

Research Article

Synthesis and Degradation of the Major Allergens in Developing and Germinating Soybean Seed

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Abstract

Gly m Bd 28K, Gly m Bd 30K and Gly m Bd 60K are the major soybean (*Glycine max* (L.) Merr.) allergens limiting the consumption of a good protein source for sensitive individuals. However, little is known about their temporal-spatial expression during seed development and upon germination. The present data shows that soy allergens accumulated in both the embryonic axes and cotyledon, but expression patterns differed depending on the specific allergen. Allergens accumulated sooner and to a greater level in cotyledons than in embryonic axes. Gly m Bd 28 began at 14 d after flowering, 7 to 14 d earlier than Gly m Bd 30K and Gly m Bd 60K. Comparatively, their degradation was faster and more profound in embryonic axes than in cotyledons. Gly m Bd 60K began to decline at 36 h after imbibition and remained detectable up to 108 h in cotyledons. In contrast, the Glym Bd 60K protein was reduced at 24 h, and eventually disappeared at 96 h. In cotyledons Gly m Bd 28K first declined at 24 h, then increased from 36 h to 48 h, followed by its large reduction at 72 h after seed germination. These findings provide useful information on soy allergen biosynthesis and will help move forward towards developing a hypoallergenic soybean for safer food.

Keywords: allergen; food allergy; *Glycine max*; soybean; synthesis and degradation; seed development and germination.

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Introduction

Cultivated soybean, *Glycine max* (L.) Merr. is an important oilseed and food crop, containing 20% oil and 40% protein in the seed. With many valuable proteins, soybean seed has been recognized as the largest source of protein meal in the world (USDA-ERS 2003). However, soybean ranks among the eight most significant food allergens (Zeece et al. 1999; Pedersen et al. 2008). The wide use of soybean in the food industry makes soybean difficult to eliminate from the diet of soy-sensitive individuals. Allergies to soybean-containing foods have been increasingly investigated over the past 20 years (Herian et al. 1990; Beardslee et al. 2000; Helm et al. 2000a,b; Hiemori

et al. 2000; Ogawa et al. 2000; Gu et al. 2001; Tsuji et al. 2001b; Xiang et al. 2004). At least 16 allergenic proteins in soybean seed have been found, which are specifically recognized by antibodies in the sera of soybean-sensitive patients (Ogawa et al. 1991, 2000). Three proteins, Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K, are shown to be the major allergenic proteins in soybean. These proteins are fractioned into the 7S globulin fraction of soybean proteins (Ogawa et al. 1991, 1993, 2000).

Gly m Bd 60K (also named Gly m Bd 68K) is identified as the α -subunit of β -conglycinin with 70 kDa (Ogawa et al. 1995). The α -subunit of β -conglycinin is a glycoprotein with a molecular weight of 57 kDa and pI of 4.9 (Thanh and Shibasaki 1977),

which affects approximately 25% of soy-sensitive patients (Ogawa et al. 1991). The protein sequence of the precursor deduced from the cDNA (pGmc76, 1971 bp in length) consists of 605 amino acid residues (Sebastiani et al. 1990).

Gly m Bd 30K, a protein of the thiol protease family (Kalinski et al. 1990), is examined as the 34 kDa oil body-associated protein (p34) (Ogawa et al. 1993). This soy allergen affects about 65% of soy-sensitive patients (Ogawa et al. 1991), exhibiting high homology with the house dust mite allergen Der p I (Chua et al. 1990). A cDNA (open reading frame (ORF), 1140 bp) of the 34 kDa oil body-associated protein has been cloned and sequenced, which encodes 379 amino acid residues (Kalinski et al. 1990; Helm et al. 1997). Gly m Bd 30K is a N-linked glycoprotein, suggesting a possible function of a defense-related protein (Okinaka et al. 2002).

Gly m Bd 28K, a member of the cupin protein family, was first isolated from soybean meal as a 28-kDa glycosylated protein (Tsuji et al. 1997). Gly m Bd 28K affects about 25% of soy-sensitive patients (Ogawa et al. 1991). *Gly m Bd 28K* is predicted to encode a polypeptide composed of 473 amino acids, which is about twice as large as anticipated (28 kDa) on its initial discovery in sodium dodecyl sulfate (SDS) gels (Tsuji et al. 2001a). It is suggested that Gly m Bd 28K proprotein is processed into two mature proteins, an N-terminal polypeptide of about 240 amino acids and a C-terminal of about 212 amino acids during the development of soybean seeds. The C-terminal 23 kDa polypeptide is also a potential allergen (Hiemori et al. 2004; Xiang et al. 2004). Gly m Bd 28K shares homology with the pumpkin MP27/MP32 protein and carrot Gea8 globulin-like protein, 54% and 45.9% identity, respectively (Hiemori et al. 2000).

To eliminate soybean allergies, a number of attempts have been made to develop hypoallergenic soybean-based foods by removing or lowering the allergenic soybean proteins through enzymatic (Hosoyama et al. 1994; Yamanishi et al. 1996) or physical-chemical treatments (Obata and Yamanaka 1994; Samoto et al. 1994). However, the most efficient method is to use a type of soybean that lacks allergens. Natural deficiency of Gly m Bd 28K is detected in a number of soybean cultivars. In Japan, 80% of soybean varieties lack Gly m Bd 28K (Yamanishi et al. 1996). Using a random set of a core collection of soybean varieties in China, Zhang et al. (2006) found that 37.8% of 412 cultivars and 13.3% of 60 wild soybeans are deficient in Gly m Bd 28K. However, all soybean accessions tested in China and the U.S. contain Gly m Bd 30K (Yaklich et al. 1999; Guan et al. 2004; Zhang et al. 2006). No soybean cultivars are identified as lacking all allergens.

One promising option is to develop non-allergenic soybean varieties using genetic modification (Takahashi and Abe 1994). A soybean mutant (Tohoku 124) was obtained by gamma-ray irradiation. Tohoku 124 lacks the α - and α' -subunits of β -conglycinin (Gly m Bd 60K) as well as Gly m Bd 28K (Samoto

et al. 1997). Gly m Bd 30k can be effectively removed from this mutant soybean by physical-chemical treatments (Samoto et al. 1996), allowing the preparation of soy products containing essentially none of the three major soybean allergens. A soybean mutant with a low level of Gly m Bd 30K is also reported (Obata and Yamanaka 1998). Herman (2003) used gene silencing techniques to develop soybean lines essentially devoid of Gly m Bd 30 K. However, there are many other hurdles that must be overcome before such genetically modified hypoallergenic soybeans could be grown commercially (Herman 2003; Fang et al. 2006).

Although the major soybean allergens have been studied to different extents by many research groups, information on the temporal and spatial expression of the allergenic proteins during seed development, upon germination, and during seedling growth is limited. Therefore, this study characterizes and compares expression patterns of the three major soybean allergens during seed maturation and germination. Elucidating the expression of soybean allergens allows for a better understanding of mechanisms controlling allergenic protein synthesis and degradation for future production of a hypoallergenic soybean.

Results

Examination of Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K in the four selected varieties

For analysis of the synthesis and degradation of the three soybean allergens in developing and germinating seeds, the soybean varieties selected were verified for their phenotypes of Gly m Bd 60 K, 30 k and 28 k in the mature seeds. Gly m Bd 60 K is easily separated and visualized by SDS-PAGE and staining. As expected, all of the four varieties contain Gly m Bd 60 K (Figure 1A).

Western blot analyses using special monoclonal antibodies were carried out to examine Gly m Bd 28K and Gly m Bd 30K in those varieties. As shown on Figure 1B, Gly m Bd 30K was detected in all four varieties while Gly m Bd 28K was found in the three varieties except for Taiwan 75, which is known to lack this protein. The data here confirm the allergen phenotypes of the soybean varieties we selected for this study.

Detection of Gly m Bd 28K in single seed

As described above, all of the soybean varieties tested contained both Gly m Bd 60 K and Gly m Bd 30 K allergens, in agreement with our previous description (Guan et al. 2004; Zhang et al. 2006). In order to identify mature seeds containing Gly m Bd 28K for analysis of the allergen degradation in germinating seeds, every seed needed to be verified. For this experiment, we added a breeding line 95-5383. Seed chips

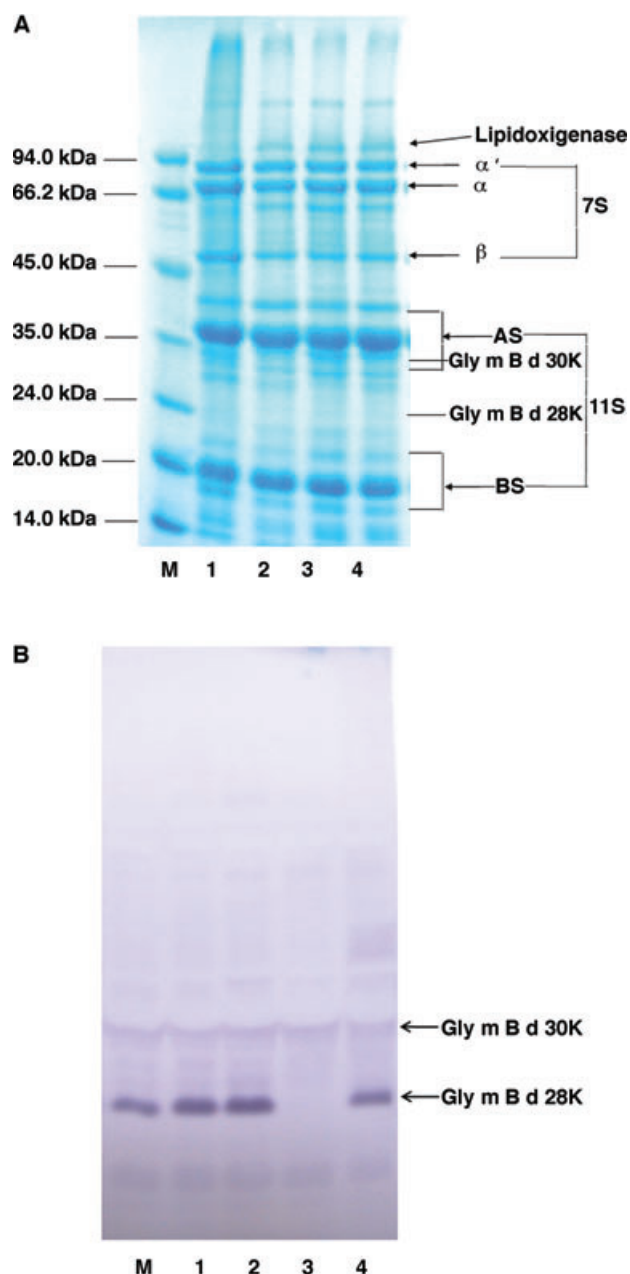


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting patterns of Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K in four soybean varieties.

The proteins (20 μ g/lane) in each extract were separated on two 10.5% polyacrylamide gels. The proteins from the extracts on one gel were stained with Coomassie Brilliant Blue R250.

(A) The proteins on the other gel were electro blotted onto a nitrocellulose membrane, and the allergens on the membrane were immunostained with the special monoclonal antibody (mAb).

(B) As described in the text. M. marker proteins; 1. cv. Zaoshu 18; 2. cv. Tiefeng 22; 3. cv. Taiwan 75; 4. cv. Huipizhi black bean.

were sampled from each dry seed for testing. As shown in **Figure 2**, Gly m Bd 28 K was detected in all seeds tested from Zhaoshu 18, whereas the allergen was not found in some seeds from Tiefeng 22, Huibuzhi black bean and 95–53838. Additionally, no Gly m Bd 28 K was detected in the seeds of Taiwan 75 (data not shown). The seeds with close to the same level of Gly m Bd 28K were subsequently germinated for identifying the degradation pattern of the allergen.

Expression patterns of the three major allergens during soybean seed development

Soybean is a dicotyledonous plant. Here, the cotyledons and embryonic axes at different stages were sampled separately to investigate the expression patterns of the three allergens at the protein level during seed development using SDS-PAGE and Western blotting. The results show that the three allergens were synthesized and increased to higher levels in later stages versus in the early stages in both cotyledons and embryonic axes. The proteins were expressed in different manners throughout seed development, along with soluble protein accumulation (**Figure 3**).

Gly m Bd 60K appeared early, at 21 DAF, in both cotyledons and embryonic axes (**Figure 3**). The protein increased gradually from 28 DAF until the peak at 49 DAF in cotyledons (**Figure 3A**, Left panel). Seven days after the appearance of Gly m Bd 60K, the protein began in embryonic axes (**Figure 3A**, right panel). The allergen level was not significantly different in both parts at the final time, 49 DAF.

Gly m Bd 30K began to accumulate at 28 DAF, and then gradually increased through 42 DAF. A significant elevation arose at 49 DAF in cotyledons (**Figure 3C**, left panel). In embryonic axes, this allergen occurred early at 14 DAF, and increased to its highest level at 49 DAF, the final stage tested (**Figure 3C**, right panel). Gly m Bd 28K was detected early at 14 DAF in both cotyledons and embryonic axes (**Figure 3B**), seven to 14 d earlier than the other two proteins, indicating that Gly m Bd 28K accumulated sooner than the Gly m Bd 60K and Gly m Bd 30K in developing soybean seed. Moreover, both Gly m Bd 28K and Gly m Bd 30K levels were higher in cotyledons than in embryonic axes.

Taken together, the three allergens exhibited differences in the timing of synthesis, rate of increase, and maximum levels of accumulation in cotyledons and embryonic axes. The result suggests a differential regulation of allergenic proteins during soybean seed development.

Degradation patterns of the major allergens during soybean seed germination and seedling growth

Generally speaking, seed storage proteins are degraded to supply nutrients for early seedling development. However, how soybean allergens are degraded in germinating seeds

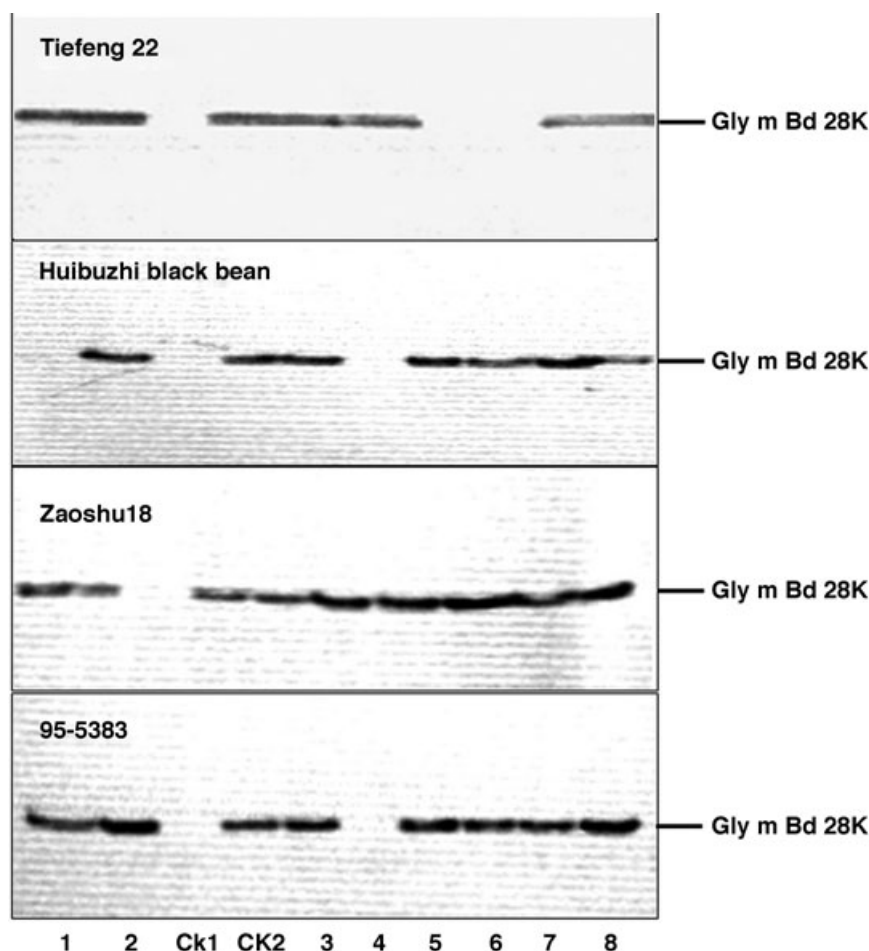


Figure 2. Seed chip-based detection of Gly m Bd 28K in single mature seed by Western blotting.

The soluble proteins were extracted from each seed chip, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and followed by transfer onto a nitrocellulose membrane. The immunoblotting analyses for allergens on the membrane were conducted with the mAb C5 specific for Gly m Bd 28K. Soybean varieties tested included Zaoshu 18, Tiefeng 22, Huipizhi black bean and 95–53838 indicated on the figure. CK1 and CK2 represent for the negative and positive controls, respectively.

unknown. SDS-PAGE and Western blot analysis were conducted to determine the degradation patterns of the three major allergens during seed germination and seedling growth. The cotyledons and embryonic axes of 25 germinating seeds were separately sampled at each time point. More than 80% of imbibed seeds showed radicle emergence after 24 h. The seeds continued to grow into seedling until 108 h after imbibition.

The SDS-PAGE (Figure 4A) revealed large changes in protein composition in the samples during germination. Soluble proteins degradation occurred earlier in embryonic axes than in cotyledons. Gly m Bd 60K started to decline early, at the 24 h time point, and was undetectable at the 96 h and 108 h time points in embryonic axes (Figure 4A, right panel). However, the allergen was detected over the entire time course tested

in cotyledons (Figure 4A, left panel), even though protein level decreased slowly after 36 h.

Western blotting analysis (Figure 4B,C) also showed that both Gly m Bd 30 K and Gly m Bd 28 K degraded faster in embryonic axes than in cotyledons, with the two allergens completely disappearing beginning at 96 h in embryonic axes (right panel) and 108 h in cotyledons (left panel), respectively. Gly m Bd 30K began to degrade at the 24 h time point in embryonic axes (Figure 4C, right panel), 12 h earlier than in cotyledons (Figure 4C, left panel). Surprisingly, Gly m Bd 28 K dramatically degraded early at 24 h in cotyledons (Figure 4B, left panel). After 24 h the protein level increased greatly over the next two time points, and then was reduced drastically again at 72 h and 96 h. By 108 h the allergen was completely degraded. However, in embryonic axes Gly m Bd 28 K decreased gradually from

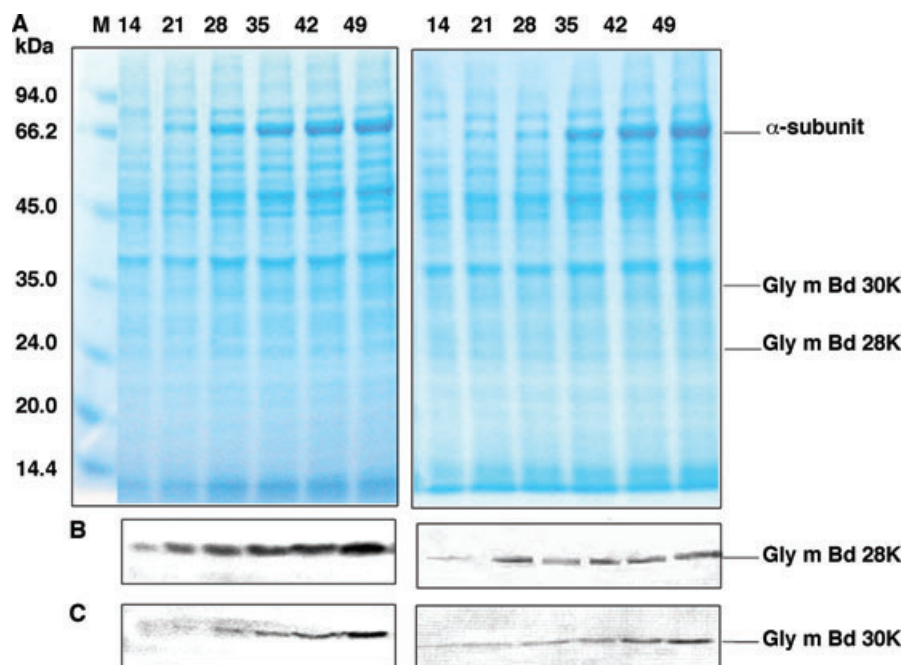


Figure 3. Synthesis and accumulation of the major soybean allergens in developing seed.

The soluble proteins were isolated from cotyledons (left panel) and embryonic axes (right panel) of soybean seeds (cv. Tiefeng 22) at various time points (days after flowering, d), samples were subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Panel A) and Western blotting (panels B, C) with monoclonal antibodies (mAb) C5 and F5 specific for Gly m Bd 28K and Gly m Bd 30K, respectively.

the 24 h time point to the 72 h time point (**Figure 4B**, right panel). These results suggest that the three soybean allergens were degraded in a different manner and probably by different proteases during seed germination.

Expression of the three allergen genes during soybean seed development

To determine tissue specificity of gene expression, total RNA was isolated from roots, stems, leaves, flowers and developing seed of the soybean variety “Zhoshu 18”. The transcripts of the three allergen genes were only detected in developing seeds, and no target fragments were amplified by RT-PCR in other tissues tested (data not shown). Moreover, no mRNA of the three genes was detected in germinating seed and seedlings (data not shown). For quantitative analysis of mRNA expression of the three genes during seed development, Real-time PCR was performed using total RNAs prepared from soybean seeds at different development stages. As shown in **Figure 5**, the transcripts of the three genes accumulated in a similar pattern in both cotyledons and embryonic axes. Expression begins at early stages (14 DAF), gradually increases to peak levels in middle stages (from 28 and 35 or 42 DAF), and then declines in the late stages (from 42 to 49 DAF). However, the peak

levels and the period of the highest increasing rate of mRNAs were different for the three genes in both parts tested. The highest levels in cotyledons occurred at 28 for Gly m Bd 28 K, Gly m Bd 30 K and 35 DAF for Gly m Bd 60 K, while their peak expressions appeared at 35 and 42 DAF in embryonic axes. The mRNAs expression of Gly m Bd 28 K and Gly m Bd 60 K was higher in cotyledons than in embryonic axes. In contrast, transcription of Gly m Bd 30 K accumulated more in embryonic axes than in cotyledons. These data indicate that the expressions of the three genes were spatially and temporally regulated during seed development.

Discussion

Soybean storage proteins are highly valuable for human nutrition and health. However, a number of proteins have been identified as allergens to humans, compromising the health of soy-sensitive individuals. Little is known about the synthesis and degradation of soybean allergens in seed formation and germination, although many studies have focused on their allergenic nature and removal from the processed foods (Ogawa et al. 2000; Fang et al. 2006). The present study was undertaken to characterize the three major soybean allergens, Gly

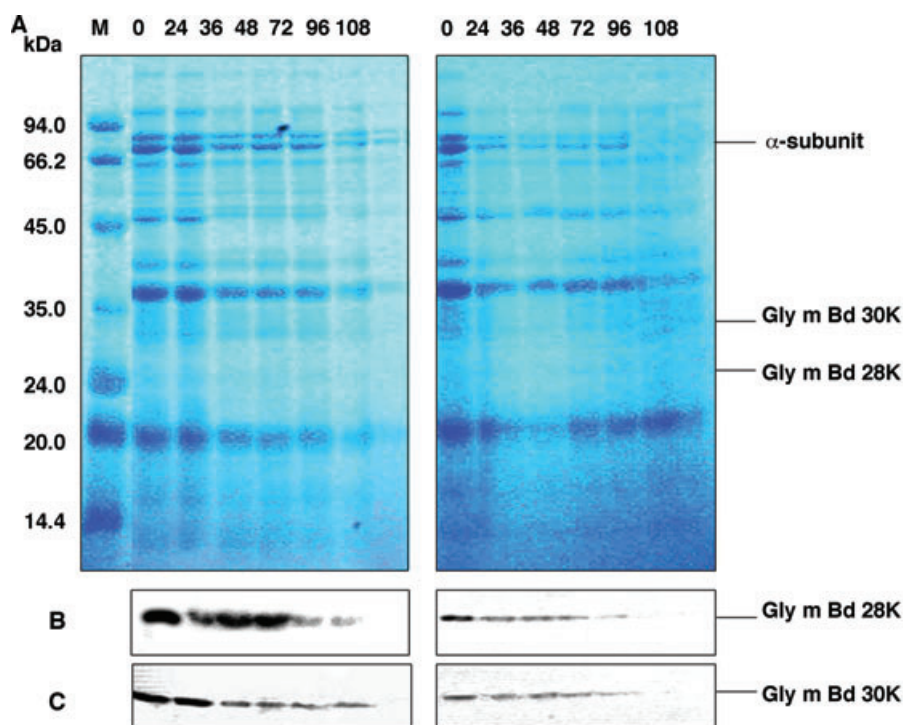


Figure 4. Degradation of three major allergens at the protein level during soybean seed germination and seedling growth.

(A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the soluble proteins prepared from cotyledons (left panel) and embryonic axes (right panel) of germinating seeds (cv. Tiefeng 22) at various time points (hours after imbibition, h); Western blot analysis for Gly m Bd 28K(**B**) and Gly m Bd 30K(**C**), respectively, using C5 and F5 mAb, respectively.

m Bd 60K, Gly m Bd 30K and Gly m Bd 28K, biosynthesis and degradation in developing and germinating seeds.

As discussed above, hydrolysis of seed storage proteins could provide amino acids for new protein synthesis in growing seedling. Protein degradation happens only after a period of rest and imbibition by water, when seeds germinate and seedlings start to grow (Shutov et al. 2003). Germination is considered as finished when the radicle breaks through the seed coat. In legume seeds, germination takes place approximately 24 h after imbibition (Bewley and Black 1994). In our case, more than 85% of the soybean seeds were germinated after 24 h imbibitions at 25 °C in the dark.

Following germination and seedling growth, the three allergens were observed to be degraded in an allergen- and tissue-specific pattern (Figure 4). Comparatively, soybean allergens degraded faster in embryonic axes than in cotyledons. The level of Gly m Bd 60K began to decline at 24 h, and was undetectable by 96 h in embryonic axes (Figure 4A, right panel). In contrast the Gly m Bd 60K protein degradation delayed to 36 h in the embryonic axis, and the protein was still detected up to the 108 h time point in cotyledons (Figure 4A, left panel). Previous reports showed that protein mobilization in the axes precedes storage protein breakdown in cotyledons (Schlereth et al. 2000;

Tiedemann et al. 2000). Consequently, the decline in most seed storage proteins in cotyledons and embryonic axes is not synchronous. Faster degradation of the three allergens in the axes than in cotyledons might be a consequence of a slower turnover of these proteins in cotyledons.

Mature storage proteins are stably accumulated in developing seeds and degraded during germination into small peptides or amino acids, which are subsequently transported to the growing seedling (Goldberg et al. 1989; Shewry et al. 1995). Our present data reveal that the three soybean allergens are synthesized and accumulated along with seed development (Figure 3). Protein expression patterns are heterogeneous and depend on the specific soybean allergen. For instance, Gly m Bd 28K was first synthesized at 14 DAF in cotyledons (Figure 3B), 7–14 d earlier than Gly m Bd 60 K and Gly m Bd 30 K. Gly m Bd 60 K slowly increased from 28 DAF until 49 DAF in cotyledons, but significantly increased at 35 DAF in embryonic axes despite the protein appearing at 21 DAF in both parts (Figure 3A). Furthermore, the peak protein level was also different between allergens. In cotyledons and embryonic axes Gly m Bd 60 K accumulation was greater than Gly m Bd 28 K. Both Gly m Bd 60 K and Gly m Bd 28 K protein levels exceeded Gly m Bd 30 K in each organ type.

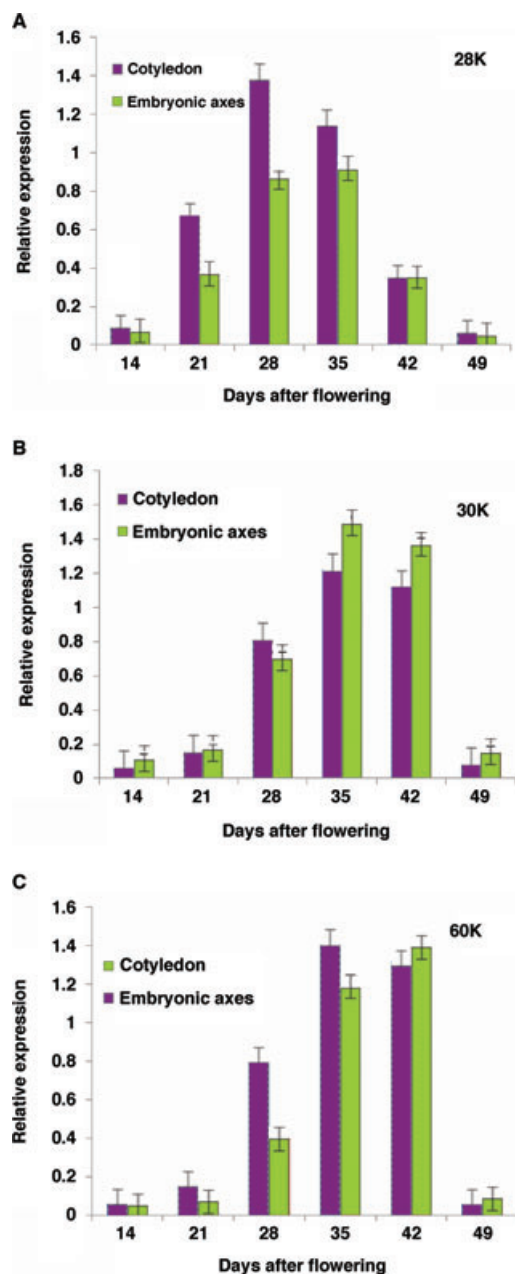


Figure 5. Expression of the three major soybean allergen genes in embryonic axes and cotyledons during seed development.

Real-time polymerase chain reaction (PCR) was performed for *Gly m Bd 28K*, *Gly m Bd 30K* and *Gly m Bd 60K* genes with mRNA extracted from embryonic axes and cotyledons at six seed developmental stages (14, 21, 28, 35, 42, and 49, DAF) of soybean variety “Zhaoshu 18”. Signal values obtained from each gene were normalized using the soybean β -tubulin signal value. Data presented as allergen transcripts per β -tubulin transcript. Data bars represent the means $\pm$ SD level of relative transcript abundance from five experiments with independent RNA extractions.

All three allergens accumulated in both the embryonic axes and cotyledons (Figure 3). However, a difference at the protein level was observed for the allergens between embryonic axes and cotyledons. Gly m Bd 28 expressed more in cotyledon than in embryonic axes, while Gly m Bd 68K accumulated more in embryonic axes than in cotyledons. The profiles of mRNA (Figure 5) showed seed-specific expression. No expression of these three allergens could be detected in roots stems, flowers or germinating seeds. Based on their function as major seed storage proteins, these results are not surprising. However, the expression patterns were somewhat different from each other, suggesting spatial and temporal regulation throughout seed development. The highest level of mRNAs occurred in the middle seed development stages, but allergen proteins accumulated to maximum levels at the mature stage. The expression patterns of mRNA did not completely coordinate with allergen accumulation patterns, suggesting protein accumulation was subjected to posttranscriptional regulation. Our spatial-temporal results for protein accumulation are consistent with reports for storage proteins in *Arabidopsis* seed development (Kroj et al. 2003). Other legumes also show a differential expression of seed protein genes. (Hauxwell et al. 1990). In soybeans, a wave of transcriptional activity of storage protein genes was detected from the outer surface to the interior of cotyledons (Goldberg et al. 1989; Perezgrau and Goldberg 1989). In peanuts, three allergens were found to be synthesized and accumulated to different levels in the axes and cotyledons. (Kang et al. 2007).

Interestingly, Gly m Bd 28K increased at 36 h and 48 h time points in cotyledons, although protein reduction was observed at the 24 h time point (Figure 4B, left panel). After such an increase, the Gly m Bd 28K protein declined again at 72 h and was undetectable at 108 h. Allergen enhancement over the course of seed germination and seedling growth was also identified in peanuts (Kang et al. 2007). The 22 kDa isoform of Ara h 3 was present at almost the same level throughout seedling development. The 14 kDa polypeptide of Ara h 3, however, increased as other seed storage protein levels decreased. The increase of the 14 kDa Ara h 3 peanut allergen might be the result of an accumulation of the processed Ara h 3 isoforms (Kang et al. 2007). The different degradation patterns for peanut allergens were also identified in work by Liao et al. (2004).

The mechanism underlying the allergen-specific degradation patterns, including the increase of Gly m Bd 28 K, in soybean seed germination is unknown. For legume seeds, Cystein proteinases are thought to be the major proteinases responsible for storage globulin breakdown (Wilson et al. 1986; Shutov and Vaintraub 1987; Muntz 1998). Multiple mechanisms modulate proteinase activity during seed germination. Most likely, different protease in the seed during germination and changes in their activity contribute to the three abundant allergens degrading in different manners over the course of

soybean germination. This question remains to be addressed. The present data demonstrate that all three allergens disappeared by 108 h after seed germination. Therefore, bean sprouts, after 5 d of seed germination, could be safe food for the soy-allergic population. The use of bean sprouts would require a demonstration of no allergic reaction in soy-sensitive individuals through the use of food allergy tests.

Conclusion

Gly m Bd 60 K, Gly m Bd 30 K and Gly m Bd 28 K are the three major allergens in the soybean seed and soy-based food. The present study, characterized the temporal and spatial expression patterns of the three allergenic proteins during seed development germination and seedling growth. The three allergens express in a different manners with regard to their synthesis and degradation, indicating that they are differentially regulated in seed development and germination. Major allergens are completely degraded after 3 d of growth. Soybean seedlings, excluding cotyledons, could be safe for food use after 3 d of germination and growth. The present data provides fundamental information for better understanding of the mechanism controlling soybean allergen synthesis and degradation, as well as toward the development of a hypoallergenic soybean.

Material and Methods

Plant material

Five soybean *Glycine max* (L.) Merr cultivars were selected from the Soybean Germplasm Bank at the Chinese Academy of Agricultural Science for the present experiment (Ma et al. 2006). Taiwan 75, a variety lacking Gly m Bd 28K was used as the negative control soybean for examining Gly m Bd 28K. Zaoshu 18, a variety containing Gly m Bd 28K, was used for the positive control for Gly m Bd 28k detection. Tiefeng 22, 95-5383 and HuiBuzhi black bean (a local landrace in Shanxi, China) were selected for detecting the phenotypes of the allergens (Gly m Bd 60, 30 and 28K) (Table 1).

Sampling of developing and germinating seeds

To make sure the selected seeds contain Gly m Bd 28K, many mature seeds of each variety were firstly chipped. Protein was extracted from the seed chip and used for a Western blot. Based on the results of the blot, the seed chip containing 28K was determined. Simultaneously, the relative content of Gly m Bd 28K on the Western blot was quantified using computer software. At least 25 seeds containing Gly m Bd 28K were

Table 1. Soybean varieties tested and allergens identified

Variety	Phenotypes of the allergens		
	28K	30K	60K
Taiwan 75	0	1	1
Zaoshu 18	1	1	1
Tiefeng 22	1/0	1	1
95-5383	1/0	1	1
Huibuzhi black bean	1/0	1	1

0: Deficiency in the allergen; 1: Containing the allergen; 1/0: Mix of allergen-containing and -lacking seeds.

selected for germination to ensure three samples could be harvested for each time point.

All varieties were grown in a greenhouse under 16:8 h light : dark cycle at 25 °C and 21 °C. Developing seeds were determined and harvested according to days after flowering (DAF). Fresh seed samples were frozen immediately in liquid nitrogen for later use.

For seed germination, the mature dry seeds from each variety were surface sterilized with Chlorine gas (100 mL NaClO + 4 mL HCl) for 2 h, subsequently rinsed three times with distilled water, and then imbibed in double distilled H₂O for 2 h. Imbibed seeds were spread onto two layers of moistened Whatman No. 1 filter paper on a mesh tray (Chemostat) and germinated under aseptic conditions at 25 °C in the dark. Samples were taken every 12 h following imbibition. The mature seeds imbibed for 20 min were used as the control 0 h time point. Fresh weight and soluble protein content were measured for seeds from each time point. 25 germinating seeds/seedlings per variety were sampled at each time point.

Soybean protein extraction and SDS-PAGE

Samples were ground to a powder, 5 mg samples from cotyledons and embryonic axes were placed in a 1.5 mL centrifuge tube to which 500 µL SDS-Urea extract solution was added. The solution consists of 5 M urea, 0.2% SDS, 5% β-mercaptoethanol, 20% glycerol, and 50 mM Tris-HCl buffer (pH 6.8). Tubes were mixed well and then incubated for 15 min at room temperature. After centrifugation at 7 000 g for 5 min at room temperature, the supernatant was subsequently centrifuged at 10 000 g for 15 min at room temperature to remove residual traces of oil and insoluble particles. The final S/N extracts were stored at -20 °C until use. The concentration of the soluble protein extracted from soybean samples was determined by the methods of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10.5% polyacrylamide gel with a Mini Protean II system (Bio-Rad, Hercules, CA, USA)

according to the method described previously by Guan et al. (2004) and Zhang et al. (2006). Briefly, equal volumes of the soluble proteins from each sample were loaded on the gel, and the electrophoresis was conducted in the running buffer for 3–4 h at 150 V. Molecular weight markers were obtained from Beijing Biosynthesis Biotechnology CO. LTD (Beijing, China). Protein on the gels was stained with 0.1% Coomassie Brilliant Blue R-250 in a solution of methanol, acetic acid, and water (5:2:2, v/v/v).

Western blot analysis and detection of allergen by mAb

The detection of Gly m Bd 28 and Gly m Bd 30K was performed using the methods described by Samoto et al. (1994) and Tsuji et al. (1997). Monoclonal antibodies (mAb) for Gly m Bd 28K (C5) and Gly m Bd 30K (F5) were kindly provided by Dr Makita Hajika (National Institute of Crop Sciences of Japan). Gly m Bd 60K was detected by SDS-PAGE only. Briefly, after separating the proteins using the SDS-PAGE gel, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion-P, Millipore, Schwalbach, Germany). The allergen on the membrane was immunostained for Gly m Bd 28K and Gly m Bd 30K separately using the C5 and F5 mAb (monoclonal antibody), respectively. After three 10 min washes with the buffer, membranes were incubated with the secondary antibody (goat anti-rabbit immunoglobulin G (IgG)-whole molecule peroxidase conjugate, Sigma, St. Louis, MO, USA). The membranes were washed again and antigen-antibody complexes were detected by chemiluminescence using the enhanced chemiluminescence (ECL) plus reagent (Amersham-Pharmacia Biotech, Arlington Heights, Illinois). Membranes were imaged using Biomax mL film (Eastman Kodak, Rochester, NY, USA).

RT-PCR and quantitative PCR

Total RNA from soybean (cv. Zhaoshu 18) seedlings (cotyledon and embryonic axes, respectively) was isolated using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). The first strand cDNA was synthesized by SuperScript III Kit (Invitrogen, Carlsbad, CA), and subsequently used as the template for reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. The *Gly m Bd 28 K* gene specific primers were 5'-TGGAACCGTATTTGGGAAG-3'/5'-TTACATGAGGTGCCAACATT-3'. The *Gly m Bd 30K* gene specific primers were 5'-TGAGCAT GGACGTGTCTACC-3' / 5'- GTTGGCCATTTTGATTGCT-3'. The *Gly m Bd 60K* gene specific primers were 5'-TCAATTCAACACCCGTCAAA-3'/5'-CCCAGTAAGCAATGCCAAAT-3'.

Expression of the three genes in seeds at different developmental stages was determined by RT-PCR. Transcript level was measured by quantitative PCR (qPCR). The

qPCR assays were performed using the iTaq SYBR Green Supermix with ROX (Bio-Rad) on an Applied Biosystems StepOne Real-Time PCR System following thermal cycling conditions recommended by the manufacturer.

The expression of soybean β -tubulin is known to be fairly uniform and was therefore used as the control gene. The soybean β -tubulin gene specific primers were 5'-CGCACCCTTAAGCTCACCACC-3'/5'-TTTCCACGGACATCGCAGAAGC-3'. Expression of *tubulin* was used as an internal control to normalize the expression of *Gly m Bd 28K*, *Gly m Bd 30K* and *Gly m Bd 60K*. The specificity of each primer pair was verified by determining the melting curve of PCR products at the end of each run and by gel electrophoresis of the PCR products.

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