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Original Article

Soybean β-conglycinin Induces Intestinal Immune Responses in Chicks

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ABSTRACT

β-conglycinin from soybean has been recognized as one of the major feed allergens. This study investigated the effects of β-conglycinin-induced allergic sensitization on chicks' small intestines. A total of 40 7-day-old (100 g) chicks were divided into four groups as control, β-conglycinin 1 h, β-conglycinin 6 h, and β-conglycinin 12 h. All treatment groups were administered 60 mg of β-conglycinin/chick and small intestine samples were collected. β-conglycinin-induced allergic sensitization marginally damages the epithelium lining of the duodenum villi and, in addition, significantly increases the accumulation of mast cells in the lamina propria and crypt of the duodenum. Moreover, the TNF-α level significantly increased in all β-conglycinin groups. IL-8 and IL-2 were significantly downregulated in the 1 h group; however, there were increases for the 6 h and 12 h groups. These results suggest that β-conglycinin may lead to an inflammatory response in the chicks' small intestines.

INTRODUCTION

Soybean, a member of the legume's family, is a good protein source because of a high protein composition (37-42%) with a rich and good balance of amino acids as well as a nutritional and healthy food (Radder & Husen 2017). However, they have a high content of storage proteins that inhibit their nutrient function and can also cause inflammation and allergic reactions (Boehm *et al.* 2017). It reduces the nutritional value of soybeans. Most storage proteins consist of globulin (70%), the main components being β-conglycinin (7S) and glycinin (11S) (Wu *et al.* 2017). These are known as allergy-causing proteins in soybeans. β-conglycinin and glycinin were administered to animals and it was discovered that the former had a greater influence on the immune response of the small intestine (Zhao *et al.* 2008). There have been many attempts to extract β-conglycinin from soybeans and to feed rodents, pigs and fishes. In the experiments feeding the purified β-conglycinin from soybean, it reduced animal growth and increased feed requirement (Zhao *et al.* 2008), histamine and IgE levels (Hao *et al.* 2009), induced passive cutaneous anaphylaxis, upregulated CD4+ lymphocyte ratio, and increased cytokine concentrations of plasma and spleen (Guo *et al.* 2007). Based on these results, β–conglycinin stimulated the immune response and induced an allergic reaction through the digestive tract.

However, studies on chickens are very rare and the fact that chickens have very different digestive organs, compared to other monogastric animals, this provides a good reason for more research.

In this study, isolated β-conglycinin from soybean was confirmed by SDS-PAGE and β-conglycinin was treated to chicks. Afterwards, the small intestine's histology and mRNA expression which affect the mechanisms of inflammatory reactions on chicks were analysed.

MATERIALS AND METHODS

Isolation of β-conglycinin and glycinin

Soybean was purchased from an agricultural cooperative of Soyang in Korea. Soybean flour (60 mesh) was defatted with hexane (1:8, w/v, soybean flour/hexane). The β-conglycinin (7S) and glycinin (11S) were isolated according to the modified method of Thanh *et al.* (1975).

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

SDS-PAGE was performed with whole soybean protein (WSB), β-conglycinin, and glycinin for comparison and confirmation. The protein concentration of soybean fractions was determined with the DC protein assay kit (Biorad, USA) using bovine serum albumin as a standard, following the manufacturer's instructions. Electrophoresis of soybean fractions was performed by 12% acrylamide (the acrylamide/bis-acrylamide ratio was 29:1) using Mini-Protein tetra cell (Biorad, USA). Protein separation was carried out at 20 mA. Afterwards, the gels were stained using Coomassie blue R-250 (R = reddish hue) and scanned by Bio-5000 plus (Microtec Co.).

Experimental animals

A total of 40 7-day-old broiler chicks with an average weight of 100 g were used in this experiment. Ten chicks per group were randomly allocated: the control group (C) caught first; those caught 1 h after administration (1 h); 6 h after administration (6 h); 12 h after administration (12 h). β-conglycinin that was isolated from soybean for oral administration was prepared in phosphate buffered saline (PBS) (60 mg β-conglycinin/100 µl PBS/chick). After 24 h of fasting, ten chicks from the control group (C) were administered with only PBS, and the other thirty chicks of β-conglycinin treatment groups (1h, 6h and 12h groups) were administered β-conglycinin. All chicks received water ad libitum. Then randomly six chicks were selected for liver sample per treatment group. Small intestine samples were collected and separated into two types: one was put into 10% neutral buffered formalin (NBF) for histology and the other was immediately put into liquid nitrogen for qRT-PCR.

Histological analysis

The small intestine (duodenum) of the experimental chick was excised for histopathological examination. Samples were immediately fixed in 10% neutral buffer formalin (NBF). After 24 h fixation, the samples were trimmed and processed using an auto processor (Excelsior ES, Thermo Scientific, USA) and embedded in paraffin wax. Sections of 5 µm duodenum tissue were stained with hematoxylin and eosin (H&E) and mounted on a glass slide. Digital images were picked up using a Leica DM2500 microscope (Leica Microsystems, Germany) at fixed 100 × magnifications.

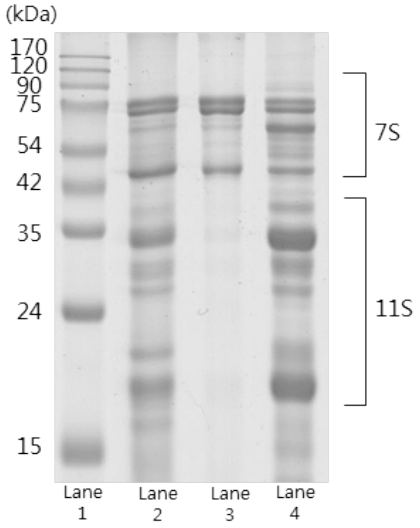

Figure 1 – SDS-PAGE of glycinin and β-conglycinin from soybean. Lane1: marker, Lane2: whole soybean, Lane3: β-conglycinin soybean, Lane4: glycinin from soybean.

Mast cell counting

The duodenum of experimental chicks was excised and immediately fixed in 10% NBF. After 24 h fixation, the samples were trimmed and processed by using the auto processor (Excelsior ES, Thermo Scientific, USA) and embedded in paraffin wax. 5 um sections of intestinal tissue were stained with toluidine blue (0.1%) for identification of mast cells and mounted on a glass slide. Digital images were picked up using Leica DM2500 microscope (Leica Microsystems, Germany) at fixed 100 x magnifications. The number of total mast cells (violet color/red purple color) was counted manually.

qRT-PCR

Total RNA was extracted from the duodenum using the AccuZol total RNA extraction reagent (Bioneer, Korea) according to the manufacturer's manual; quality and concentration of extracted RNA were determined with a microplate reader (NanoDrop, Thermo Fisher). cDNA was synthesized with AccuPower Cycle Script RT Premix (dT20) (Bioneer, Korea). Primer sequences are shown in Table 1. All primers were designed by the Primer 3 (v. 0.4.0) software and synthesized by Bioneer Corporation (Bioneer, Korea). The realtime qPCR

mixture was prepared using SYBR green fluorescence (SsoFast™ EvaGreen® Supermix, Biorad, USA) and quantification was performed by CFX96™ Real-Time PCR detection system (Biorad, USA) following the manufacturer's protocol. Every gene expression was analyzed and normalized using GAPDH (reference gene) with a duplicate.

Table 1 – Composition of basal diets.

Items	Composition
Ingredients, g/kg	
Corn	504.3
Corn gluten meal	35.0
Soybean meal	357.2
Soybean oil	60.0
DCP	18.6
Limstone	15.4
99%-Methionine	1.5
Salt	2.0
Vitamin-mineral premix	5.0
Sodium bicarbonate	1.0
Total	1000
Nutrients*	
Metabolisable energy, kcal/kg	3113.56
Crude protein, %	23
Lys	1.19
Met+Cys	0.85
Met	0.50
Ca	1.00
Available phosphorus	0.45

*Calculated value based on the analysed data of experimental diets.

Statistical analysis

The results were presented as means ± standard error (SE). All data were analyzed using an ANOVA (analysis of variance) procedure with the SAS software version 9.4 (SAS institute Inc.). Statistical significance was indicated at $p<0.05$, based on Duncan's Multiple Range Test. TNF-α, IL-8, IL-2, and IL-1 were statistically evaluated by Student's t-test for unpaired comparisons.

RESULTS AND DISCUSSION

β-conglycinin and glycinin isolation

In this experiment, a method for isolating and purifying soybean insoluble proteins, β-conglycinin and glycinin, was constructed for the specification experiment using the insoluble protein β-conglycinin. The proteins were separated by SDS-PAGE, and the positions of the same proteins were compared and confirmed by previous studies (Wang *et al.* 2014, Yamada *et al.* 2014, Perrechil *et al.* 2015). Compared with previous studies, β-conglycinin was well separated, but the glycinin was not separated purely.

Modification of duodenum histology of chicks sensitized with β-conglycinin

To evaluate the effects of β-conglycinin on the small intestine of chicks after oral administration, modifications of the duodenum's histological structure were observed and revealed that they were marginal in experimental chicks compared to the control group (Figure 2). We found that the epithelium lining of the duodenum tips' villi was slightly damaged by β-conglycinin-induced allergic sensitization. β-conglycinin is the key storage protein of soybeans which is a prospective diagnostic indicator for allergic reactions to soy (Wu *et al.* 2016, Ashaolu & Yupanqui 2017). Evidence showed that histological damages of small intestines are a common feature in animal oral gavages of soybean allergens (Liu *et al.* 2008, Zhang *et al.* 2013). β-conglycinin originating from undigested soybean meal may cause damage of the intestinal epithelial mucosa and concurrently disturb nutrient absorption (Dunsford *et al.* 1989). Serum immunoglobulins (IgE and IgG1) were associated with the allergic sensitization of chicks. These antibodies stimulated the intestinal mast cells' degranulation and caused releases of elevated plasma histamine that leads to intestinal damages and contributes to systemic anaphylaxis (Kumar Gupta *et al.* 2016, Taketomi *et al.* 2017).

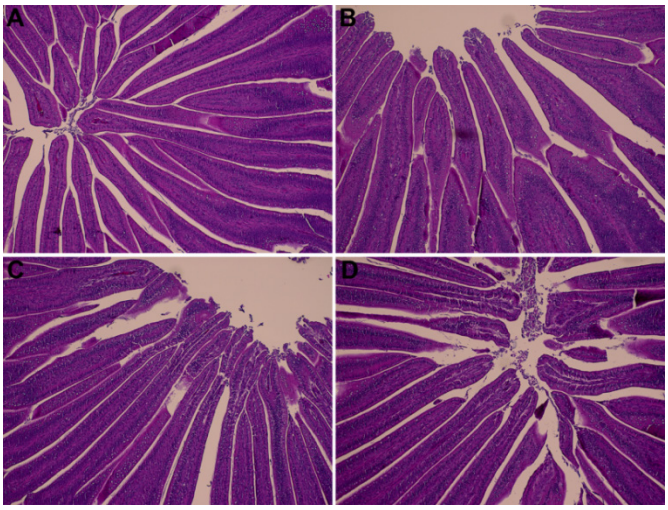

Figure 2 – β-conglycinin prompts damages to the duodenum's histological structure in experimental chicks (A. control, B. 1 h, C. 6 h, and D. 12 h).

β-conglycinin provokes the mast cell number in the duodenum

Mast cell number in the small intestine is an important marker of allergic sensitization (Hao *et al.* 2010). The activates and inactivates of mast cells and histamine are closely linked and we can divide them into two processes. First, the mast cell is activated by the antigen-specific IgE and release hiatamine. Second,

Table 2 – Real time quantitative primer sequences.

Gene name	Product size (bp)	strand	Sequence (5′-3′)
TNF-α	395	Sense	AGA TGG GAA GGG AAT GAA CC
		antisense	ACT GGG CGG TCA TAG AAC AG
IL-8	371	Sense	GCT CTG TCG CAA GGT AGG AC
		antisense	GCG TCA GCT TCA CAT CTT GA
IL-2	212	Sense	ACC GGA AGT GAA TGC AAG AT
		antisense	AGT GGT CCC AGA ATG GAC AG
IL-1	263	Sense	GCA TCA AGG GCT ACA AGC TC
		antisense	CAG GCG GTA GAA GAT GAA GC
GAPDH	133	Sense	AGA ACA TCA TCC CAG CGT CC
		antisense	CGG CAG GTC AGG TCA ACA AC

the released histamine concentration causes the mast cell to degranulation and secrete cytokines (Guo *et al.* 2008). Therefore, we determined their total number in the duodenum. A number of mast cells markedly increased at 6 h and 12 h in the β-conglycinin treated group as compared to the control (Figure 3). According to our results, upregulated mast cells can release histamines. The intestinal damages allow antigens to pass first into the systemic circulation, prompting the release of specific cytokines. Evidence exists that histamine levels in β-conglycinin-sensitized animals were significantly elevated as a result of mast cell accumulation in the small intestine (Liu *et al.* 2008, Sun *et al.* 2008). Consistent with such reports, we found an increased number of mast cell accumulations in the chicks' duodenum after β-conglycinin administration. However, (Liu *et al.* 2008) showed that the mast cell from BALB/c mice significantly decreased after 14 days after sensitized by β-conglycinin, caused by histamine release. It can be inferred that this is the result of the second processing by histamine.

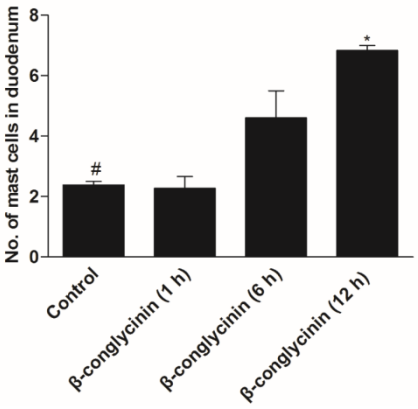

Figure 3 – Quantification of duodenum mast cells in β-conglycinin-treated chicks. #,*: Columns with different superscripts differ (p<0.05).

TNF-α, IL-8, IL-2, and IL-1 expression by β-conglycinin

To investigate the effect of β-conglycinin on chicks, inflammation-related cytokines such as TNF-α, Il-8, Il-2, and Il-1 genes were analyzed by qRT-PCR (Figure 4). TNF-α, IL-8, IL-2 and IL-1 are cytokines, small proteins, that mediate inflammatory responses and that activate macrophages and releases by cells to send signals to other cells and are indicators to pro-inflammatory. TNF-α is related to allergies and asthma induction (Nguyen *et al.* 2016). The level at which TNF-α increased depended on time. This result indicated that inflammation occurred in the small intestine. Increased IL-8 levels were found in patients' inflammatory areas and associated with cancer, acute myeloid leukemia, chronic obstructive pulmonary disease, and asthma (Beigelman *et al.* 2015). The levels of IL-8 and IL-2 decreased at the beginning of the β-conglycinin administration. However, depending on time, an increment of the IL-8 and IL-2 levels showed allergic and inflammatory reactions. However, over time, the IL-1 levels decreased significantly, and its expression diminished consistently.

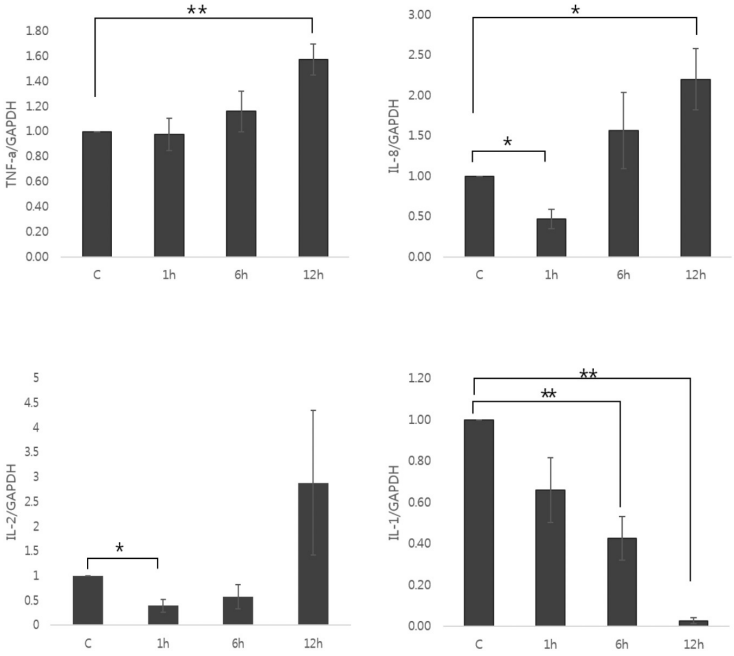

Figure 4 – Expression of TNF-α, IL-8, IL-2, and IL-1 mRNA levels in the intestines of β-conglycinin treated chicks.

Many studies have examined the genes involved in the immune response after feeding the animals with extracted or purified β-conglycinin. Various animal species were analyzed such as fish, rodents, pigs and others. According to (Zhang *et al.* 2013), TNF-a significantly increased in the intestine after feeding β-conglycinin to the fish, and IL-8 and IL-1 decreased. In addition, TNF-a and IL-1 significantly increased in juvenile turbot (Li *et al.* 2017) and Mitten crab (Han *et al.* 2019) contained in fish species. In mouse epithelia cell treated with β-conglycinin, IL-8, IL-6, and IL-2 significantly increased (Xu *et al.* 2010), and in other experiments using rats, IL-2 decreased and IL-4, IL-5 and TNF-α increased (Guo *et al.* 2007). Overall, in the results from the published papers, β-conglycinin-fed animals showed negative effects on the intestine, which is consistent with this study. However, depending on the species and experimental type (in vivo or in vitro), the expression patterns of immune responsive genes or factors were different.

In conclusion, we analyzed β-conglycinin-induced allergic reactions in chicks' small intestines. β-conglycinin was isolated from soybean flour and analyzed by SDS-PAGE. A total of 24 chicks were divided into four groups such as control, β-conglycinin 1 h, β-conglycinin 6 h, β-conglycinin 12 h – and the small intestine was analyzed. In the histology analysis, we observed that the β-conglycinin-induced allergic sensitization groups' duodenum villi epithelia were slightly damaged depending on time. Also, mast cell numbers significantly increased in β-conglycinin-sensitized groups within the duodenal lamina propria and crypt. Moreover, the mRNA expression of TNF-α, IL-8, and IL-2 increased in β-conglycinin treatment depending on the sensitization time. These results indicate that β-conglycinin may lead to immune responses by allergic sensitization in small intestines of chicks.

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