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EFFECTS OF GLYCININ AND β -CONGLYCININ ON INTEGRITY AND IMMUNE RESPONSES OF MOUSE INTESTINAL EPITHELIAL CELLS

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ABSTRACT

Antigen protein contained in soybeans are the major cause of allergic reaction in young animals. In this study, glycinin and β-conglycinin were purified material and experimentally tested at concentration of 0.1, 5, and 10 mg/ml for their effects on integrity and immune responses of mouse intestinal epithelial cells. At 5 mg/ml or above, glycinin and β-conglycinin undermined the integrity of intestinal epithelial cells, inhibited epithelial cell proliferation, and significantly increase the levels of LDH and GOT released to the culture medium. The administration of glycinin and β-conglycinin also significantly promoted the secretion of inflammatory cytokines (IL-2, IL-6, IL-8), a key feature in allergic reactions.

Key words: Glycinin, β-conglycinin; intestinal epithelial cells; integrity; cell factor.

INTRODUCTION

Soybeans are a nutrient-rich food, with high protein content and a good balance of amino acids (Dudek, 2001, Morrison and Hark, 1999). Thus, soybeans and soybean meal are a high-quality plant protein source for livestock and poultry. However, some proteins in soybeans also act as antigenic proteins that can cause allergic reactions in young animals. Sissons and Smith. (1976) pointed glycinin and β-conglycinin as the main antigenic components that caused allergic reaction in weanling pigs. Both of the proteins are mainly found in the cotyledons of the soybean seed.

Glycinin, also known as 11S protein, is composed of six subunits. Each subunit contains an acidic chain and a basic chain, linked to each other through a disulfide bond (Golubovic *et al.*, 2005). The β-conglycinin, also known as 7S protein, belongs to a class of glycosylated proteins (Hou and Chang, 2004) It has three subunits that associate with each other by hydrophobic interactions. The relative molecular mass of β-conglycinin is 180KDa and the protein contains 4 to 5% carbohydrate (Ogawa *et al.*, 1995, Yamauchi *et al.*, 1975)

Both glycinin and β-conglycinin cause allergic reaction in piglets, calves and other young animals, but their effects are especially more pronounced in weaned piglets, which show symptoms of diarrhea (Li *et al.*,1990). In the present study, purified glycinin and β-conglycinin were tested in cultured mouse intestinal epithelial cells to determine their effects on cell viability, supernatant level of lactate dehydrogenase (LDH), aspartate aminotransferase activity (GOT), cell proliferation and integrity. The levels of cytokines like IL-2, IL-6 and IL-8 in the supernatant were also assayed to assess effects of glycinin and β-conglycinin on immune status of mouse intestinal epithelial cells.

MATERIALS AND METHODS

Animals: 10-day-old healthy Kunming mice were obtained from the National Experimental Teaching Demonstration Center of Animal Science (Ya'an,China).

Main Reagents: Glycinin and β-conglycinin were extracted and isolated in the laboratory. The extraction method was according to read Wang *et al.,* (2003). Purity coefficient of glycinin and β-conglycinin was 93.8 and 67.2 percents. L-glutamine, insulin, epidermal growth factor (EGF), collagenase, collagen, benzyl penicillin, streptomycin sulfate, D-sorbitol, HEPES and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Dulbecco's minimum essential medium (DMEM), Phosphate buffered saline (PBS), Hanks balanced salt solution (HBSS) and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA).

Instruments and equipment: A CO_2 incubator (Heraeus Inc., Germany), enzyme-linked immunoassay instrument (USA Thermo Corporation), UV spectrophotometer (Beckman Inc. USA), Clean Benches (Harbin East Joint Electronic Technology Development Co., Ltd.), inverted microscope (Zeiss, Germany company), centrifuges, micro-plus kind of vehicles, etc.

Cell culture: Isolation of intestinal epithelial cells was essentially as described by Evans *et al.*, (1992), with some improvements. Firstly, the duodenum and jejunum were cut with eye scissors, and shredded into small pieces of approximately $1mm^3$. The shredded pieces were then digested with collagenase and neutral protease, the residual enzyme solution was discarded, and the cells

were washed and resuspended. The end result was a culture of intestinal epithelial cells in a fluid suspension with a concentration of 10^5 cells/ml. A 500µl volume of cell suspension was inoculated into each well of a 24-well cell culture plate. After 48h incubation, the original culture medium was replaced with fresh culture medium, and this was again changed after every 48h. The cells were observed every day until the culture plate bottom showed a large colony. Then the cells were incubated with serum-free DMEM and various concentrations of antigen proteins.

Table 1 The experimental design of trial

Treatment groups	Controls	β-conglycinin			Glycinin		
		1-1	1-2	1-3	2-1	2-2	2-3
Replicates	4	4	4	4	4	4	4
Soybean antigen protein concentration (mg/ml)	0	1	5	10	1	5	10

The experimental design of trial: A single-factor experimental design was used in this study. Six treatment groups were used with 4 replicates per treatment group (the cell survival test had 10 replicates), with each culture plate well representing a replicate. The six treatment groups and experimental design of the trial are shown in Table 1.

INDICATOR STUDY

Determination of cell culture fluid index: The cell culture medium after treatment with antigen protein for 36 h was collected from three culture plates and stored in a -20 ^{0}C freezer for subsequent determination of LDH and GOT activities and IL-2 , IL-6 and IL-8 levels. The concenterations were determined by using commercial kits following the manufacturer's instructions and using a DU-800 UV spectrophotometer or a fully automatic microplate reader.

Determination of cell viability: Cell viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay. Cells were treated with soybean antigen proteins for 36 h, then the cell culture medium was removed. The cells were washed once with 150µl serum-free DMEM culture medium, and then 150µl of 0.4 µg/µl MTT in serum-free DMEM culture medium was added, followed by culture at 37 ^{0}C for 6h. The culture medium containing MTT was then discarded, the cells were washed once with 150µl serum-free DMEM medium and 150µl of dimethyl sulfoxide was added to dissolve the intracellular formazan crystals. The absorbance at 540 nm was measured with an enzyme-linked immunoassay instrument.

Data processing and statistical analysis: Single-factor analysis was performed with SPSS11.0. Analysis of variance was applied using the Duncan multiple comparison method to test the differences between the results. Data are presented as means ± standard deviation and significant differences were further analyzed by regression analysis of indicators.

RESULTS AND DISCUSSION

Effects of soybean antigen proteins on cell viability: The influence of soybean β-conglycinin and glycinin extracts on the MTT viability assay of mice IEC in primary culture is shown in Table 2. None of the protein had any effect at concentrations of 0.1 mg/ml (treatments 1-1 and 2-1). At higher concentrations of 5 and 10 mg/ml, significant decreases in cell viability were observed for both β-conglycinin (treatments 1-2 and 1-3, respectively) and glycinin (treatments 2-2 and 2-3, respectively). It was also found that Glycinin reduced cell viability at the higher concentrations to a greater extent than did β-conglycinin ($P<0.05$)

Table 2 The influence of soybean β-conglycinin (Group 1) and glycinin (Group 2) on viability of mice intestinal epithelial cells in primary culture

Groups	MTT OD
Controls	0.527 ± 0.017^{Aa}
1-1	0.508 ± 0.018^{Aa}
1-2	0.463 ± 0.022^{Bb}
1-3	0.444 ± 0.023^{Bb}
2-1	0.503 ± 0.020^{Ab}
2-2	0.418 ± 0.017^{Bc}
2-3	0.410 ± 0.027^{Bc}

Note: The difference between data with different capital letters was significant among groups ($P<0.01$), and the difference between data with different small letters was significant among groups ($P<0.05$) and the same letters was not significant ($P>0.05$).

Effects of soybean antigen protein on LDH and GOT activities in IECs: As seen in Table 3, neither β-conglycinin nor glycinin had any significant effect on LDH or GOT activities when supplied at 0.1 mg/ml. However, at 10 mg/ml, addition of either of the protein significantly increased the levels of LDH and GOT in the supernatant.

The effect of soybean protein antigen on the release of IL-2, IL-6, and IL-8 from mouse intestinal epithelial cells in primary culture: As seen in Table 4, addition of either β-conglycinin or glycinin resulted in increased secretion of IL-2, IL-6 and IL-8 in the extracellular culture medium, particularly for the addition at 10 mg/ml. Glycinin (group 2) at 10 mg/ml appeared to be more effective than β-conglycinin (group 1) at promoting release of cytokines from IECs.

The effect of soybean antigen protein on the integrity of intestinal epithelial cells: An animal's survival and growth depends on the maintenance of normal cell morphology. Soy antigen proteins have been reported to damage the structure of animal intestinal epithelial cells in in vivo studies, thus affecting the integrity of intestinal cells. LDH is an important enzyme in cell metabolism, but when the cell membrane is damaged in an in vitro situation, LDH is released into the cell culture medium resulting in the increase in its concentration. In the present study, treatment of cultured mouse IECs with different concentrations of soybean β-conglycinin extract resulted in a gradual increase in the levels of LDH activity in the culture medium. The release in response to 10mg / mL β-conglycinin was significantly higher than that seen for the control (P <0.01) and clearly indicated that β-conglycinin could significantly undermine the structure and integrity of mouse IECs. Glycinin also had similar effects.

Table 3 The effects of soybean β-conglycinin and glycinin on the activity of extracellular LDH and GOT in mouse intestinal epithelial cells in primary culture

Groups	activity of LDH (IU/L)	activity of GOT(IU/ml)
Controls	648.3 ± 17.3^{Aa}	8.61 ± 0.50^{Aa}
1-1	670.5 ± 14.7^{Aab}	9.55 ± 0.72^{ABa}
1-2	695.5 ± 12.9^{ABb}	10.80 ± 0.84^{Bb}
1-3	736.4 ± 40.4^{Bc}	12.58 ± 0.57^{Cc}
2-1	654.8 ± 17.1^{A}	9.61 ± 0.91^{ABab}
2-2	703.9 ± 18.7^{B}	11.06 ± 1.18^{BCbc}
2-3	718.8 ± 28.5^{B}	11.92 ± 1.10^{Cc}

Note: The difference between data with different capital letters was significant among groups (P<0.01), and the difference between data with different small letters was significant among groups (P<0.05) and the same letters was not significant (P>0.05).

Table 4 The effect of soybean β-conglycinin and glycinin on IL-2, IL-6, and IL-8 levels in the extracellular culture medium of mouse intestinal epithelial cells in primary culture.

Groups	IL-2 content(ng/ml)	IL-6 content(ng/ml)	IL-8 content(ng/ml)
Controls	176.4 ± 56.2^{Aa}	138.4 ± 21.1^{Aa}	111.9 ± 18.6^{Aa}
1-1	164.3 ± 35.4^{Aa}	142.7 ± 13.3^{Aab}	128.9 ± 21.1^{ABa}
1-2	291.4 ± 51.8^{Bb}	167.9 ± 19.4^{Ab}	227.3 ± 17.9^{Bb}
1-3	283.0 ± 30.9^{Bb}	209.9 ± 11.6^{Bc}	169.2 ± 30.6^{Cc}
2-1	159.5 ± 49.8^{Aa}	135.2 ± 17.9^{Aa}	119.4 ± 29.7^{Aa}
2-2	306.5 ± 41.2^{Bb}	173.6 ± 15.5^{ABb}	210.4 ± 49.2^{Bb}
2-3	354.7 ± 54.6^{Bb}	191.7 ± 20.5^{Bb}	178.7 ± 38.1^{ABb}

Note: Capital letters denote significant differences among groups (P<0.01). Lowercase letters indicate differences among groups (P<0.05). Data with the same letters are not significantly different (P> 0.05).

Li *et al.*, (1991) and Qiao *et al.*, (2003) have previously reported that high dietary concentrations of soybean antigen protein can be harmful to piglet intestinal integrity and immune function, thereby inhibiting the growth of piglets. Burrells *et al.*, (1999) reported that high concentrations of soy protein undermine the integrity of salmon gut, thus inhibiting growth Li *et al.*, (1991) reported that soybean protein extract fed to 7-day-old piglets resulted in a daily weight loss of 6 g over 5 days. At 21 days of age after weaning, piglets fed with soy protein added to the corresponding weaning diets showed a relatively high titer of anti-glycinin and β-conglycinin antibody in their serum. Seven days after weaning, the measured intestinal epithelium villus height was significantly lower than the control group fed with milk, and crypt depth and villus width were significantly lower than those of the control group. Electron microscopy of the villus development showed that, compared with pigs fed milk, the pigs fed with soy protein had incomplete brush border epithelial cells and showed a phenomenon of cell fusion.

The present study also showed that soy antigen proteins had significant negative effects on mouse IEC viability, as measured by the MTT assay, while activities of LDH and GOT activity gradually increased. Since most cellular GOT is found in the mitochondria, the loss of this protein does not represent normal membrane leakage or excretion, but indicates severe damage to the mitochondria or even disintegration of the cell. GOT measurement is therefore important when evaluating cytotoxicity of exogenous toxins, as it is an important indicator of mitochondrial integrity. Mitochondrial energy metabolism is now recognized as only one important role of these organelles, as mitochondria are

also substantially involved in the regulation of intracellular Ca^{2+} concentration and in the induction of apoptosis. (Andre *et al.*, 2002, Ramachandran and Levonen, 2002). When the mitochondrial outer membrane ruptures, this can release pro-apoptotic proteins, such as cytochrome and apoptosis-inducing factor, into the cytoplasm, resulting in induction of apoptosis and necrosis. (Cande *et al.*, 2002, Kuwano *and Maeyama*2002, Suleiman *et al.*, 2001). Mitochondrial damage is thought to be the primary initiator of cellular apoptosis and necrosis. The loss of GOT following treatment of mouse IECs with soybean antigen protein extract clearly indicated damage to mitochondrial membranes, suggesting that these proteins may not only affect energy metabolism in mice IEC but may also launch IEC apoptosis and necrosis.

The effect of soybean antigen protein on immune function of mouse intestinal epithelial cells: The body's immune cells and non-immune cells can synthesize and secrete small molecule peptide factors such as the cytokines IL-2, IL-6, IL-8, IFN-γ, that regulate other cells or physiological functions. Each cytokine involved in an immune response is modulated by a series of biological events. As the concentration of the target cell type increases and at the emergence of different phases, this may lead to beneficial anti-inflammatory or inflammatory responses in the development of these two diametrically opposite effects. In this experiment, soybean antigen protein increased secretion of IL-2 and IL-6 from mouse IECs. IL-2 is known to generate a large number of inflammatory cells and to amplify inflammation. (Jones *et al.*,2002).

In the present experiments, treatment with 5mg/ml soybean antigen protein resulted in a significant increase in IL-2, in line with previous reports. IL-2 normally increases due to large expansion caused by inflammatory cells in the body, tissues, and organs resulting in the inflammatory response. A Th1/Th2 cell imbalance is now generally agreed to result in a Th2 cell response that is a hyper-IgE-mediated allergic reaction. Th2 cells are regulated by an important class of humoral immune cells; when their number increases, the end result is that hyperthyroidism-induced allergen-specific B lymphocytes produce high levels of IgE (pl. give reference).

IL-6 is a representative of the cell cytokines and results in positive feedback that promotes further differentiation of the cells, thus mediating the rapid onset of allergy responses (Steinke and Borish, 2001). IL-6 can promote B-cell secretion of IgE antibodies and the promotion of expression of high-affinity IgE receptor in mast cells and basophils. The appearance of IL-2, IL-6, and IL-8 in the cell medium in the present study might have stimulated electrolyte secretion from intestinal cells, resulting in the allergic reactions in the body.

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