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Effect of Glycinin and β-conglycinin on the Absorbing Capacity of Mouse Intestinal Epithelial Cells

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ABSTRACT

Glycinin and β-conglycinin in soybeans can reduce the absorption of nutrients by young animals and result in watery diarrhea. In this study, self-prepared purified glycinin and β-conglycinin were applied to intestinal epithelial cells of primary cultured mice. The effects of purified glycinin and β-conglycinin (0.1, 5 and 10 mg mL^{-1}) on Na^+-K^+-ATP enzyme, PEPT1 and DMT1 expression in mouse intestinal epithelial cells were studied. Glycinin and β-conglycinin could enhance epithelial cell Na^+-K^+-ATP enzyme activity but reduced the amount of PEPT1 and DMT1. The effect of β-conglycinin was more pronounced than that of glycinin.

Key words: Glycinin, β-conglycinin, Na^+-K^+-ATP enzyme, PEPT1, DMT1

INTRODUCTION

Soybean is nutrient-rich, not only because of its high protein content but because it also contains more balanced amino acids (Liener,1994; Naismith, 1955). Thus, soybeans and soybean meals are high-quality plant protein sources for livestock and poultry. However, soy protein that contains antigens can cause allergic reactions and reduce the absorption of nutrients by young animals. Sissons and Smith (1976) reported that the main allergy-causing antigenic components of soy protein for weanling pigs are glycinin and β-conglycinin. Glycinin and β-conglycinin, which exhibit antigen activity in soybean, primarily belong to cotyledon proteins. Glycinin, also known as 11S protein, is composed of six subunits; each subunit contains an acidic chain and a basic chain linked with each other through a disulfide bond (Golubovic *et al*., 2005). β-conglycinin, also known as 7S protein, is a class of glycosylated protein (Hou and Chang, 2004) that contains three subunits associated with each other by hydrophobic interactions; it has a relative molecular mass of 180 Kda, containing 4-5% of carbohydrates (Ogawa *et al*., 1995; Yamauchi *et al*., 1975).

Peptide transporter 1 (Pept1), is a member of the family of solute carriers that rely on the production of a peptide (POT) and plays a key role in the absorption of a peptideDivalent metal transporter 1 (DMTl), also known as soluble carrier family 11 number 2 (SLC11A2), is the ion transporter in the protons of mammals. Zheng *et al*. (2011) DMTl can transport Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, and so on. No studies on the effect of glycinin and β-conglycinin on the expression of PEPT1 and DMTl in young animals have been reported. In this study, therefore, purified glycinin, β-conglycinin and *in vitro* cultured mouse intestinal epithelial cells were used as experimental materials. The effects of glycinin and β-conglycinin on the absorbing capacity of mouse intestinal epithelial cells were investigated.

MATERIALS AND METHODS

Test animals: Healthy 10-day-old Kunming mice were obtained from the Center of Animal Science, Si Chuan Agricultural University.

Reagents: Glycinin and β-conglycinin were extracted and isolated in the laboratory.

DMEM high glucose medium (GIBCO U.S. companies), trypsin XI, medium protease I, insulin, epidermal growth factor, fetal calf serum, sorbitol, L-glutamyl ammonium, HEPES, pyruvate, penicillin, streptomycin, etc. were purchased from Sigma (USA). The Minim Na-K ATP enzyme test kit was bought from Nanjing Jiancheng Bioengineering Institute, while the SV Total RNA Isolation system, SYBR Premix Ex Taq II, DNase (RNase-free), gel extraction kit, Taq DNA Polymerase, SDS, proteinase K and dNTP were purchased from Takara Bio Co., Ltd. The reverse transcription kit was purchased from Promega Corporation.

Instruments and equipment: The instruments and equipment used were as follows: CO_2 incubator (Heraeus, Germany), enzyme-linked immunoassay analyzer (Thermo Corporation USA), UV spectrophotometer (Beckman Inc.), Clean Benches (Harbin East Union Electronic Technology Development Co., Ltd.), inverted microscope (Zeiss, Germany company), ordinary centrifuge, micro pipette, real-time PCR instrument (Bio-Rad company), protein nucleic acid detector (BeckmanCoulter) and gel imaging system (Bio-Rad).

The isolation of intestinal epithelial cells was conducted following the methods of Evans *et al.* (1992) with modifications. First, the duodenum and jejunum were cut using eye scissors and then shredded into small pieces of approximately 1 mm^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 fluid suspension with a concentration of 10^5 cells mL^{-1}. Cell suspension of 500 μL volume was inoculated into each well of a 24 hole cell culture plate. The original culture medium was replenished with a new culture medium every 48 h. Cells were observed every day until the culture plate bottom showed a large colony and then the cell culture plates were covered with the end of the forthcoming. Processing was then implemented.

Design of trials: A single-factor experimental design with six treatment groups was used. Each treatment group consisted of 4 replicates (the cell survival test for 10 repetitions) for each well. The six treatment groups were designated as 1-1, 1-2, 1-3, 2-1, 2-2 and 2-3; soybean globulin and β-conglycinin extract were added at concentrations of 0, 1, 5 and 10 mg mL^{-1}. The design of the trials is shown in Table 1.

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Na-K ATP enzyme determination: Soy protein antigen-treated cells were cultured for 36 h, then collected. Cell samples were prepared and stored in a refrigerator at

Table 1: Experimental design of the trials

		β-conglycinin			Glycinin		
Treatment groups	Controls	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^{-1})	0	1	5	10	1	5	10

-20°C. Determination of medium Na-K ATP enzyme was conducted according to the operating instructions kit of the DU-800 UV spectrophotometer.

Determination of PEPT1 and DMT1 expression

Primer synthesis: The primers were designed using the Premier 5.0 software. According to the PEPT1 gene sequence, a pair of primers for the amplification length of 136 bp is the upstream Primer F1: 5'-CAGTATCTCCAAATGCCCAGGAA-3'; Primer R1: 5'-TTTGGCCCTAAACCAACATATCAAC-3'. According to the DMT1 gene sequence, a pair of primers for the amplification length of 90 bp is the upstream primer F2: 5'-CTGTCCGGCCTCAACGATCTA-3'; Primer R2: 5'-TGGCATGCTGGTGAAAGTCAA-3'. According to the GenBank sequence of mouse β-actin mRNA internal reference sequence primers designed to amplify a length of 171 bp, the upstream primer F3 is: 5'-CATCCGTAAAGACCTCTATGCCAAC-3'; downstream primer R3 is: 5'-ATGGAGCCACCGATCCACA-3', with an amplification length of 171 bp. The design of the primers was determined by NCBI BLAST web tool search. The initial validation of specificity was also searched. The primers were synthesized by TaKaRa (Dalian) Company. Total RNA extraction, reverse transcription and real-time quantitative PCR were performed according to the kits' instructions.

Data processing and statistical analysis: Single-factor analysis was performed on the data obtained for Na-K-ATP enzyme determination using SPSS11.0 Analysis of variance with Duncan's multiple comparison method was used to test the differences between the results. Data were presented as means±standard deviation and significant differences were further analyzed by regression analysis of indicators. Gene expression in the sample with β-actin as an internal reference was analyzed using the ΔΔ Ct method: the average relative concentration = $2^{-\text{average }\Delta\Delta\text{ Ct}}$, which was calculated using the time at which each genome copy number template was initiated for the average relative content of PEPT1 and DMT1.

RESULTS

Effect of soybean antigen protein on cell survival rate: Soybean β-conglycinin and glycinin extracts had significant effects ($p<0.01$) on the MTT OD of mouse IEC in primary culture (Table 2). The effect on the control group MTT OD was significantly higher than that on the 1-2, 1-3, 2-2 and 2-3 groups ($p<0.01$); the effect on the 1-1 group MTT OD was significantly higher than that on the 1-2 and 1-3 groups ($p<0.01$); the effect on the 2-1 group MTT OD was significantly higher than that on the 2-2 and 2-3 groups ($p<0.01$).

Table 2: Soybean β-conglycinin and glycinin in cultured mouse intestinal cells Na-K-ATP enzyme activities

Treatment group	Added concentration (mg mL^{-1})	Sample	Cell Na^+, K^+-ATP enzyme activity (U mg^{-1} protein)
Control	0	4	0.23 ± 0.02^{Aa}
1-1	1	4	0.26 ± 0.04^{Aab}
1-2	5	4	0.31 ± 0.05^{ABbc}
1-3	10	4	0.39 ± 0.06^{Bc}
2-1	1	4	0.27 ± 0.03^{Aa}
2-2	5	4	0.36 ± 0.03^{Bb}
2-3	10	4	0.47 ± 0.03^{Cc}

Different superscript capital letters indicate level of significance = 0.01. Different lowercase letters indicate level of significance = 0.05, whereas same letters indicate no significant difference ($p>0.05$)

Table 3: PEPT1 mRNA expression dynamics

Groups	Dosage (mg mL^{-1})	Average CT value of internal reference	PEPT1 average	Log ($2^{\Delta\Delta}$CT)
Control	0	18.69±0.21	30.87±0.31	1.00
β-conglycinin	1	18.51±0.41	28.51±0.32	0.66
	5	19.80±0.38	30.54±0.55	0.43
	10	19.81±0.65	31.34±0.31	0.19
Glycinin	1	18.91±0.39	28.27±0.46	0.85
	5	19.17±0.70	29.52±0.42	0.55
	10	18.98±0.31	29.67±0.53	0.45

Table 4: DMT1 mRNA expression dynamics

Groups	Dosage (mg mL^{-1})	Average CT value of internal reference	DMT1 average	Log ($2^{\Delta\Delta}$CT)
Control	0	18.69±0.21	30.90±0.67	1.00
β-conglycinin	1	19.80±0.38	30.61±0.28	0.42
	5	18.51±0.41	29.39±0.46	0.40
	10	19.81±0.65	31.34±0.40	0.20
Glycinin	1	18.91±0.39	29.50±0.27	0.49
	5	19.17±0.70	29.82±0.21	0.47
	10	18.98±0.31	30.04±0.32	0.34

Soy protein antigen PEPT1, DMT1 mRNA expression of genes: Glycinin and β-conglycinin inhibited the expression of PEPT1 and DMT1 genes in mouse intestinal epithelial cells (Table 3 and 4).

As antigen protein concentration increased, the inhibition of PEPT1 and DMT1 gene expression gradually increased. A protein antigen concentration of 5 mg mL^{-1} decreased PEPT1 gene expression by 7%; at an antigen protein concentration higher than 5 mg mL^{-1}, the inhibition by β-conglycinin was lower than 11% and that by glycinin was significantly reduced. The effect of the antigen on DMT1 gene expression suggests that at low doses (5 mg mL^{-1}) of the antigen, DMT1 gene expression could be reduced by 50%, whereas at high doses (5 mg mL^{-1} above), the inhibition effect was even more pronounced.

DISCUSSION

Li *et al*. (1990) 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*. (1990) 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.

With an increase in the added soy protein antigen, PEPT1 expression decreased. Correlation analysis shows that PEPT1 expression, as well as soybean β-conglycinin and glycinin dosage showed a significant negative correlation (R = -0.94), indicating that soy protein reduces the antigen expression of PEPT1, thereby reducing the absorption of small peptides in the intestinal epithelial cells. Studying the human colon cancer cell line Caco-2. Baron-Delage *et al*. (1996) showed that when transfected with the proto-oncogene, the intestinal cell-specific hexose transporter (SGLT1), specific brush border enzymes and association with hair growth gene expression significantly decreased. The relationship between soy protein antigen and PEPT1 gene expression may be due to several reasons.

First, any expression or function of the carrier's protein expression regulator has its own substrate. PEPT1 substrates for transport are mainly dipeptides and tripeptides and β-conglycinin and glycinin may have corresponding binding sites. However, the two proteins on the intestinal epithelial cells are antigens, which triggered cellular immune responses when combined with epithelial cells. Consequently, the expression of PEPT1 was reduced, as well as the antigenic protein and cells, thereby reducing cell damage. Second, as the β-conglycinin and glycinin dosages increased, cell damage also gradually increased, which caused cells to mobilize stored energy and proteins for damage repair and immune functions, such as cytokine secretion and Na-K-ATP enzyme accelerated synthesis. These cost substantial energy and amino acid cells, resulting in reduced nutrient absorption and cell gene expression. Third, the intestinal epithelial cell function may have been altered after reaction with the antigen, including nutrient absorption. In addition, some severely damaged cells die, to a certain extent, affected the absolute expression of PEPT1.

DMT1 gene expression may be regulated by many factors, such as inflammatory mediators, TNF-α, IFN-γ, protein kinase C and developmental stage, among others. Ludwiczek *et al.* (2003) confirmed that proinflammatory cytokines and lipopolysaccharides affect DMT1 expression in macrophages. In the present paper, increasing the amount of soy protein antigen resulted in decreased DMTl gene expression. DMTl gene expression and the quantity of β-conglycinin added exhibited a negative correlation (R = -0.78). Similarly, DMT1 and the quantity of soybean globulin were negatively correlated (R = -0.75). After the addition of soy antigens, the integrity of cell damage (part of the antigen protein in the cell) directly interfered with DMTl of gene transcription. Meanwhile, epithelial cells exhibiting allergic reactions increased in inflammatory cytokines; cell metabolic disorders and the self-regulation of DMTl expression may have affected the expression of DMTl. Reduced DMTl gene expression will lead to a decrease in the absorption of divalent metal ions, such as Zn^{2+}, which are necessary for RNA transcription enzymes; these, in turn, reduces the expression of DMTl genes. The Na-K-ATP enzyme functions as Na-K pump and enzyme, indicating that it is involved in energy metabolism, material transport, oxidative phosphorylation and other important biochemical processes, as well as ion balance regulation. Thus, Na-K-ATP activity changes can affect many important cellular functions.

In this study, the addition of soy protein antigen in mouse intestinal cells increased Na-K-ATP enzyme activity. The self-protection mechanism of the cells may result to the increased consumption of a large amount of energy. Thus, the cell membrane Na and K-ATP enzyme are in a highly active metabolic state, leading to decreased levels of ATP within the cell membrane. These decreased levels affect other important intracellular biochemical reactions and ultimately influence the nutrient absorption of normal cells (Lytton *et al.*, 1991).

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