Effects of soybean antigen proteins on intestinal permeability, 5-hydroxytryptamine levels and secretory IgA distribution in the intestine of weaned piglets

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ABSTRACT
In this experiment, the effects of soybean antigen proteins on intestinal permeability, 5-hydroxytryptamine (5-HT) levels and secretory IgA (sIgA) distribution in the intestine of weaned piglets were evaluated. Thirty piglets (21 d of age) were randomly divided into three groups: control group, glycinin-sensitised group and β-conglycinin-sensitised group. Piglets in the control group were fed a basal diet, and in two sensitised groups were fed the diet with 4% glycinin or β-conglycinin. Piglets in glycinin and β-conglycinin groups were sensitised twice on 21–27 d and 32–34 d period, respectively. Blood samples were collected and analysed for D-lactic acid, diamine oxidase (DAO) and 5-HT levels on d 21, 28 and 35. At the end of the trial, five piglets per group were sacrificed and the small intestine was collected to evaluate intestinal sIgA distribution and the optical density (OD) of the relative staining positivity. The results revealed that the piglets in glycinin and β-conglycinin groups had higher D-lactic acid, DAO and 5-HT levels than those of the control group (p < 0.01). Similarly, OD values were higher in the treatment groups than in the control group (p < 0.01). The intestinal sections, in order of decreasing OD value, were middle jejunum > duodenum > proximal jejunum and distal ileum > distal jejunum. These results suggest that soybean antigen proteins induce allergic reactions, damage the intestinal mucosa, increase intestinal permeability and promote sIgA synthesis in weaned piglets.

Introduction
Soybean, which is a low-cost, high-quality protein, is provided as a feed to a wide range of animals (Guo et al. 2007; Sun et al. 2008, 2013). However, researchers have reported that anaphylaxis in young animals is mainly attributed to allergens in soybean, especially soybean antigen proteins (van de Lagemaat et al. 2007; Liu et al. 2008a; Lin & Ji 2011). According to their centrifugal sedimentation rate, there are four types of soybean antigen proteins: glycinin, α-conglycinin, β-conglycinin and γ-conglycinin. Among the soybean antigen proteins, glycinin and β-conglycinin are the most predominant (65–80% of total soybean protein) (Zhu et al. 2011; Kou et al. 2014).

Soybean allergy in animals causes mucosal lesions in the gastrointestinal tract. Symptoms of soybean allergy include abnormal bowel patterns, including increased crypt cell mitosis, plasma protein leakage, goblet cell mucus exudation, intestinal villous atrophy, cell fusion and cellular apoptosis (Xu et al. 2010; Wang et al. 2012, 2014). In our previous study (Wu et al. 2016), it showed that a direct sensitisation by injecting glycinin could cause a decrease of growth performance, an increase of serum IgG and IgE levels and damage intestinal mucosal integrity in piglets. Intestinal mucosal immunity is the first line of defence against pathogens. In impaired intestinal epithelial mucosa, bacterial strains and toxins are absorbed and cause damage. D-lactic acid and diamine oxidase (DAO), which are indicators of intestinal permeability, can be used to diagnose intestinal mucosal injury and function (Nielsen et al. 2011). The gastrointestinal tract contains approximately 90% of the body 5-hydroxytryptamine (5-HT) (Willemen et al. 2012). Intestinal peristalsis and contraction are mediated by 5-HT receptors. Secretory IgA (sIgA) is the principal antibody of mucosal immunity (Kang et al. 2012).
By now, there are many reports about the allergic reactions in young animals induced by soybean antigen proteins, but the effects of soybean antigen proteins on intestinal permeability and mucosal immunity are scarce. In order to further explore the allergic mechanism of soybean antigen proteins, the effects of glycinin and β-conglycinin on intestinal permeability and sIgA distribution and expression in weaned piglets were evaluated.

Materials and methods

The experimental use of animals and procedures followed were approved by the Anhui Agricultural University Animal Care Committee (Hefei, China).

Characterisation of soybean antigen proteins

Purified glycinin and β-conglycinin powder were kindly donated by Professor Shuntang Guo from the Food Institute of China Agricultural University (patent number: 200,410,029,589.4, China). Following lyophilisation, glycinin and β-conglycinin protein contents were identified and their purity was analysed according to a previous report (Hao et al. 2009). The purity of glycinin and β-conglycinin were 90.6% and 90.0%, respectively.

Piglets and diets

Thirty cross-bred [(Duroc × Landrace) × Yorkshire] piglets (21 d of age) were selected from three litters in the Antai Agricultural Group in Anhui (China). Ten piglets were kept in each pen, which was equipped with a feeder and a waterer to allow ad libitum access to feed and water. The piglets were housed in a mechanically ventilated nursery room with a lighting cycle of 12 h light and 12 h darkness, the temperature was maintained at 26–28°C and the relative humidity was set at 50–60%.

During the experiment, the diet was provided (Table 1) and the diet was formulated to meet or exceed nutrient requirements suggested by the National Research Council (NRC 1998).

Experimental design and samples collection

The experiment had a completely randomised design. All the piglets were randomly divided by weight and gender into a control group, a glycinin-sensitised group and a β-conglycinin-sensitised group, with 10 piglets per group. The piglets of the control group were fed a basal diet. The piglets in the sensitised groups were fed the diet with 4% glycinin or β-conglycinin per kg dietary. The glycinin and β-conglycinin groups were sensitised twice by feeding glycinin or β-conglycinin on d 21–27 and d 32–34. All piglets were weaned at the age of 21 d.

From all piglets, blood samples were collected from the anterior vena cava on d 21, 28 and 35, transferred to ethylenediaminetetraacetate (EDTA)-containing tubes, and allowed to stand for 2 h at room temperature. Plasma samples were obtained after centrifugation at 2000 g for 15 min at 4°C and analysed immediately. At the end of the trial, five piglets from each group were randomly selected by taking advantage of SPSS function, and slaughtered by an intracardiac injection of sodium pentobarbital (50 mg/kg BW) followed by jugular exsanguination. The standard histological procedure was performed as previously described with some slight modification (Li et al. 1999). The intestinal tissues of the duodenum, proximal jejunum, middle jejunum, distal jejunum and distal ileum were fixed in 3–5 volumes of 4% paraformaldehyde solution for 24 h, dehydrated with gradient ethanol and embedded in paraffin. The paraffin sections were used for sIgA determination.

Determination of plasma D-lactic acid, diamine oxidase and 5-HT levels

The plasma D-lactic acid, DAO and 5-HT levels were analysed by using the swine Enzyme-Linked

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**Table 1.** Ingredients and nutrient contents of experimental diets.

| Ingredients, % | Nutrient levels, % unless otherwise stated |
|----------------|------------------------------------------|
| Corn           | 60.85                                    |
| Soya bean meal | 25.00                                    |
| (expanded)a    |                                          |
| Whey powder    | 5.00                                     |
| Fish meal      | 5.00                                     |
| Calcium        | 2.20                                     |
| Hydrogen       | 0.69                                     |
| Limestone      | 0.37                                     |
| Bran           | 0.25                                     |
| NaCl           | 0.49                                     |
| Premixb        | 0.49                                     |
| Choline chloride | 0.15                                  |
| Total          | 100.00                                  |

Nutrient levels, % unless otherwise stated

| Crude protein | 20.80 |
| Calcium       | 0.64  |
| Phosphorus    | 0.51  |
| Lysine        | 1.06  |
| DE, MJ/kg, calculated | 13.50 |

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aExpanded for eliminating the antigen proteins.

bProvided per kg diet: vitamin A, 5250 IU; vitamin D₃, 1050 IU; vitamin E, 4.5 mg; vitamin K₃, 1.2 mg; vitamin B₁₂, 0.375 mg; vitamin B₁, 1.8 mg; vitamin B₂, 0.15 mg; vitamin B₃, 7.5 μg; niacin, 6 mg; Ca-pantothenate, 3.75 mg; folic acid, 0.15 mg; biotin, 7.5 μg; lysine, 0.75 mg; antioxidant (ethoxyquin), 45 μg; enzyme preparation (mixture of amylase, prolase, lipase and phytase), 1000 mg; flavour agents, 40 mg; sweet agents, 40 mg; neomycin, 20 mg; Cu, 15 mg; Fe, 144 mg; Zn, 110 mg; Mn, 10.18 mg; I, 0.4 mg; Se, 0.3 mg.
Immunosorbent Assay Kit (ELISA kit; Rapidbio Company, CA, USA) and the ELISA reader was a microplate reader (Bio-Rad 680, Hercules, CA, USA) as described by the manufacturer.

**Distribution and expression of intestinal sIgA**

The sIgA distributions in the mucosa of the duodenum, proximal jejunum, middle jejunum, distal jejunum and distal ileum were assessed by immunohistochemistry (method PV6000). The intestinal paraffin sections were de-waxed and dehydrated. The antigen of sIgA in each paraffin section was patched in a microwave, blocked with 10% bovine serum albumin, and incubated overnight with primary antibody (1:200) at 4°C. Following the overnight incubation, the antigen was washed three times with 0.1 mol/l phosphate buffer solution (PBS) (5 min each time), incubated with horseradish peroxidase secondary antibody (IgA) for 20 min at 37°C, and washed three times with 0.1 mol/l PBS, pH 7.4 (5 min each time). Following the addition of diaminobenzidine and haematoxylin, the sample was mounted on an Olympus microscope (Olympus, Tokyo, Japan) with neutral resin. The negative control was prepared with PBS buffer as opposed to the primary antibody.

The expression of intestinal sIgA was analysed by the Image-Pro plus 6.0 Analysis Software (Media Cybernetics, USA) according to the images obtained from the immunohistochemistry experiments. The average optical density (OD) value of the relative staining positivity was evaluated for intestinal sIgA positive expression. Five images were randomly selected from each of the three groups and analysed by image analysis software for investigating the OD value of sIgA positive expression. The final data of each group is the average OD value of sIgA expression of five images from five piglets ($n=5$). A tissue can produce many slices, and the optimal slice was selected according to the integrity of cells and tissues in positive expression zone.

**Chemical analysis**

Samples of all feeds were analysed for their nitrogen, calcium and total phosphorus contents using the methods of the AOAC (1990). Nitrogen was analysed using the Kjeldahl method (AOAC official method 988.05), calcium by titration with 0.1 N KMnO$_4$ (AOAC official method 927.02) and the total phosphorus was determined colourimetrically using a molybdovanadate reagent (AOAC official method 965.17). Lysine contents in feed were determined using high-performance liquid chromatography (Shimadzu LC 10 Liquid Chromatograph, Kyoto, Japan).

**Statistical analysis**

Statistical analyses were performed with SPSS 17.0 for Windows statistical software package (SPSS, Inc., Cary, NC). The differences between the groups were examined using analysis of variance. Differences between means were considered statistically significant for values of $p<0.05$.

**Results**

**D-lactic acid levels**

The effects of glycinin and β-conglycinin on plasma D-lactic acid levels are shown in Table 2. On d 21, there were no significant differences in D-lactic acid levels among the three groups ($p>0.05$). However, on d 28 and 35, D-lactic acid levels in the glycinin and β-conglycinin groups were higher than those in the control group ($p<0.01$); the β-conglycinin group had higher D-lactic acid levels than the glycinin group ($p<0.01$).

**Diamine oxidase levels**

The plasma DAO levels are presented in Table 2. On d 21, there were no significant differences in DAO levels among the three groups ($p>0.05$). On d 28 and 35, the glycinin and β-conglycinin groups had higher DAO levels than the control group ($p<0.01$); the β-conglycinin group had higher DAO levels than the glycinin group ($p<0.01$).

**5-Hydroxytryptamine levels**

On d 21, 5-HT levels were not different between the three groups (Table 2). On d 28 and 35, the glycinin and β-conglycinin groups had higher plasma 5-HT levels than the control group ($p<0.01$); the β-conglycinin group had higher DAO levels than the glycinin group ($p<0.01$).

| Item                  | Control | Glycinin-sensitised | β-conglycinin-sensitised | SEM | p value |
|-----------------------|---------|---------------------|-------------------------|-----|---------|
| D-lactic acid, g/l    |         |                     |                         |     |         |
| 21 d                  | 14.5    | 14.64               | 14.54                   | 0.10| n.s     |
| 28 d                  | 14.50A  | 15.34B              | 16.28C                  | 0.08| 0.002   |
| 35 d                  | 14.59A  | 15.51B              | 16.18C                  | 0.06| 0.001   |
| Diamine oxidase, U/l  |         |                     |                         |     |         |
| 21 d                  | 148.30  | 135.45              | 135.45                  | 1.60| n.s     |
| 28 d                  | 144.61A | 176.60B             | 183.36C                 | 1.81| 0.005   |
| 35 d                  | 141.80A | 173.42B             | 190.40C                 | 3.59| 0.006   |
| 5-HT, pg/ml           |         |                     |                         |     |         |
| 21 d                  | 251.86  | 248.00              | 250.00                  | 1.21| n.s     |
| 28 d                  | 254.65A | 291.03B             | 295.36B                 | 1.60| 0.007   |
| 35 d                  | 245.93A | 276.08B             | 286.57C                 | 1.72| 0.009   |

Means not sharing the same superscript in the same row are significantly different at $p<0.01$. 

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group had higher plasma 5-HT levels than the glycinin group on d 35 ($p < 0.01$).

**Intestinal secretory IgA positive distribution**

The immunohistochemistry results of sIgA distribution are shown in Figures 1 and 2. In this study, sIgA-positive cells had brown filamentous regions or pellets around the nuclei; sIgA-negative cells had blue nuclei, unstained or pale blue cytoplasm and colourless cell membranes.

Distribution of sIgA was detected in the duodenum, proximal jejunum, middle jejunum, distal jejunum and distal ileum, mainly between the lamina propria and gland joints (Figures 1C, E, F, H, and 2F) and slightly in the intestinal villi (Figure 1A). Most of the ileum sIgA was distributed in the ileal lamina propria; less sIgA was expressed around the glands (Figures 2G–I). Most of the sIgA-positive cells were brown yellow and round. As shown in Figure 2(B), the jejunal structure disappeared and there were brown yellow cells. Plasma cells synthesise sIgA. There were no colour changes in the negative control group (Figure 1B).

**Expression of secretory IgA**

To investigate the OD value of sIgA expression, five images were randomly selected from each of the three groups and analysed by image analysis software. The results are shown in Table 3. The OD value of the middle jejunum was significantly higher than that of other intestinal segments ($p < 0.01$). The OD value of the β-conglycinin group was significantly higher than that of the glycinin or control groups ($p < 0.01$). In the duodenum and middle jejunum, the OD value of the glycinin group was significantly higher than that of the control group ($p < 0.01$). Similarly, in the proximal jejunum, distal jejunum and distal ileum, the OD value of the glycinin group was higher than that of the control group ($p < 0.05$). The OD value of the duodenum was significantly higher than that of the proximal...
jejunum, distal jejunum or distal ileum ($p < 0.01$). The distal jejunum had the lowest OD value.

**Discussion**

D-lactic acid is the final end-product of bacterial fermentation in the gastrointestinal tract. Increased plasma D-lactic acid levels reflect changes in intestinal permeability (Liu et al. 2008b). DAO is an enzyme that serves as an indicator of intestinal epithelial integrity: when the intestinal mucosa is damaged, serum DOA levels increase (Chang et al. 2012). In this experiment, the piglets were sensitised twice with soybean antigen proteins. The results of this study revealed that compared to the control group, the glycinin and β-conglycinin groups had higher D-lactic acid and DAO levels, consistent with a previous report (Liu et al. 2012). Therefore, the treatment groups had intestinal mucosal damage and serum lactic acid build-up, which represent secondary effects of food allergy that could lead to death in piglets.

**Table 3.** The OD values of sIgA positive expression in different intestinal tissues

| Intestine          | n  | Control     | Glycinin-sensitised | β-conglycinin-sensitised | SEM  | p value |
|--------------------|----|-------------|---------------------|--------------------------|------|---------|
| Duodenum           | 5  | 0.24$^a$    | 0.35$^b$            | 0.39$^c$                 | 0.04 | 0.001   |
| Proximal jejunum   | 5  | 0.20$^{ab}$ | 0.24$^{ab}$         | 0.32$^b$                 | 0.01 | 0.030   |
| Middle jejunum     | 5  | 0.41$^a$    | 0.42$^b$            | 0.44$^b$                 | 0.01 | 0.002   |
| Distal jejunum     | 5  | 0.19$^a$    | 0.21$^a$            | 0.28$^a$                 | 0.01 | 0.045   |
| Distal ileum       | 5  | 0.20$^{ab}$ | 0.23$^{ab}$         | 0.32$^b$                 | 0.03 | 0.016   |

Means not sharing the same superscript in the same row are significantly different. The indicated significance level of capital letters and lower case letters is $p < 0.01$ and $p < 0.05$, respectively.
More than 90% of 5-HT is stored in intestinal enterochromaffin cells; 8–9% is present in platelets (Willemen et al. 2012). Following an allergic reaction, enterochromaffin cells are damaged, thereby releasing 5-HT, which increases the strength and rate of intestinal peristalsis, thereby contributing to diarrhoea (Kojima et al. 2012). Following the first sensitisation test, 5-HT levels of the glycinin and β-conglycinin groups were significantly higher than those of the control group. However, following the second sensitisation test, the 5-HT levels decreased, and there were no significant differences between the glycinin and β-conglycinin groups. At 35 d, 5-HT levels decreased, consistent with the findings of another study (Tong et al. 2010). The glycinin and β-conglycinin groups had higher 5-HT levels than the control group. Therefore, soybean antigen proteins can indirectly stimulate the cells to secrete 5-HT in piglets.

SIgA is synthesised by plasma cells in the lamina propria, which is widely distributed on mucosal surfaces. SIgA is the main antibody in the intestinal mucosa. When SIgA is released into the intestinal lumen, it binds to antigens, inhibits the proliferation of bacteria, neutralises toxins, protects the intestinal mucosa and resists the effect of fibrinolytic enzymes (Curi et al. 2005; Bertolo & Burrin 2008). The SIgA distribution in the duodenum, proximal jejunum, middle jejunum, distal jejunum and distal ileum was assessed with an immunohistochemical method. The results revealed that SIgA-positive cells were mainly localised in the middle jejunum. The β-conglycinin group had high SIgA expression levels and OD values, which may be attributed to the allergenicity of β-conglycinin.

The results revealed that SIgA expression was mainly localised close to the enteraden, consistent with other reports (Wang et al. 2000). In normal circumstances, plasma cells are present in the jejunum; SIgA is mostly released around the glands, which explains why the OD values of the middle jejunum were higher than those of other intestinal sections. Moreover, the distribution of SIgA-positive cells in this study was similar to that reported by Shen et al. (2014). Even though SIgA levels were determined, the correlation between SIgA/allergenic substances and the mucosal immune system is not fully understood; further studies are needed.

Conclusions

Glycinin or β-conglycinin protein could increase the plasma levels of D-lactic acid, DAO and 5-HT, and enhance the SIgA distribution and average OD values of SIgA positive expression in the duodenum, proximal jejunum, middle jejunum, distal jejunum and distal ileum, cause allergic reactions in piglets, resulting in intestinal mucosal damage. Additionally, by damaging the intestinal mucosa, there was increased intestinal permeability. The intestinal mucosal damage induced by β-conglycinin protein was higher than that of glycinin protein in weaned piglets.

Disclosure statement

The authors report no conflicts of interest. The Authors alone are responsible for the content and writing of this article.

Funding information

This study was jointly supported by the National Natural Science Foundation of China [No. 31472250] and the National ‘Fu Min Qiang Xian’ Program of China [No. 2012-745].

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