to the wells of a 96-well microtiter plate (Nunc-Immuno Plate MaxiSorp, Denmark) and incubated overnight at 4 °C.

Construction of the pET28a-28K Plasmid. A 720 bp cDNA sequence of the mature *Gly m* Bd 28K peptide (GenBank AB046874.1) was optimized for expression in *E. coli* and synthesized by Sangon Biotech Co., Ltd. (China). The full-length of the *Gly m* Bd 28K cDNA was amplified by PCR using *EasyPfu* DNA polymerase (TransGen Biotech Co., Ltd., China). The primers containing *Nde*I and *EcoR*I restriction sites were designed according to the cDNA sequence of the mature *Gly m* Bd 28K peptide as follows: the forward primer (28K-F), 5′-GGAATTCCATATGTTCCATGATGATGAGGGTG-3′ (*Nde*I site underlined), and the reverse primer (28K-R), 5′-CGGAATTCTCAATTCACCTTCCCAAATACGG-3′ (*EcoR*I site underlined). The reaction mixture (50 μL) consisted of *Gly m* Bd 28K cDNA (1 ng), primer 28K-F (10 pM), primer 28K-R (10 pM), 10× *EasyPfu* buffer (5 μL), dNTP mixture (4 μL), *EasyPfu* DNA polymerase (2.5 U), and sterile water (37 μL). The mixtures were then subjected to PCR using the Techne TC-512 Gradient Thermal Cycler (Duxford, Cambridge, UK). The PCR products were electrophoresed on a 1% agarose gel. The target DNA band was purified and digested with *Nde*I and *EcoR*I and ligated into a similarly digested pET-28a vector to generate a recombinant pET28a-28K plasmid, and its expected expression product contains one amino acid segment of MGSSHHHHHHSSGLVPRGSHM from pET-28a; the theoretical molecular weight of the recombiant target protein was 29.71 kDa. The plasmid was then transformed into *E. coli* BL21 (DE3). The *Gly m* Bd 28K cDNA sequence on the resulting plasmid was examined using the T7 promoter and the T7 terminator primers (Sangon Biotech Co., Ltd., China).

Expression of *Gly m* Bd 28K in *E. coli*. *E. coli* BL21 (DE3) carrying the pET28a-28K plasmid was cultured with shaking overnight at 37 °C in 5 mL of Luria−Bertani (LB) medium containing 50 μg/mL of kanamycin (Kan). The preculture (5 mL) was inoculated into 500 mL of fresh Terrific Broth (TB) medium containing 50 μg/mL Kan and was cultured with shaking at 37 °C until the OD_{600} was 0.5−0.8, at which time protein expression was induced by adding 0.2 mM IPTG. After 6 h of culture at 28 °C, the cells were harvested by centrifugation at 5000 rpm for 30 min at 4 °C.

Purification of the Recombinant *Gly m* Bd 28K Protein. The cells were suspended in 50 mL of 0.01 M PBS (pH 7.4). The cell suspension was sonicated on ice for 20 min with an Ultrasonic Crasher Noise Isolating Chamber (SCIENTZ, Ningbo Science Biotechnol Co. LTD., China). It was then centrifuged at 12 000 rpm for 10 min at 4 °C to separate the soluble and insoluble protein fractions. The supernatant was stored at 4 °C as the soluble fraction. The pellet was resuspended in 50 mL of the insoluble protein lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 8 M urea, 1 mM PMSF, pH 7.9). The lysis suspension was then centrifuged at 12 000 rpm for 10 min, and the supernatant fraction was stored at 4 °C as the insoluble fraction. The two protein fractions were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R_{250}.

The fraction carrying the recombinant *Gly m* Bd 28K protein was loaded on a His-Bind Resin column (Novagen, USA), which had been pre-equilibrated with the lysis buffer. The loaded column was extensively washed with the eluents (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, and 6 M urea) containing 10, 20, 60, 100, 160, 250, 300, or 1000 mM imidazole. The eluents were analyzed by 12% SDS-PAGE, and the eluents that contained the recombinant *Gly m* Bd 28K protein were pooled. The protein solution was dialyzed using sterile water and lyophilized with a freeze-dryer (ALPHA 1-2 LD plus, Christ, Germany). The purity of the recombinant *Gly m* Bd 28K protein powder was determined by SDS-PAGE and the Geliance 200 imaging system (Perkin Elmer, USA).

Preparation of pAB. Two New Zealand white rabbits (named A and B), weighing 2 kg (Beijing CoWin Biotech Co., Ltd., China), were immunized with the purified recombinant *Gly m* Bd 28K by three subcutaneous injections. The first injection contained 600 μg of recombinant *Gly m* Bd 28K emulsified with complete Freund's Adjuvant (PIERCE Biotechnology, USA). The two subsequent injections contained 400 μg of recombinant *Gly m* Bd 28K emulsified with incomplete Freund's Adjuvant and were given every 2 weeks. The rabbits were then bled by cardiac puncture 10 days after the final injection, and the serum antibody titers were determined using an iELISA and the recombinant *Gly m* Bd 28K protein. The serum chosen for the next trial was referred to as pAB-28K.

SDS-PAGE and Western Blotting Analysis. Western blotting was performed to assess the specificity of the pAB-28K for *Gly m* Bd 28K. The recombinant *Gly m* Bd 28K and the total soybean meal proteins were dissolved in 0.01 M PBS. The recombinant *Gly m* Bd 28K (10 μg/well) and the soybean meal proteins (45 μg/well) were subjected to SDS-PAGE using a mini-gel apparatus (BioRad Laboratories, Hertfordshire, UK) with a 12% acrylamide separating gel and a 5% acrylamide stacking gel.

The separated proteins were eletrophoretically transferred from the gel to a 0.45 μm polyvinylidene fluoride (PVDF) microporous membrane (Millipore Corp., USA) in a semidry transfer cell (Bio-Rad, USA). The semidry blotting was performed at 12 V for 40 min. The membrane was blocked overnight at 4 °C in Tris-buffered saline (TBS-T) (10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 and 5% BSA. The membrane was rinsed four times for 10 min each in TBS-T and incubated for 2 h at room temperature (RT) with pAB-28K diluted 1:4000 in TBS-T/1% BSA. After being washed with TBS-T, the membrane was probed with the secondary antibody (goat antirabbit IgG (H+L)-AP, Beijing CoWin Biotech Co., Ltd., China) diluted 1:5000 for 30 min at RT. After being washed with TBS-T four times, the immunocomplexes were detected with the BCIP/NBT Chromogenic Substrate Kit (Tiangen biotech Co., Ltd., China).

iELISA Development. *i. iELISA Protocol.* 100 μL of recombinant *Gly m* Bd 28K or soybean sample extracts in 0.05 mM bicarbonate buffer (pH 9.6) was added to the wells of a 96-well microtiter plate (Nunc-Immuno Plate MaxiSorp, Denmark) and incubated overnight

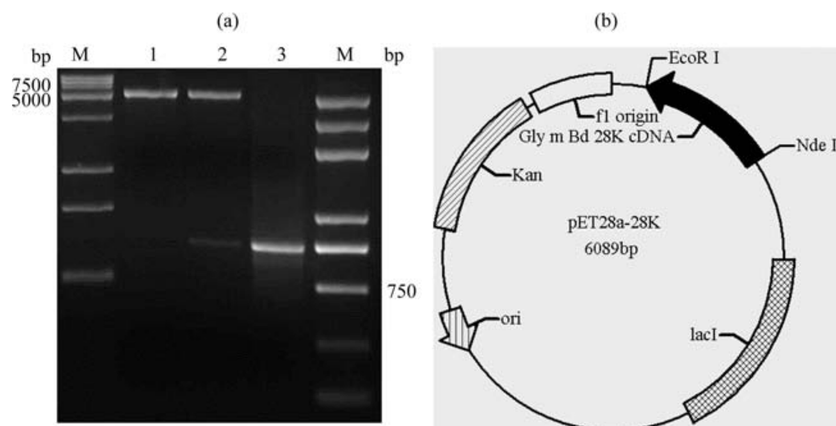

Figure 1. Schematic diagrams of the pET28a-28K plasmid and the restriction enzyme digestion. (a) The restriction enzyme digestion. (b) The schematic diagram of the pET28a-28K plasmid. (1) The pET-28a (+) plasmid. (2) The pET28a-28K plasmid/*Nde*I, *Eco*RI. (3) The *Gly m* Bd 28K gene. (M) Molecular weight ranges.

at 4 °C. After three washes with 300 μL/well of 0.01 M PBST (0.01 M PBS containing 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 5% BSA. Following an additional three washes, 100 μL of pAB-28K (diluted with 1% BSA in 0.01 M PBST) was added to each well and incubated for 1.5 h at 37 °C. After washing, 100 μL of goat antirabbit IgG (H+L)-HRP was diluted 1:5000 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 the addition of 50 μL of 2 M H_2SO_4 to each well. The absorbance of each well at 450 nm was determined by an automatic ELISA plate reader (Beijing PuLang New Technology Ltd., China).

ii. Optimal Dilution Factor for pAB-28K. The recombinant *Gly m* Bd 28K was diluted using 0.05 M bicarbonate buffer to concentrations of 0.08, 0.125, 0.25, 0.50, 1.00, and 2.00 μg/mL. The primary antibody, pAB-28K, was diluted from 1:1000 to 1:729 000 by 3-fold serial dilutions. The optimal dilution factor for pAB-28K for the iELISA was determined by the above iELISA protocol.

iii. Development of the iELISA. The recombinant *Gly m* Bd 28K was diluted in 0.05 M bicarbonate buffer to concentrations of 0.08, 0.125, 0.25, 0.50, 1.00, and 2.00 μg/mL. The primary antibody, pAB-28K, was diluted to the optimal dilution factor in the assay buffer (1% BSA in 0.01 M PBST). The standard curve was built by plotting the absorbance obtained from each reference standard against its concentration in μg/mL by the iELISA method.

iv. iELISA Spiked Recovery. To assess the iELISA reliability, the assay was evaluated by performing eight replicates of the recombinant *Gly m* Bd 28K samples diluted to 0.2, 1, and 2 μg/mL. The recovery was determined by iELISA with the above standard curve.

Determination of the *Gly m* Bd 28K Content in Soybeans and Soybean Meal. Substances from soybean seeds, soybean meal, and fermented soybean meal were diluted in assay buffer to 1 mg/mL prior to assessment. The immunoreactive *Gly m* Bd 28K content from these soybean products was analyzed by the iELISA based on the recombinant *Gly m* Bd 28K and the pAB-28K. Each sample was assayed eight times per plate, and the plates were performed in triplicate.

RESULTS

Construction of the Recombinant Plasmid pET28a-28K. The PCR fragment containing the *Gly m* Bd 28K cDNA was digested with the restriction enzymes *Eco*RI and *Nde*I and ligated into the pET-28a vector that had been digested with the same pair of restriction enzymes (Figure 1b). As shown in Figure 1a, the recombinant pET28a-28K plasmid was successfully constructed and identified by PCR and restriction enzyme digestion. The nucleotide sequencing confirmed that the construct was in the correct reading frame. The positive recombinants were chosen for the next expression trial.

Expression of *Gly m* Bd 28K in *E. coli* BL21 (DE3). The expression of the recombinant *Gly m* Bd 28K protein in *E. coli* BL21 (DE3) was induced after 6 h by adding 0.2 mM IPTG at 28 °C. To analyze the expression and the solubility of the recombinant proteins, the lysates, the soluble and insoluble fractions were examined by SDS-PAGE gels. As shown in Figure 2, the molecular weight near 30 kDa of the target protein band on the stained SDS-PAGE gel was consistent with its theoretical MW of 29.71 kDa, and the bulk of the recombinant *Gly m* Bd 28K protein was present in the insoluble fraction (lane 3). The inclusion body could further be dissolved in 6 M urea for other detection methods.

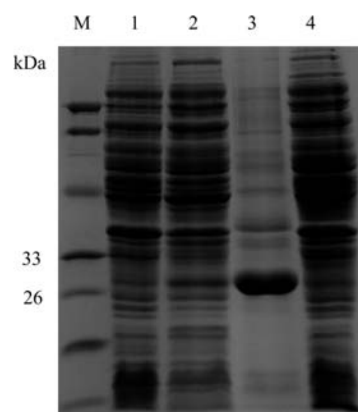

Figure 2. SDS-PAGE analysis of the expression pattern of the recombinant *Gly m* Bd 28K protein. (M) Molecular weight ranges. (1) The soluble fractions of pET28a-28K/BL21 (DE3). (2) The lysates of pET28a-28K/BL21 (DE3). (3) The insoluble fractions of pET28a-28K/BL21 (DE3). (4) The lysates of *E. coli* BL21 (DE3).

Purification of Recombinant *Gly m* Bd 28K. As shown in Figure 3, the purification of the recombinant *Gly m* Bd 28K was achieved with a His-Bind Resin column, and the single *Gly m* Bd 28K band was found in the eluents with 100, 160, and 250 mM imidazole (lanes 5, 6, and 7, respectively). The target band of the recombinant *Gly m* Bd 28K was over a 90% pure protein, and no other bands were observed on the gel following purification and refolding (Figure 4a, lane 1 and Figure 4b, lane

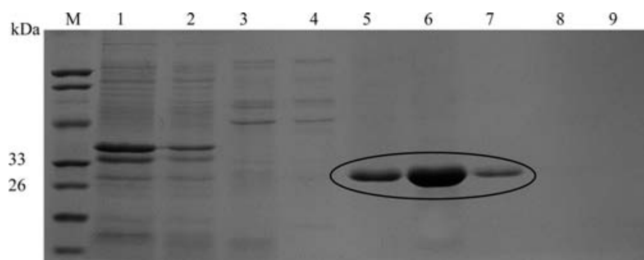

Figure 3. SDS-PAGE analysis of the purification of the recombinant *Gly m* Bd 28K protein. (M) Molecular weight ranges. (2)−(9) The eluents containing 0, 10, 20, 60, 100, 160, 250, 300, and 1000 mmol/L imidazole.

1). These results indicated that the *Gly m* Bd 28K was successfully produced in the *E. coli* expression system.

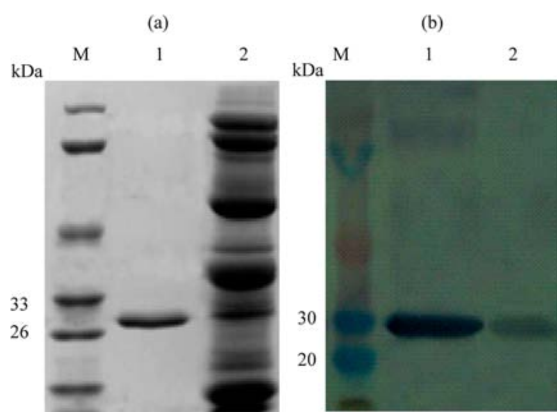

Figure 4. SDS-PAGE and Western blotting of the recombinant *Gly m* Bd 28K protein and the extruded soybean meal protein using the pAB-28K antibody. (a) SDS-PAGE analysis of the purified recombinant *Gly m* Bd 28K and soybean meal proteins. (b) Corresponding Western blot analysis using pAB-28K. (M) Molecular weight ranges. (1) The purified recombinant *Gly m* Bd 28K (10 μg/well). (2) The soybean meal (45 μg/well, Sanhe Huifu Foodstuff and Oil, Ltd., China).

Preparation of pAB. *i. Titers of Rabbit Sera.* The serum titers of two rabbits were detected by iELISA using the recombinant *Gly m* Bd 28K as the coating antigen. When the rabbit serum titers were below 27 000, their positivity/negativity (P/N) ratios were higher than 2.1, and their *P* values were higher than 0.1 (Figure 5). Both of the rabbits met the requirements for antibody donation, but the serum of rabbit B displayed a better response and was chosen as the source of the primary *Gly m* Bd 28K antibody, referred to as pAB-28K.

ii. Specificity of pAB-28K. The Western blotting analysis showed that pAB-28K could specifically recognize *Gly m* Bd 28K but could not recognize other soybean meal proteins (Figure 4b). This indicated that the polyclonal antibody pAB-28K could specifically identify the target antigen and, thus, could be used to develop an immunoassay for the detection of *Gly m* Bd 28K from soybean products.

Indirect ELISA. *i. The Optimal Dilution Factor for pAB-28K.* It was important to optimize the dilution factor for the primary antibody for the iELISA. When the pAB-28K was diluted 1:3000, a linear relationship was found between the primary antibody and the coating antigen (recombinant *Gly m* Bd 28K) at concentrations of 0.08 and 2 μg/mL (Figure 6). Therefore, 1:3000 was chosen as the optimal dilution of pAB-28K for the iELISA.

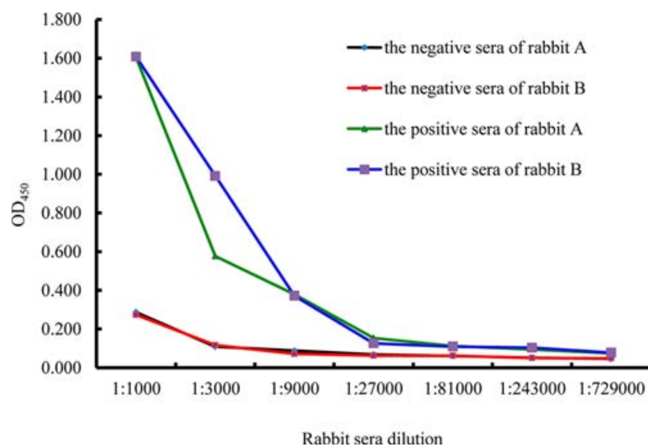

Figure 5. The determination of the rabbit serum titers by iELISA.

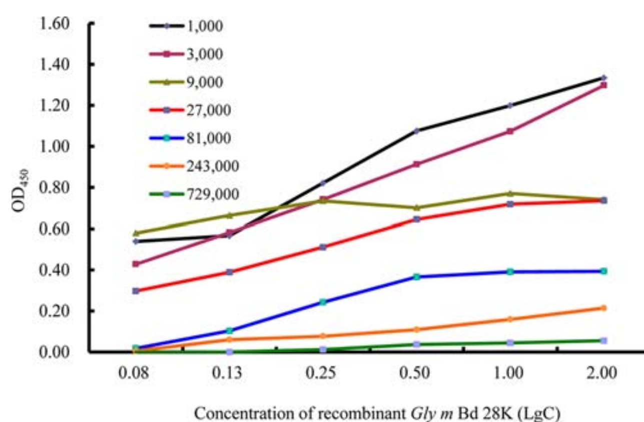

Figure 6. The relationship between the absorption values and the serum titers of rabbit B.

ii. Standard Curve for the iELISA. A calibration curve for the iELISA was established on the basis of the optimal dilution factor for pAb-28K and the optimal coating concentrations of the recombinant *Gly m* Bd 28K. As shown in Figure 7, the linear formula of the method was $y = 0.5563x + 0.4686$ ($R^2 = 0.9910$), and its high linear relationship would meet its possible application special for assay of target *Gly m* Bd 28K in soybean products.

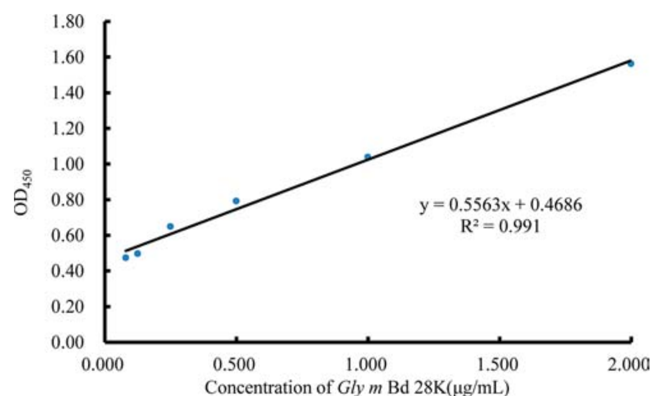

Figure 7. The standard curve of the iELISA for the detection of *Gly m* Bd 28K in soybeans and soybean products based on the pAB-28K and the recombinant *Gly m* Bd 28K protein.

iii. Spiked Recovery of the iELISA. To validate the recombinant *Gly m* Bd 28K and pAB-28K used for the iELISA, spiked recovery tests were performed using 0.2, 1, and 2 μg/mL of the recombinant *Gly m* Bd 28K. As shown in Table 1, the average recoveries of *Gly m* Bd 28K at the three concentrations were 95.50% (varying from 85.00% to 103.00%). The relative standard deviation (RSD) ranged from 13.71% to 20.59%, which was similar to the value (5.3−20%) of the sandwich ELISA used by Bandos,[15] despite that better optimization should be done in future work. The recoveries and the RSD of the iELISA were within the acceptable ranges, indicating that the *Gly m* Bd 28K- and pAB-28K-based iELISA was sensitive, reliable, and reproducible.

Table 1. Recovery Analysis of the iELISA Based on the Recombinant *Gly m* Bd 28K and the pAB-28K

Gly m Bd 28K (μg/mL)	mean (μg/mL)[a]	SD	recovery (%)	RSD (%)
2	1.97	0.27	98.50	13.71
1	1.03	0.17	103.00	16.50
0.2	0.17	0.035	85.00	20.59

[a]The data represent the means of eight replicate subsamples.

Detection of *Gly m* Bd 28K in Soybean Seeds and Soybean Meal. The iELISA was used to determine the *Gly m* Bd 28K content of soybean seeds, soybean meal, and fermented soybean meal (FSM). As shown in Table 2, the unprocessed soybean seeds had the highest content of the immunoreactive *Gly m* Bd 28K at 2.96 μg/mg, while the protein content of the soybean meal was much lower at 1.21 μg/mg. The reduction of *Gly m* Bd 28K in the soybean products may have been due to treatments such as heating, expanding, and aqueous alcohol extracting.[17−19,26] The *Gly m* Bd 28K content in fermented soybean meal was significantly reduced with increased fermentation time by a variety of fermenting agents. When the soybean meal was fermented for 24 and 48 h by *Bacillus subtilis*, the *Gly m* Bd 28K content was reduced to 0.46 and 0.23 μg/mg, respectively. When FSM samples were fermented for 24 and 48 h by *Geotrichum candidum*, the *Gly m* Bd 28K content was reduced to 0.43 and 0.21 μg/mg, respectively. With 24 and 48 h of *Endomycopsis fibuligera* fermentation, the *Gly m* Bd 28K content was 0.42 and 0.15 μg/mg, respectively. *Gly m* Bd 28K content was 0.39 and 0.13 μg/mg, in FSM fermented by *Candida utiliz* for 24 and 48 h, respectively. Finally, fermentation with *Saccharomyces cerevisiae* for 24 and 48 h reduced the levels to 0.33 and 0.14 μg/mg, respectively. In total, the reduction in *Gly m* Bd 28K content by each fermenting agent at 24 and 48 h was 61.98−72.73% and 80.99−89.25%, respectively. Furthermore, the *Gly m* Bd 28K from the samples that were fermented for 72 h could hardly be detected (Table 2). These results indicated that the allergen *Gly m* Bd 28K could effectively be reduced in soybean meal by solid-state fermentation with a variety of microbes, and this could be a feasible approach for diminishing *Gly m* Bd 28K levels in soybean meal.

Table 2. Detection of the *Gly m* Bd 28K Protein in Soybean Seeds and Soybean Meal by the iELISA Based on the Recombinant *Gly m* Bd 28K and the pAB-28K[a]

samples	replicates (*n*)	*Gly m* Bd 28K content (μg/mg)	SD	CV (%)
soybean seeds[b]	8	2.96	0.41	13.90
soybean meal[c]	8	1.21	0.18	14.62
Soybean Meal Fermented by *Bacillus subtilis*[d]				
24 h	8	0.46	0.07	14.51
48 h	8	0.23	0.04	15.35
72 h	8	−	−	−
Soybean Meal Fermented by *Geotrichum candidum*[e]				
24 h	8	0.43	0.06	14.88
48 h	8	0.21	0.03	15.48
72 h	8	−	−	−
Soybean Meal Fermented by *Endomycopsis fibuligera*[f]				
24 h	8	0.42	0.06	14.28
48 h	8	0.15	0.02	15.36
72 h	8	−	−	−
Soybean Meal Fermented by *Candida utiliz*[g]				
24 h	8	0.39	0.04	10.27
48 h	8	0.13	0.02	13.71
72 h	8	−	−	−
Soybean Meal Fermented by *Saccharomyces cerevisiae*[h]				
24 h	8	0.33	0.04	11.35
48 h	8	0.14	0.01	10.97
72 h	8	−	−	−

[a]The data represent the means of eight replicate subsamples. −: Not detected. [b]Zhonghuang 13 soybean seeds were obtained from the Crop Research Institute, Chinese Academy of Agricultural Sciences. [c]Soybean meal was obtained from Sanhe Huifu Foodstuff and Oil, Ltd. [d]Soybeans were fermented by *Bacillus subtilis* at the Genetic Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences. [e]Soybeans were fermented by *Geotrichum candidum* at the Genetic Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences. [f]Soybeans were fermented by *Endomycopsis fibnligera* at the Genetic Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences. [g]Soybeans were fermented by *Candida utilis* at the Genetic Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences. [h]Soybeans were fermented by *Saccharomyces cerevisiae* at the Genetic Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences.

■ DISCUSSION

Gly m Bd 28K is a minor component of the soybean seed 7S globulin fraction and constitutes less than 0.5% (w/w) of the total seed protein.[27] However, together with P34 and the α subunit of β-conglycinin, it represents one of the three most common seed allergens of soybean-sensitive patients[28] and is regarded as the predominating soybean allergen. The purpose of this study was to develop a method for the rapid detection of the *Gly m* Bd 28K protein in soybean products utilizing a recombinant *Gly m* Bd 28K and a pAB.

In a previous study, the *Gly m* Bd 28K protein was purified from defatted soybean flakes by a series of protocols, including isoelectric precipitation, mAb-C5-CM-cellulofine chromatography, DEAE-Sepharose CL-6B chromatography, and gel filtration on a Sephacryl S-200 column.[29] A total of 19 potentially allergenic proteins were separated by one- and two-dimensional gel electrophoresis and screened by Western blotting with the sera from soy-allergic or soy-sensitive patients. Ten allergenic proteins, including the *Gly m* Bd 28K, were then identified by mass spectrometry.[30] These methods, while effective, require time-consuming cleanup steps and expensive equipment. By comparison, a prokaryotic expression system, such as *E. coli*, is more practical for recombinant proteins due to the ease of culture, rapid cell growth, and simpler purification procedures.[31] The recombinant *Gly m* Bd 28K protein was

highly immunologically reactive, indicating that both techniques yield a protein with similar epitope structures. Therefore, the method of extracting the native *Gly m* Bd 28K protein could be replaced by an *E. coli*-based recombinant protein expression system for its detection via iELISA. A further advantage of the *E. coli* system is that the recombinant *Gly m* Bd 28K protein can easily be prepared in large quantities.[32] This solution should be encouraged in similar work in the future. Our iELISA is also more advantageous than the previously published sandwich ELISA and cELISA methods aimed at native soy proteins (P34, *Gly m* Bd 28K, β-conglycinin, glycinin, and the α subunit of β-conglycinin)[15,16,21,22,24,25] because they relied on monoclonal antibodies that were difficult to produce. By comparison, a rabbit pAB against the recombinant *Gly m* Bd 28K protein was produced quickly and inexpensively. Western blotting analysis revealed that the antibody reacted strongly with the purified recombinant *Gly m* Bd 28K and produced a single band corresponding to that of native *Gly m* Bd 28K in soybean, and so we deduced they contain the same epitope (Figure 4). This indicated that the pAB-28K was able to specifically bind to the *Gly m* Bd 28K protein and thus could be used to develop immunoassays for the detection of *Gly m* Bd 28K. The R^2 of the iELISA based on the binding of the pAB-28K to the recombinant *Gly* m Bd 28K was 0.9910, and its recovery was between 85% and 103%, which indicated that the assay was accurate and reliable enough for use as a *Gly m* Bd 28K detection method. The RSDs that range from 13.71% to 20.59% of the iELISA developed in our work should be reducced via more and better optimization in future work to increase the precision as high as possible, despite that our RSD values were similar to RSDs in the range of 5.3−20% of the sandwich ELISA for detection of *Gly m* Bd 28K in soybean and soybean products.[15] Basically, the iELISA assay developed for the detection of *Gly m* Bd 28K in this study is competitive with the sandwich ELISA and cELISA methods.

In the previous study,[15] the *Gly m* Bd 28K protein content in various soybean products, such as soybean protein isolates, tofu, kori-dofu, and yuba, was observed using the sandwich ELISA. The protein content in soybean seeds was 4.35 ± 0.47 μg/g fresh weight (FW) or 66.4 μg/g nitrogen, and its content in soy milk and abura-age was low. In fermented products, such as natto, soy sauce, and miso, and in processed foods with soybean protein, the allergen was not detected,[15] which strongly suggested that the fermentation process destroyed the *Gly m* Bd 28K protein. Our results revealed that the *Gly m* Bd 28K content in soybean seeds and soybean meal was 2.96 μg/mg and 1.21 μg/mg, respectively (Table 2), which indicated that the process of fat extraction can destroy 59.12% of the antigen. Importantly, the protein content in FSM samples that were fermented by *Bacillus subtilis*, *Geotrichum candidum*, *Endomycopsis fibuligera*, *Candida utiliz*, or *Saccharomyces cerevisiae* for 24 or 48 h showed a sharp decline, and the protein was not detectable after 72 h of fermentation (Table 2). This indicated that the allergen *Gly m* Bd 28K can be destroyed by fermentation, and this conclusion is consistent with previous reports on other fermented soy products.[15] Solid-state fermentation is an effective process for the eradication of allergenic proteins in soy, while maintaining or improving bioavailability, functionality, and nutritional value in the subsequent products.[16,21,33−35] Therefore, we highly recommend the use of fermentation in the feed and food industries, as we estimate that 500 thousand tons of FSM products will be used for piglet feed in China per year.

An immunoassay based on a monoclonal antibody can only detect a substance that contains the single given epitope and, thus, is very likely to show no response if the structure of the particular epitope or allergen is destroyed or modified during processing.[25] Tsuji et al. (1997) reported that the *Gly m* Bd 28K protein was converted to a more acidic protein with the same molecular mass during the purification process, suggesting that the allergen is unstable. In addition, previous studies have shown that manufacturing conditions, such as heat treatment, fermentation, and extrusion, can result in the modification of the structure and immunoreactivity of certain allergens.[23] It is unlikely that other derivatives of an antigen can be specifically detected by an ELISA that uses an antibody that is rooted in a single predicted epitope. Conversely, the ELISA based on a polyclonal antibody can cover multiple epitopes and shows a lower detection limit.[13,16] The polyclonal antibody based ELISA results can reflect a variety of different potential allergenic structures on the same antigen. From the viewpoint of food/feed safety and quality control, a pseudopositive result from a polyclonal antibody is better than a pseudonegative result from a monoclonal antibody. For this reason, we devised an iELISA for soy *Gly m* Bd 28K after the iELISA for the soy allergenic protein P34.[16] This iELISA assay should be popular for quality evaluation of soy food and feed products in the future. Currently, the development of rapid and reliable assays is urgent for the establishment of FSM as a standardized protein source in the Chinese feed industry.

In conclusion, the *Gly m* Bd 28K protein was successfully expressed in *E. coli* as an inclusion body by IPTG induction at low temperature. The specific and immunogenic pAB-28K antibody was isolated from the serum of rabbits that had been immunized with the recombinant *Gly m* Bd 28K protein. We then developed an iELISA based on the recombinant *Gly m* Bd 28K and the pAB-28K. The iELISA was indirectly validated for the detection of the *Gly m* Bd 28K content of soybean products. Therefore, this assay provides some important technical bases of materials, methods, and design to access to our final aim for the development of a feasible and sensitive protocol for the detection of *Gly m* Bd 28K in soybean products and a higher safety assurement level in soy products.

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Notes

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