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Role of soybean β -conglycinin subunits as potential dietary allergens in piglets

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ABSTRACT

β -Conglycinin, a major seed-storage protein in soybeans, is one of the primary antigenic proteins responsible for soybean-meal hypersensitivity in weaned piglets. The protein is a heterotrimer composed of subunits α , α' and β . It is currently unknown which of the β -conglycinin subunits are allergenic for piglets. The aim of this study was to identify potential allergenic subunits of β -conglycinin for soybean sensitive piglets and to characterise these subunits by immunoglobulin (Ig) G and E immunoblotting, ELISA, 'skin prick' and whole blood histamine-release testing. The IgG and IgE binding capabilities of the purified α , α' and β subunits of β -conglycinin were determined by immunoblot analysis and ELISA with sera from β -conglycinin sensitised piglets. Skin prick testing and whole blood histamine release testing were also performed to detect the activated effector cell response to specific allergens. Specific IgG and E antibodies were identified that recognised all three subunits of β -conglycinin in the sera of β -conglycinin sensitised piglets. All three subunits of β -conglycinin elicited positive skin test and specific histamine release responses from the whole blood of β -conglycinin sensitised piglets. These results suggest that all three β -conglycinin subunits are potential allergens for piglets.

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Introduction

Soybeans are one of the most important sources of high quality food protein globally due to their well-balanced amino acid profile, which complements the amino acid pattern in cereal grains (Friedman and Brandon, 2001). However, soybeans also acts as allergens for human beings (Foucard and Malmheden Yman, 1999), pigs (Li et al., 1990), cattle (Kilshaw and Sissons, 1979; Dawson et al., 1988; Gardner et al., 1990), mice (Mowat and Ferguson, 1981), and salmon and trout (Nordrum et al., 2000; Buttler et al., 2001). Of these species, piglets are the most commonly affected, often developing transient hypersensitivity following weaning onto a soybean diet (Li et al., 1990, 1991; Bailey et al., 1993; Friesen et al., 1993; Dreau et al., 1995). This hypersensitivity response results in decreased growth rate and reduced general health in the immediate post-weaning period, and is mediated by immunoglobulin (Ig) E produced by Th2 lymphocytes (Sun et al., 2008). It has been speculated that the intestinal damage that occurs in piglets post-weaning, may be caused by antibody-mediated immune reactions to soybean proteins (Hall and Byrne, 1989).

β -Conglycinin, a main 'storage' protein, accounts for approximately 30% of all seed proteins (Maruyama et al., 2001) and is considered to be the primary antigen responsible for soybean-meal hypersensitivity in

weaned pigs (Li et al., 1990; Zhao et al., 2008; Hao et al., 2009). β -Conglycinin consists of three subunits: α (68 kDa), α' (72 kDa) and β (52 kDa) (Thanh and Shibasaki, 1977), which share extensive amino acid sequence homology (Harada et al., 1989; Maruyama et al., 1998). Despite these similarities, it is the α subunit, and not the highly homologous α' and β subunits, that induces immunoglobulin (Ig) G production in sensitised piglets (Fu et al., 2007).

To date, there has been limited research focussing on the immunogenicity/allergenicity of β -conglycinin constituent subunits in the pig. It is important to elucidate which subunits serve as allergens if we are to better understand the transient hypersensitivity to this protein that occurs in pigs. The aims of the present study were to assess the allergenic potential of each of the β -conglycinin subunits by immunoblot analysis, ELISA, skin prick testing and whole blood histamine-release testing.

Materials and methods

Animal selection and experimental protocol

All experiments were approved by the Jilin Agricultural University Animal Care and Use Committee (reference number 2008-30430520-01). Three pregnant Landrace sows were fed a soybean-free diet from day 108 of pregnancy until the end of their lactations to limit colostral transfer of maternally-derived anti-soybean antibodies to their piglets (Li et al., 1990). The crossbred (Yorkshire $\times$ Landrace) piglets used in this experiment were weaned at 28 days old and housed in an environmentally controlled room. One week after weaning, four piglets from each of the three litters were randomly allocated to one of two treatment groups. A total of 12 piglets

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(35 days old; 10.72 ± 1.99 kg bodyweight) were selected from the three litters; each of the two treatment groups contained six animals. Each piglet was individually penned; each pen was equipped with a feeder and nipple drinker so that the animals had ad libitum access to food and water.

Since the present study was focused on the assessment of the allergenicity of β -conglycinin constituent subunits, purified β -conglycinin was used to sensitise the piglets to avoid interference from other allergenic components in the soybean meal. The purified β -conglycinin used in the animal diets was extracted from soybean cultivar fengjiao 7607 by a simplified purification method (Thanh and Shibasaki, 1976). The control diet, devoid of soybean protein, contained 4% spray-dried porcine plasma, 8% fish meal and 10% corn-gluten meal on an air-dry basis as protein sources. The purified β -conglycinin substituted for all of the above proteins in the test diet. Both diets consisted of 66% corn on an air-dry basis as an energy source and were formulated to meet or exceed National Research Council (1998) requirements (Table 1).

The six piglets in the exposed group were orally sensitised and challenged with the experimental diet from days 0–10 (sensitisation) and days 18–20 (challenge), and were then challenged with the experimental diet at 1 week intervals, each for three consecutive days; they were fed the control diet at all other times. The six piglets in the control group were fed the soybean-free control diet at all times.

All piglets were weighed on days 0 and 10 to determine average daily weight gain (ADWG). Any episodes of diarrhoea were recorded daily throughout the experiment; faecal consistency was graded at four levels (0, solid; 1, semi-solid; 2, semi-liquid; and 3, liquid) (Yuan et al., 1996; Azevedo et al., 2006). Diarrhoea was defined as grade 2 or 3 faecal consistency over a minimum of two consecutive days (Taras et al., 2006). The prevalence of diarrhoea (%) was calculated as the percentage of the number of piglets with diarrhoea divided by the total number of piglets (Taras et al., 2006).

A blood sample (8 mL) from each piglet was collected by vena caval puncture on the morning after the final oral challenge (day 40), and the serum was separated and stored at -80°C for ELISA and immunoblot analysis. Heparinised venous blood (2 mL) was taken at the same time from all animals for whole blood histamine release testing.

Table 1
Composition of β -conglycinin sensitising and control diets.

	Diet	
	Control	Sensitising
<i>Composition (%)</i>		
Corn	66.0	66.0
Corn gluten meal	10.0	0.0
Fish meal	8.0	0.0
Spray-dried porcine plasma	4.0	0.0
Soybean β -conglycinin	0.0	18.0
Whey powder	6.0	6.0
Corn oil	2.0	2.0
Premix ^a	1.0	1.0
Choline chloride	0.1	0.1
Corn starch	1.0	3.8
MDCP ^b	0.4	1.2
Limestone	0.8	1.0
Salt	0.0	0.3
L-Lysine-HCl	0.60	0.15
DL-Methionine	0.00	0.20
L-Threonine	0.10	0.25
<i>Chemical analysis</i>		
Crude protein (%) ^c	20.10	20.17
Calcium (%) ^c	0.69	0.70
Phosphate (%) ^c	0.59	0.59
Ileal digestible lysine (%) ^d	1.14	1.12
Ileal digestible methionine and cysteine (%) ^d	0.64	0.68
Ileal digestible threonine (%) ^d	0.66	0.68
Ileal digestible tryptophan (%) ^d	0.21	0.73
Ileal digestible valine (%) ^d	0.80	0.76
Ileal digestible isoleucine (%) ^d	0.67	0.72
Ileal digestible leucine (%) ^d	1.79	1.62
Metabolisable energy (Mcal/kg) ^d	3.26	3.25

^a Premix/kg of complete diet: vitamin A 2200 IU; vitamin D3 220 IU; vitamin E 16 IU; vitamin K 0.5 mg; vitamin B1 1 mg; vitamin B2 3.5 mg; vitamin B6 1.5 mg; vitamin B12 0.175 mg; pantothenic acid 10 mg; niacin 15 mg; folic acid 0.3 mg; biotin 0.05 mg; copper 150 mg; iron 100 mg; zinc 100 mg; manganese 4 mg; selenium 0.3 mg; iodine 0.14 mg.

^b MDCP, monocalcium and dicalcium phosphate.

^c Analysed value.

^d Calculated value.

Western blot analysis

β -Conglycinin was extracted from soybean cultivar fengjiao 7607 by isoelectric point precipitation (Liu et al., 2007) and the α , α' and β subunits were purified using a combination of ion exchange and metal affinity chromatography (Zheng et al., 2012). Electrophoresis was performed on slab gels consisting of a 12% acrylamide resolving gel and a 5% stacking gel. Purified subunit samples were dissolved in $1 \times$ sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer (50 mmol/L Tris–HCl, 2% sodium dodecyl sulphate, 10% glycerol, 1% β -mercaptoethanol, 0.1% bromophenol blue, pH 6.8) and heated in a boiling water bath for 6 min. An aliquot (10 μL) of the sample solution was loaded onto the gel and resolved by SDS–PAGE. Gels were then stained with 0.1% Coomassie brilliant blue.

For immunoblot analysis of IgG against the purified subunits of β -conglycinin, serum proteins resolved by SDS–PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). This membrane was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.3) for 2 h at room temperature and then incubated with appropriately diluted individual sera from sensitised or control piglets. Following three washings with TBS containing 0.05% Tween-20 (TBST), the membrane was incubated with a 1:3000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-pig IgG secondary antibody (Beijing Biosynthesis Biotechnology). The membrane was then washed three times with TBST and any immunoreactive proteins were detected with 3,3'-diaminobenzidine (DAB) substrate (Tiangen Biotech).

For immunoblot analysis of IgE, the PVDF membrane containing blotted proteins was blocked with 3% BSA in PBST and then incubated with appropriately diluted individual sera from sensitised or control piglets for 18 h at 4°C with gentle rocking. Following four washings with PBST, the membrane was incubated with biotinylated anti-pig IgE (RapidBio) and HRP-conjugated streptavidin (Beijing Biosynthesis Biotechnology). The membrane was then washed a further four times and visualised by chemiluminescence (Super Enhanced Chemiluminescence Detection Kit, Applygen Technologies).

ELISA, skin prick and histamine release tests

An indirect ELISA was used to quantify IgG against the β -conglycinin subunits in sera (Helm et al., 2002). An ELISA plate was coated with a subunit solution (5 $\mu\text{g}/\text{mL}$ protein in 100 mmol/L sodium carbonate buffer, pH 9.6), then 20-fold diluted serum from a piglet was added. After incubation with HRP-conjugated rabbit anti-pig IgG (Beijing Biosynthesis Biotechnology), phenylenediamine was used to visualise antibody binding. Absorbance was measured at 492 nm; data are expressed in optical density (OD) units.

The ability of the purified subunits to activate dermal mast cells in vivo was determined by skin prick testing (Dreau et al., 1994; Sun et al., 2008). This test was performed on day 30 by intradermal injection of 0.5 mg of the various purified subunits in physiological saline solution into previously shaved flank regions of individual piglets. Thirty minutes after injection, the diameters of erythematous areas were measured using Vernier calipers.

The histamine-release capacity of the purified β -conglycinin subunits was determined using the method of Batanero et al. (1999). Heparinised blood was diluted 1:7 in buffer consisting of 25 mmol/L piperazine-1,4-bis(2-ethanesulphonic acid), 110 mmol/L NaCl, 5 mmol/L KCl and 2.0 mmol/L CaCl_2 pH 7.35, then 100 μL of diluted blood was incubated with 50 μL purified β -conglycinin subunits (3 $\mu\text{g}/\text{mL}$) at 37°C for 45 min in 96-well microtitre plates. The reaction was terminated by placing the plates on ice. Blood cells were then centrifuged at 300 g at 4°C , and any histamine in the cell-free supernatant was measured using a pig histamine ELISA kit (RapidBio). Spontaneous histamine release was measured by incubating the diluted blood in the buffer without purified proteins under the same conditions. The total amount of histamine released into the supernatant was determined after cell lysis in distilled water and freeze–thawing of the blood cells. The whole blood histamine-release rate was calculated using the following equation:

$$\text{Histamine release ratio (\%)} = \frac{([\text{allergen-stimulated histamine release} - \text{spontaneous release}]/[\text{total histamine release} - \text{spontaneous release}]) \times 100\%}{}$$

Statistical analysis

Differences in growth rate, antibody levels, erythematous reactions and histamine release rates between the groups were analysed by using Student's *t* test in SAS. Differences in the occurrence of diarrhoea between treatment groups were analysed by the χ^2 contingency test. *P* values <0.05 were considered to be significant.

Results

Growth rates and occurrence diarrhoea

Sensitised piglets had lower ADWG ($P = 0.017$), but higher feed-to-weight gain ratios (F:G) ($P = 0.003$), compared with controls,

although the average daily feed intake (ADFI) was similar ($P = 0.697$) (Table 2). Two pigs from the sensitised group produced semi-liquid or liquid faeces on at least two consecutive days, whereas none of the controls had diarrhoea; the difference between the groups was not statistically significant.

Antibody production

Using Western blot analysis, serum IgG (Fig. 1) and IgE (Fig. 2) from sensitised pigs reacted specifically with all three β -conglycinin subunits (α , α' and β), whereas serum from control pigs did not react with any of the three subunits. Using an indirect ELISA to quantify IgG, serum from sensitised piglets exhibited strong IgG binding to all three β -conglycinin subunits (Table 3). Levels of IgG against all three β -conglycinin subunits were significantly higher than in control pigs ($P = 0.011$, 0.047 and 0.008 , respectively).

Table 2

Effects of β -conglycinin supplementation on growth performance during the sensitisation period and occurrence of diarrhoea in β -conglycinin sensitised and control piglets.

Parameter	Controls	Sensitised group	<i>P</i> value ^a
ADWG (g/day)	550.4 $\pm$ 106.9	402.4 $\pm$ 69.0	0.017
ADFI (g/day)	991.4 $\pm$ 149.0	958.3 $\pm$ 136.7	0.697
F/G	1.80 $\pm$ 0.16	2.38 $\pm$ 0.28	0.003
Occurrence of diarrhoea (%)	0 (0/6)	33.3 (2/6)	>0.05

ADWG, average daily weight gain; ADFI, average daily feed intake; F/G, feed to weight gain ratio.

Data are presented as group means $\pm$ standard deviations of six piglets/group.

^a χ^2 contingency test.

Skin prick and histamine release responses

The diameters of areas of skin erythema measured 30 min following intradermal injection of the purified β -conglycinin subunits were significantly larger in sensitised piglets compared to control piglets ($P = 0.004$, 0.036 and 0.010 for the α , α' and β subunits, respectively) (Table 4).

Rates of histamine release from whole blood induced by the three β -conglycinin subunits were significantly higher in sensitised piglets relative to the control group ($P = 0.006$, 0.009 and 0.001 for the α , α' and β subunits, respectively) (Table 5).

Discussion

Hypersensitivity to soybean protein represents a significant animal health problem (Miller et al., 1984a; Gardner et al., 1990; Nordrum et al., 2000; Buttle et al., 2001), occurring particularly frequently in weaned piglets (Armstrong and Clawson, 1980; Li et al., 1990, 1991). β -conglycinin is a major allergenic soybean protein that transiently sensitises piglets abruptly weaned onto such diets, resulting in reduced growth performance and diarrhoea (Newby et al., 1984; Li et al., 1991; Zhao et al., 2008; Hao et al., 2009). The severity of this post-weaning diarrhoea is a criteria used to evaluate the degree of piglet sensitisation (Miller et al., 1984b; Helm et al., 2002; Brandt et al., 2003; Herman, 2005; Sun et al., 2008). Although previous studies have demonstrated reduced weight gain and lowered feed efficiency in piglets fed β -conglycinin, in the present study there were no significant differences in the occurrence of diarrhoea between the groups, probably because of the small amounts of purified β -conglycinin fed to piglets.

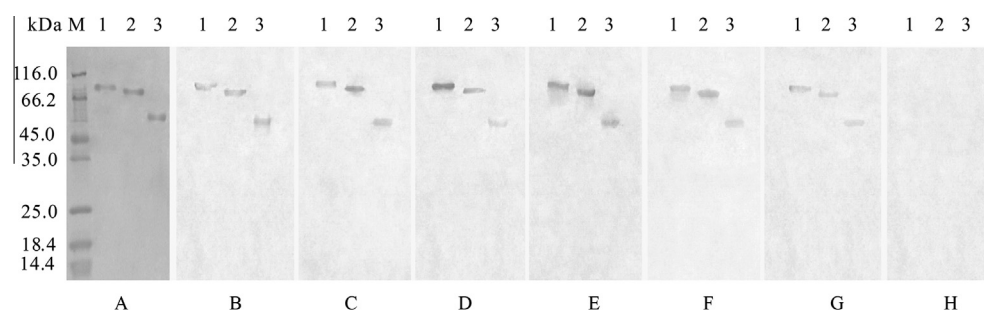


Fig. 1. SDS-PAGE and immunoglobulin (Ig) G immunoblotting of the α , α' and β subunit proteins of soybean β -conglycinin. (A) Purified subunits resolved on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue. (B–G) Proteins in panel A were transferred to PVDF membranes and probed with individual sera from sensitised piglets. (H) Representative immunoblot probed with sera from control piglets. IgG-binding proteins were identified using HRP-conjugated anti-pig IgG followed by DAB and detected by chemiluminescence. Lane M, protein marker; lanes 1–3, purified α , α' and β subunit proteins, respectively.

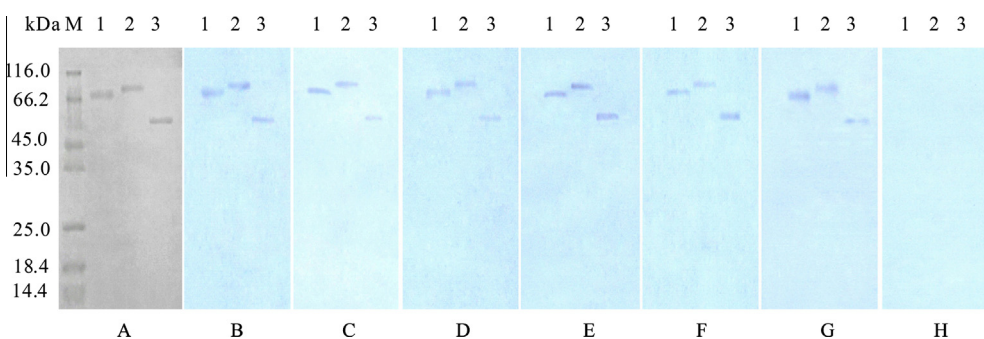


Fig. 2. SDS-PAGE and immunoglobulin (Ig) E immunoblotting of the α , α' and β subunit proteins of soybean β -conglycinin. (A) Purified subunits resolved on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue. (B–G) Proteins in panel A were transferred to PVDF membranes and probed with individual sera from sensitised piglets. (H) Representative immunoblot probed with sera from control piglets. IgE-binding proteins were identified using HRP-conjugated anti-pig IgE followed by DAB and detected by chemiluminescence. Lane M, protein marker; lanes 1–3, purified α , α' and β subunit proteins, respectively.

Table 3

Specific immunoglobulin (Ig) G levels by ELISA in the sera of β -conglycinin sensitised and control piglets (OD_{492nm}).

Groups	β -Conglycinin subunit proteins		
	α Subunit	α' Subunit	β Subunit
Sensitised group	0.61 $\pm$ 0.21	0.46 $\pm$ 0.17	0.57 $\pm$ 0.16
Controls	0.29 $\pm$ 0.06	0.28 $\pm$ 0.08	0.30 $\pm$ 0.07
P value ^a	0.011	0.047	0.008

Data are presented as group means $\pm$ standard deviations of six piglets/group.

^a P values for two-group Student's *t* test.

Table 4

Skin prick test responses of β -conglycinin sensitised and control piglets to β -conglycinin subunit proteins.

Groups	β -Conglycinin subunit proteins		
	α Subunit	α' Subunit	β Subunit
Sensitised group	6.1 $\pm$ 2.4	5.2 $\pm$ 3.1	5.6 $\pm$ 2.3
Controls	1.6 $\pm$ 0.9	1.7 $\pm$ 1.0	2.1 $\pm$ 1.4
P value ^a	0.004	0.036	0.010

Diameter of areas of erythema (mm) triggered by intradermal injection of subunit proteins.

Data are presented as group means $\pm$ standard deviations of six piglets/group.

^a P values for two-group Student's *t* test.

Table 5

Whole blood histamine release responses of β -conglycinin sensitised and control piglets to β -conglycinin subunit proteins (%).

Groups	β -Conglycinin subunit proteins		
	α Subunit	α' Subunit	β Subunit
Sensitised group	35.8 $\pm$ 13.6	29.8 $\pm$ 11.8	32.6 $\pm$ 9.4
Controls	11.7 $\pm$ 3.7	10.8 $\pm$ 4.0	9.3 $\pm$ 3.2
P value ^a	0.006	0.009	0.001

Data are presented as group means $\pm$ standard deviations of six piglets/group.

^a P values for two-group Student's *t* test.

The similarities among the amino acid sequences of the three β -conglycinin subunits (α , α' and β) are 86.8% between α and α' , 75.5% between α and β , and 71.4% between α' and β (Maruyama et al., 1998) respectively. Previously, IgG directed against the α subunit, but not the α' or β subunits, was demonstrated in the sera of soybean sensitised piglets (Fu et al., 2007). In the present study, piglets fed a soybean diet developed IgE directed against all three subunits of the β -conglycinin.

The presence of allergen-specific antibodies does not always correlate with clinical signs (Atkins et al., 1985; Bernhisel-Broadbent et al., 1992). The ELISA and Western blot data reflect the systemic humoral response, whereas the skin prick test provides information relating to the direct activation of specific effector dermal mast cells and the whole blood histamine release test provides information relating to the activation of a serum effector cell response (Du Buske, 1993). The results of the skin prick and whole blood histamine release tests supported our serological findings that all three protein subunits of β -conglycinin are allergenic.

Although allergen avoidance is effective in preventing allergic disease (Zeiger, 2003), this has to be balanced against the advantages of using soybean meal in pig rations, given its relatively well-balanced composition of amino acids and its relatively low cost. However, the production and use of hypoallergenic soybean products may be feasible (Li et al., 1991) through the application of food processing techniques (Ogawa et al., 2000), selection of mutant strains (Samoto et al., 1996; Joseph et al., 2006) and genetic

engineering (Herman et al., 2003). Further studies focussed on the precise identification and characterisation of the allergenic components of soybeans at a molecular level may assist in providing a clearer understanding of this disease.

Conclusions

This study demonstrated that all three subunits of soybean β -conglycinin (α , α' and β) reacted with both serum IgG and IgE, and induced positive skin prick and histamine release responses in piglets experimentally sensitised to this protein. These findings suggest all three subunits are potentially allergenic for pigs.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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