

Effects of soybean glycinin on performance and immune function in early weaned pigs

Peng Sun, Defa Li*, Bing Dong, Shiyan Qiao and Xi Ma

National Key Laboratory of Animal Nutrition, China Agricultural University, Beijing, P. R. China

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Glycinin, a major storage protein in soybean, has been identified as an important food allergen. The present study was conducted to investigate the effects of soybean glycinin on the local and systemic immune responses using a swine model specific for glycinin allergy. Performance, peripheral blood lymphocyte proliferation and CD4⁺ and CD8⁺ subsets in the plasma of naive and sensitised piglets were determined. In addition, the concentrations of IgA, IgG, IgM, interleukin-4 and interleukin-6 in the jejunum mucosa were measured. Our results showed that dietary supplementation of glycinin reduced piglet performance ($p < 0.05$), while increasing lymphocyte proliferation and CD4⁺/CD8⁺ ratio ($p < 0.01$). Intestinal mucosal immune responses to glycinin were enhanced with high levels of IgA ($p < 0.01$) as well as interleukin-4 and interleukin-6 in the jejunum mucosa in glycinin-treated piglets compared with the control ($p < 0.01$). There were no differences due to treatment in the densities of IgG and IgM. In conclusion, it was found that glycinin stimulated local and systemic immune responses in allergic piglets and had negative effects on piglet performance. The severity of the immune reactions depends on the dose of glycinin with higher doses causing more severe symptoms.

Keywords: glycinin; piglets; IgA; immune response; cytokine

1. Introduction

Soybean is a high-quality protein due to its good nutritional, processing and functional properties and can be found in a wide range of food products (Hou and Chang 2004; Golubovic et al. 2005). However, soybean allergy is a serious health threat to individuals sensitive to soybean (Foucard and Yman 1999). To date, allergies to soybean protein are common among infants (Duke 1934) and adults (Burks et al. 1988). In addition, with an increasing use of soy protein in the feed industry, more and more people are interested in its allergenic properties because of its potential for causing hypersensitivity in young animals such as piglets (Li et al. 1990, 1991; Dréau et al. 1994) and calves (Lallès et al. 1996).

Previous studies have shown that the allergenic properties of soybean protein are mainly due to the presence of glycinin, a primary storage protein in soybean (Shibasaki et al. 1980; Zeece et al. 1999). As a dietary allergen, glycinin has been shown to cause changes in intestinal morphology and disorders of immune function, resulting in growth

*Corresponding author. Email: defali@public2.bta.net.cn

depression and diarrhea in young animals (Li et al. 1990, 1991; Dréau et al. 1994; Qiao et al. 2003; Sun et al. 2007).

The gastrointestinal tract is a large mucosal immune system, which protects the body from damage from offending food and helps to scavenge antigens. Mucosal lesions of the gastrointestinal tract are typically observed in the animals suffering from soybean allergy, with symptoms varying from villous atrophy to focal necrosis and exfoliation (Li et al. 1990, 1991; Guo et al. 2007). However, the relationship between food allergy and immune responses in the intestine mucosal system is still unknown.

Although food allergy is well documented as an antigen-specific Th2 type immune response (van Halteren et al. 1997; van Wijk and Knippels 2007), some studies have shown that food allergy in experimental animals is not strictly mediated by Th2 responses, but involves a mixed Th1/Th2 response, which has been found both in human (Kolopp-Sarda et al. 2001) and in rats (de Jonge et al. 2007). However, the role of the systemic immune system, including lymphocyte proliferation as well as differentiation of T cells, in response to soybean glycinin in allergic piglets after oral sensitisation is not clear.

The objective of the present study was to investigate the alterations in immune function, including systemic immune responses and local immune responses in the intestinal mucosa of allergic piglets induced by soybean glycinin. Here we used an experimental piglet model of glycinin allergy, which was established in a previous study (Sun et al. 2007). Purified glycinin was fed to eliminate potential interference from other components found in soybean meal or whole soybean protein extract.

2. Materials and methods

2.1. Characterisation of purified soybean glycinin

Purified glycinin suspension was kindly donated by Professor Shuntang Guo at the Food Institute of China Agricultural University (Patent number, 200410029589.4, Beijing, China). The purity of glycinin was measured and the sample contained more than 85% glycinin.

2.2. Piglets and diets

All animals used in this experiment were maintained according to the principles of the China Agricultural University Animal Care and Use Committee. Twenty-four crossbred (Large White $\times$ Landrace) barrows were purchased from the Zhonghe Breeding Farm in Beijing (China). The piglets were weaned at 14 days of age and had an average weight of 4.98 ± 0.67 kg.

Each piglet was individually housed in a 0.7×1.1 m stainless steel metabolic crate equipped with a plastic-coated, totally slatted floor. Each pen was equipped with a feeder and a waterer to allow the pigs unlimited access to water and feed at all times. The pigs were housed in a mechanically ventilated nursery room with the lighting set on a cycle of 12 h light: 12 h dark, the temperature maintained at $26\text{--}28^\circ\text{C}$ and the relative humidity set at 50–60%.

After four days of adaptation, the piglets were randomly allotted to one of four treatments ($n = 6$). The corn-based control diet, devoid of soybean protein, contained skim milk, fishmeal, whey powder, casein and spray dried porcine plasma as protein sources. Three experimental diets were formulated by adding 2%, 4%, or 8% purified glycinin at the expense of skimmed milk powder and casein. All diets were formulated to provide similar levels of crude protein (23.0%), lysine (1.6%) and energy

(DE, 14.2 MJ/kg). Additional details regarding diet composition and chemical analysis have been published previously (Sun et al. 2007).

2.3. Experimental design and sample collection

The experimental design was as described in our previous study (Sun et al. 2007). Briefly, the experimental pigs were fed the diets containing 2%, 4% or 8% glycinin from days 0–10 (sensitisation period) and days 16–18 (challenge period). Thereafter, the pigs were challenged after two weeks from the final day of the challenge period (on day 32 only). The pigs were fed the control diet at all other times. Pigs were weighed at the beginning and the end of the trial (day 32) while feeders were weighed at the beginning and end of the trial as well as when a diet change was conducted in order to calculate weight gain, feed intake and feed conversion.

Blood (7 ml) was collected in 9-ml heparinised vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) from the precaval vein 3 hours after the final challenge (day 32). Subsequently, piglets were slaughtered and a 10 cm section of the jejunum was removed and flushed by physiological saline to remove the gut contents. The intestinal tissue was then stored in liquid nitrogen until needed for analysis.

2.4. Isolation and preparation of peripheral blood mononuclear cells

Heparinised whole blood samples were obtained as described above. The peripheral blood mononuclear cells were isolated by density gradient centrifugation. Briefly, blood samples were added into Lymphocyte Separation Medium (HyClone Laboratories Inc. Logan UT) and centrifuged at 2500 g for 30 min. The mononuclear cell layer was collected and washed twice with RPMI-1640 medium (HyClone Laboratories Inc.). Thereafter, the cells were resuspended in complete RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories Inc.), 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mmol/l HEPES (Sigma, St. Louis, MO). The cells were counted and adjusted the density at $2 \cdot 10^6$ cell per ml of culture medium for proliferation assay.

2.5. Assay of lymphocyte proliferation

Peripheral blood mononuclear cell proliferation was determined using a colorimetric test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Inc., St Louis, MO, USA) as described by Guo et al. (2007) with slight modifications. The cells were incubated in the presence or absence of 100 µg/ml glycinin for 72 h at 37°C and 5% CO₂. Concanavalin A (Sigma, St Louis, MO) served as the positive stimulus. Ten millilitre MTT solution and 100 µl of 10% sodium dodecyl sulfate in 0.04 mol/l HCl solution were added at 66 h and 70 h during the incubation period, respectively. After incubation, the plates were read on an automated ELISA reader (Sunrise, Tecan Asai, Singapore) at 570 nm and the result was expressed as a stimulation index.

2.6. CD4⁺ and CD8⁺ lymphocyte subset analysis by flow cytometry

Peripheral blood lymphocyte CD4⁺ and CD8⁺ subset phenotypes were analysed by flow cytometry as described by Lai et al. (2005). The primary antibodies used were IgG2b mouse anti-pig-CD4 FITC-labelled and IgG2a mouse anti-pig-CD8α PE-labelled (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and two-colour flow

cytometric analysis was conducted with a Coulter Epics XL (Beckman Coulter Corp., Miami, FL).

2.7. Analysis of intestinal antibodies by ELISA

Approximately 0.5 g of intestinal mucosal sample was resolved in phosphate buffered saline containing 1 mM phenylmethanesulfonyl fluoride. After homogenisation, the sample was centrifuged at 10,000 *g* at 4°C for 10 min, and the supernatant was obtained. Protein was quantified with Coomassie Brilliant Blue Protein Assay Kit (Jiancheng Institute of Biological Technology, Nanjing, China).

The measurement of IgA levels was performed by ELISA as described by Burks et al. (1988) with some modifications. The upper two rows of a 96-well microtitre plate (BioFil, Canada) were coated with goat anti-pig IgA-affinity purified antibody as the standard. The remainder of the plate was coated with 5 µg/ml glycinin in 100 µl carbonate buffer (NaHCO₃/Na₂CO₃, pH 9.6). Then, 100 µl supernatant was added to each well and incubated. After being washed 5 times, goat anti-pig IgA horseradish peroxidase conjugate (Sigma, St Louis MO) and 0.4 mg/ml O-phenylenediamine-dihydrochloride buffer containing 0.015% H₂O₂ (Sigma) were added to measure the absorbance at 492 nm. The data were expressed as optical density (OD) units.

The determination of IgG and IgM was performed in a similar fashion to IgA except for the utilisation of corresponding goat anti-pig IgG- or IgM-affinity purified antibodies and goat anti-pig IgG or IgM HRP conjugate (Sigma).

2.8. Quantification of cytokines in jejunum mucosa

Levels of interleukin-4 and interleukin-6 in jejunum mucosa were determined using the swine Enzyme-Linked Immunosorbent Assay Kit (ELISA Kit; Biosource, Camarillo, CA, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

All data were analysed using the General Linear Model (GLM) procedures of SAS system (version 8.2, SAS Institute, Inc., Cary, NC, USA). Linear and quadratic polynomial contrasts were used to determine the dose-dependent effect of soybean glycinin. Values of *p* less than 0.05 were considered statistically significant.

3. Results

3.1. Piglet performance

The performance data are presented in Table 1. Average daily gain and feed conversion declined linearly (*p* < 0.05) with increasing levels of glycinin in the diets. Piglets fed 4% and 8% glycinin had lower average daily gain (*p* < 0.05) compared with those in the control and 2% glycinin group (230 and 223 g/d vs. 256 and 242 g/d). Average daily feed intake was not influenced by treatment (*p* = 0.58).

3.2. Lymphocyte proliferation, CD4⁺ and CD8⁺ lymphocyte subset phenotype

As shown in Table 2, the stimulation index of peripheral blood mononuclear cells was increased linearly (*p* < 0.01) with the elevation of glycinin in the piglet diets. Ingestion of

Table 1. Effects of different doses of glycinin on the performance of piglets*.

	Glycinin level				SEM [#]	p-value	
	0%	2%	4%	8%		Linear	Quadratic
ADG [g/d]	256	242	230	223	10.3	0.028	0.747
ADFI [g/d]	390	382	377	371	24.3	0.579	0.946
Feed:gain ratio	1.52	1.57	1.63	1.66	0.05	0.032	0.783

Notes: *Each value is the mean of data from six piglets (average initial BW of 4.98 ± 0.67 kg) per group; [#]Standard error of the mean.

Table 2. Effects of different doses of glycinin on peripheral blood lymphocyte proliferation and CD4⁺ and CD8⁺ subsets of piglets*.

	Glycinin level				SEM [#]	p-value	
	0%	2%	4%	8%		Linear	Quadratic
Lymphocyte proliferation	1.14	1.25	1.31	1.42	0.05	<0.001	0.963
CD4 ⁺ lymphocyte subset	27.16	29.49	38.25	40.31	0.98	<0.001	0.888
CD8 ⁺ lymphocyte subset	26.46	27.63	28.25	28.82	0.96	0.086	0.754
CD4 ⁺ /CD8 ⁺ ratio	1.03	1.07	1.36	1.40	0.02	<0.001	0.954

Notes: *Each value is the mean of data from six piglets (average initial BW 4.98 ± 0.67 kg per group); [#]Standard error of the mean.

increasing doses of glycinin linearly increased the percentage of CD4⁺ lymphocyte subset ($p < 0.01$), while that of CD8⁺ in the whole blood was not significantly elevated ($p = 0.09$), which resulted in the increased ratio of CD4⁺ and CD8⁺.

3.3. Antibody levels in the jejunum mucosa

To investigate the effects of glycinin on intestinal mucosal reactions in sensitised pigs, levels of IgA, IgG and IgM antibodies in the jejunum were determined (Table 3). The antibody levels in the intestinal mucosa exhibited a similar trend to that for the CD4⁺ lymphocyte subset, but there was no difference in the concentrations of IgG or IgM. The IgA levels in pigs fed increasing doses of glycinin were linearly elevated in the jejunum mucosa ($p < 0.01$), especially for those treated with 4% and 8% glycinin ($p < 0.01$).

Table 3. Effects of different doses of glycinin on concentrations of IgA, IgG and IgM in the jejunum mucosa of piglets*.

	Glycinin level				SEM [#]	p-value	
	0%	2%	4%	8%		Linear	Quadratic
IgA [μ g/g]	96.85	114.41	136.67	144.01	11.48	0.005	0.661
IgG [μ g/g]	62.43	66.58	76.64	80.76	12.03	0.085	0.658
IgM [μ g/g]	151.12	156.86	171.83	172.22	25.45	0.499	0.917

Notes: *Each value is the mean of data from six piglets (average initial BW 4.98 ± 0.67 kg per group); [#]Standard error of the mean.

Table 4. Effects of different doses of glycinin on cytokines in the jejunum mucosa of piglets*.

	Glycinin level				SEM [#]	<i>p</i> -value	
	0%	2%	4%	8%		Linear	Quadratic
IL-4 [pg/g]	19.10	25.59	42.29	50.69	5.72	<0.001	0.870
IL-6 [pg/g]	24.32	34.04	47.92	64.64	5.96	<0.001	0.565

Notes: *Each value is the mean of data from six piglets (average initial BW 4.98 ± 0.67 kg per group); [#]Standard error of the mean.

3.4. Cytokine concentrations in the jejunum mucosa

Concentrations of interleukin-4 and interleukin-6 in jejunum mucosa increased linearly ($p < 0.001$) with increasing glycinin supplementation (Table 4).

4. Discussion

The present study investigated the local and systemic responses in an experimental pig model of soybean glycinin allergy, which is an important concern related to both animal nutrition and human health. Soybean allergy represents a significant health problem, which occurs more frequently early in life (Herian et al. 1993). As one of the main storage proteins in soybean, glycinin has been recognised as the main trigger to cause hypersensitive reactions and reduce performance in soybean-sensitive subjects (Sun et al. 2007).

Many studies have been conducted using young pigs as an animal model to investigate the allergenic properties of soybean proteins. These studies typically observe disorders of immune function and reduced performance (Li et al. 1990; Guo et al. 2007). Consistent with these reports, reduced performance was also observed in glycinin-sensitised piglets and there was a dose-dependent relationship between the supplementation of glycinin and the performance of piglets. With increasing supplementation of glycinin in the diet, the average weight gain and feed conversion decreased, although feed intake was not affected in glycinin-sensitised pigs compared with the control.

Damage to the integrity of the intestine is a common phenomenon in animals consuming soybean proteins (including glycinin). Li et al. (1990, 1991) reported that pigs had lower villus height but greater crypt depth after ingestion of soybean protein. Dréau et al. (1994) observed an indication of epithelial cell hyperplasia in pigs fed antigenic soybean products. These results indicate that in addition to a predominantly Th2 type immune response, occurrence of food allergy may correlate with immune responses in the intestinal mucosa.

IgA, as the major class of antibody present in the mucosal secretions of the gastrointestinal tract, plays an important role in the defense against invasion by ingested food antigens, attenuation of inflammatory responses and alleviation of food allergic symptoms (Woof and Kerr 2004). In the present study, we found a significant increase of IgA concentration in the jejunum mucosa of piglets fed glycinin, which is consistent with the results described by Kokkonen et al. (2002).

In contrast to IgA, no difference was observed in the concentrations of IgM in the jejunum mucosa. The results of the current study agree with those obtained by Dréau et al. (1994). In addition, it is postulated that IgG plays only a small role in intestinal mucosal immunity with relatively small quantities of IgG being secreted in the mucosa. However,

glycinin that is absorbed into the blood through the intestinal epithelial cell stimulates production of large quantities of IgG antibody in the serum and plays an important role in the systemic immune response, which has been proved in many previous studies (Li et al. 1990, 1991; Helm et al. 2002; Gizzarelli et al. 2006) as well as in our previous study (Sun et al. 2007).

One of the important findings of the present study is that supplementation of glycinin resulted in increased peripheral blood lymphocyte proliferation response in glycinin-treated groups compared with the control. Since lymphocyte proliferation is an important indicator of lymphocyte function, these results suggest the enhancement of immune function by glycinin. Similar observation was obtained by Gizzarelli et al. (2006) that soybean protein extract induced spleen cell proliferation in mice.

According to their surface markers, T lymphocytes can be classified as CD4⁺ or CD8⁺ subsets (Swain 1983). CD8⁺ T-cells can directly destroy virally infected cells or antigens, thus are defined as cytotoxic T lymphocytes (Calder 2001). CD4⁺ T cells can secrete cytokines to indirectly control the immune response in humoral immunity, which are defined as helper T cells. CD4⁺ lymphocytes can be further divided into T-helper 1 (Th1) cells and T-helper 2 (Th2) cells according to secretion patterns of cytokines (Carter and Dutton 1996). Th1 lymphocytes secrete interleukin-2, tumor necrosis factor- α , and interferon- γ , and so on, to initiate cell-mediated immune responses, while Th2 lymphocytes are mainly involved in humoral immune responses by producing interleukin-4 and interleukin-6 that play important roles in allergic responses (Carter and Dutton 1996; Desmedt et al. 1998; Prescott and Hogan 2006). In this study, the CD4⁺ lymphocytes were significantly increased in percentage as the oral administration of glycinin, and in turn increased the ratio of CD4⁺ and CD8⁺.

It has been revealed that glycinin-induced food allergy in piglets is a predominantly Th2 type immune response, in which Th2 type cytokines play an important role in immunoregulation (Lee et al. 2001; Sun et al. 2007). In the present study, the increase in lymphocyte proliferation could be attributed to an increase in CD4⁺ lymphocyte subsets, which resulted in significantly increased production of interleukin-4 and interleukin-6 with supplementation of increasing doses of glycinin in piglet diets. The results are consistent with a Th2-type immune response in glycinin-induced hypersensitivity. A similar cytokine profile has been reported in previous publications (Gizzarelli et al. 2006).

Interleukin-4 has dual functions in both stimulation of IgE production (Defrance et al. 1994), and induction of naive CD4⁺ T cells to differentiate into Th2 cells rather than Th1 cells (Snapper and Paul 1987). Furthermore, interleukin-4 promotes B lymphocytes to proliferate and differentiate into plasma cells, which are the main source of IgA in the mucosa of the intestine. Interleukin-6 is another Th2 type cytokine that induces the proliferation and differentiation of B lymphocyte to secrete IgA, IgG and IgM antibodies (Carter and Dutton 1996). Therefore, monitoring the production of cytokines in type and amount can provide helpful clues to evaluate the body immune status (Abbas et al. 1996).

Taken together, the current study demonstrates positive local and systemic immune responses to soybean glycinin in glycinin-sensitised piglets, with increased IgA antibodies and concomitant production of interleukin-4 and interleukin-6 in the intestinal mucosa as well as an increase in lymphocyte proliferation and CD4⁺/CD8⁺ in the plasma. Therefore, we can draw the conclusion that, at least in part, soybean glycinin induced CD4⁺ T cells differentiation into the Th2 pathway by increasing the production of interleukin-4 and interleukin-6 that relatively inhibited Th1-type immune responses, which finally affected the immune function and reduced the performance of allergic piglets.

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