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Inhibitory Action of Soybean β-Conglycinin Hydrolysates on *Salmonella typhimurium* Translocation in Caco-2 Epithelial Cell Monolayers

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Soybean protein hydrolysates are widely used as functional foods as they have antioxidative properties able to enhance immune responses in humans. The alcalase enzymatic hydrolysates of β-conglycinin were fractionated by ultrafiltration, and two main fractions, SP1 (<10 kDa) and SP2 (10−20 kDa), were obtained. The effects of these two fractions on the growth, development of epithelial cells, and formation of intercellular tight junctions were tested on an in vitro Caco-2 cell culture system. The inhibitory effects of SP1 and SP2 on the penetration of *Salmonella typhimurium* into Caco-2 epithelial cells were also examined. The results showed that the addition of >0.05 g/L of SP2 improved epithelial cell growth and that a concentration of 0.5 g/L of SP2 increased intercellular tight junction formation, which resulted in increased of transepithelial monolayer resistance (TER) values. Moreover, a lower *S. typhimurium* count compared to control was obtained when Caco-2 cells were grown in 0.05 and 0.5 g/L of SP2. These results show that β-conglycinin hydrolysates play an important role in resisting *S. typhimurium* penetration into intestinal epithelial cells and that high molecular mass peptides (10−20 kDa) were more effective overall than low molecular mass peptides.

KEYWORDS: β-Conglycinin; hydrolysate; ultrafiltration; *Salmonella typhimurium*; bacterial translocation; Caco-2 cell monolayers

INTRODUCTION

Soybean is a traditional food in Asia and has been a part of the Asian diet for many years; it is also accepted worldwide as an important protein source. In recent years, soybean protein hydrolysates have been shown by many to manifest important physiological activities such as antioxidative activity (*1*), hypocholesterolemic effect (*2*), and immunoregulatory activity (*3*). Nevertheless, the beneficial effects of soy protein hydrolysates on the maintenance of intestinal health have raised particular focus. A study by Nakamura et al. (*4*) on the effects of soybean β-conglycinin and isoflavones on the treatment of hypercholesterolemia in ovariectomized monkeys has found that fecal counts of *Escherichia coli* and *Clostridia* of β-conglycinin-fed monkeys were significantly lower than that of isoflavones-fed monkeys and control. In addition, they found that the beneficial bifidobacteria had doubled in the fecal count instead. Nonetheless, Shen et al. (*5*) have reported no significant differences in intestinal microbiota with control in *E. coli* O138-infected mice when they were fed β-conglycinin hydrolysates after their infection. In summary, these studies have indicated that the hydrolysates from β-conglycinin are able to modulate the balance of gut microbiota, which may lead to a lower risk of pathogenic bacterial infection.

Salmonella species are food-borne pathogens that are known causative agents for gastroenteritis, typhoid fever, and other associated diseases. Bacterial translocation in *Salmonella*-infected gastroenteritis patients have been known to result in systemic infections when *Salmonella* penetrate the intestinal epithelial cells and are transported to the liver and spleen (*6, 7*). Kops et al. (*8*) conducted a study to investigate the effects of soy-derived isoflavones and phenolics components in protein hydrolysates of soybean on the growth, development, and resistance capacity for enteric bacterial infection in C2BBe cell cultures (a variant of the colonic adenocarcinoma cell lines). They found that the highly processed hydrolysates fraction (SP-A) containing low amounts of isoflavones and phenolics acids caused retardation in growth and differentiation of confluent monolayer. As evaluated by transmonolayer electrical resistance (TER) values, they had found that the development of intercellular tight junctions was also reduced. In contrast, the minimally processed hydrolysates (SP-B) containing abundant isoflavones and phenolic acids showed no signs of retardation in growth and TER of cell cultures. From these results, they

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concluded that SP-A and SP-B appear to affect in vitro intestinal epithelial cell development. Furthermore, both protein hydrolysates were able to resist penetration of *Salmonella* typhi but no effects for *S. typhimurium*. Their findings have shed significant light on the importance of soybean protein hydrolysates in intestinal health.

The storage protein of soybean is composed of two biochemically distinct subunits—glycinin and β-conglycinin. The enzymatic hydrolysates for these two subunits differ in structure and composition. Here we use β-conglycinin for further study. Our objective is to prepare the hydrolysates from β-conglycinin alcalase digested and examine the effect of the different molecular weight peptide fractions on the infection of *S. typhimurium* utilizing Caco-2 (human colonic adenocarcinoma) cells model in vitro. Through this study, we hope to understand the inhibitory effects of soybean protein hydrolysates on pathogenic bacterial infection of intestinal cells.

MATERIALS AND METHODS

Materials and Reagents. Defatted soybean flakes were obtained from Harbin Hi-Tech Soybean Food Co., Ltd. (Harbin, China). The enzyme was alcalase 2.4 L provided by Novozymes (Beijing, China). Dulbecco's modified Eagle's medium (DMEM-high glucose) was purchased from Invitrogen Corp. Fetal bovine serum (FBS) was from Biochrom. Nonessential amino acids were obtained from Hyclone. Hektonen Enteric (HE) agar medium was purchased from San-Yao Science-Technology Co. (Beijing, China) and brain heart infusion broth (BHI) from Oxoid (Hampshire, U.K.). Protein molecular mass markers (20100−3313 Da) were purchased from Yu-Yan Biotechnology Co. (Shanghai, China). All other reagents used in the study were of analytical or chromatographic grade.

Preparation of β-Conglycinin Hydrolysate. β-Conglycinin was isolated from the defatted soybean flakes according to the method of Nagano et al. (*9*). The β-conglycinin was hydrolyzed by alcalase as follows: 2% (w/v) β-conglycinin solution was preheated at 80 °C for 15 min; solution was adjusted to pH 8.0 before it was hydrolyzed at a ratio of substrate to enzyme of 100:5 (w/v) for 6 h at 55 °C. The pH was maintained by continuous addition of 1.0 M NaOH. After hydrolysis, the solution was heated in boiling water for 10 min, then adjusted to pH 4.5, and centrifuged at 3000*g* for 20 min to recover the supernatant. The supernatant was dialyzed with a dialysis bag (24 mm MWCO 500, 12.5 × 1 cm, Sorua, Germany) and freeze-dried for storage at −20 °C. The degree of hydrolysis (DH) was determined by means of the pH-Stat method (*10*).

Molecular Mass Distribution. The molecular mass distribution of the hydrolysate was determined by size exclusion chromatography using high-performance liquid chromatography (model Agilent 1100) (SE-HPLC) with a Waters Protein Pac-60 column (7.8 mm × 300 mm, bead size = 10 μm, fractionation range of 1−20 kDa). The column was equilibrated and eluted with 30 mM Tris-HCl, pH 7.4, at a flow rate of 0.5 mL/min. A sample volume of 10 μL was injected onto the column. Detection was performed at 214 nm. The areas of the peaks of the peptide fractions present in the hydrolysate were integrated by the program software (Agilent ChemStation A.09). The size of the peptide fractions was estimated using a calibration curve. The column was calibrated using protein molecular mass markers. The markers contained trypsin inhibitor (20100 Da), lysozyme (14400 Da), ABI-80 (7823 Da), ABI-81 (5856 Da), and ABI-95 (3313 Da). In addition, blue dextran (2000 kDa) was used for calibration purposes. A linear dependency of log molecular weight (MW) versus retention time (RT) was observed ($R^2 = 0.9923$, $p < 0.05$).

Ultrafiltration of Hydrolysates. β-Conglycinin hydrolysate was further fractionated using an ultrafiltration unit (Amicon, model 8200, Millipore Corp., Beverly, MA) under the conditions of 16 psi of nitrogen gas and 4 °C. One percent (w/v) hydrolysates was adjusted to pH 7.2 and first separated by a 10 kDa nominal molecular weight limit (NMWL) membrane. The retentate was further separated by a 20 kDa NMWL membrane. Three size fractions, <10, 10−20, and >20 kDa, were named SP1, SP2, and SP3. Each fraction was lyophilized. One gram of each of the fraction was dissolved in 100 mL of 0.85% NaCl solution to make a working stock solution of 10 g/L. Each stock solution was sterile filtered using 0.20 μm filter membrane (Sartorius).

Determination of Protein and Carbohydrate Concentration. The protein concentration was determined according to the method of Lowry et al. (*11*), using bovine serum albumen (BSA) as a standard. The carbohydrate concentration was determined according to the phenol−sulfuric acid method (*12*), in which glucose was used as a standard.

Analysis of Amino Acid Composition. A 75 mg portion of each sample was placed in a 20 mL ampule and mixed with 10 mL of 6 M hydrochloric acid. The ampule was sealed, and the samples were hydrolyzed at 110 °C for 24 h under vacuum. The hydrolysates were evaporated to dryness under vacuum at 60 °C. The dried sample was dissolved in 3−5 mL of sodium citrate buffer (pH 2.2), resulting in an amino acid concentration of 50−250 nmol/L. The sample was filtered, and the filtrate was loaded on a Hitachi L-8800 amino acid analyzer (Tokyo, Japan).

Caco-2 Cell Growth Assay. Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (Rockville, MD). Cells of passage 10−20 were maintained at 37 °C in flasks containing DMEM supplemented with 10% FBS and 1% nonessential amino acids, in an atmosphere of 5% CO_2. For routine culture, penicillin (100 units/mL) and streptomycin (100 units/mL) were added. Flasks were treated with 0.25% trypsin and 0.53 mmol/L EDTA to split cells. Following two washes with medium, the concentration of the cells was adjusted by counting in a hemocytometer.

The stock solution of hydrolysates was serially diluted in a 96-well microplate with complete medium to a concentration of 0.01−1.0 g/L in a total volume of 100 μL per well. The Caco-2 cells were added into each well to a final concentration of approximately 2×10^4 cells/mL. Five days after seeding, the cell proliferation rate was measured with the WST-1 Cell Proliferation and Cytotoxicity Assay Kit according to the manufacturer's instructions (Beyotime Biotechnology, Haimen, China). Each plate was optically read at 450 nm with a microplate reader (Multisan Mk3, Thermo Electron Co.).

***Salmonella typhimurium* Translocation Assay.** *Culture of Caco-2 Cell Monolayers.* Cells (5×10^4) were applied onto transwell inserts (PET Clear membrane; area = 0.33 cm^2; pore size, 3.0 μm; Corning Costar, Cambridge, MA). Cells were cultured in complete media with hydrolysates concentration of 0.5 and 0.05 g/L. Phosphate-buffered saline (PBS) solution was taken as control. The medium was added to both lower and upper chambers of the transwell inserts, with the volumes kept at 1000 and 200 μL, respectively. Cells were fed with fresh medium every other day for 2 weeks. Each treatment contained six transwell inserts. Twelve hours before the experimental assays were performed, the medium of the transwell inserts was replaced with a medium devoid of antibiotics.

Culture of Bacteria and Their Application to Caco-2 Cell Monolayers. S. typhimurium LT2 was kindly provided by Prof. Shu-Lin Liu (Department of Microbiology, Peking University Health Science Center, China). *E. coli* DH5α was obtained from Dr. Gui-Qin Qu, College of Food Science and Nutritional Engineering, China Agricultural University. Bacteria were grown on LB agar overnight at 37 °C from frozen stocks. A single colony was transferred to BHI broth and incubated overnight at 37 °C. Bacteria were harvested from the broth culture by centrifugation at 2500*g* for 15 min and washed twice with sterile PBS solution. Then the bacteria were resuspended in PBS to a final concentration of 10^9 CFU/mL by optical density measurement.

The assay of bacterial translocation was performed according to the method described by Finlay and Falkow (*13*) with slight modification. The medium in the transwell inserts was withdrawn, and 10 μL of the bacteria suspension was applied to the apical surface of the Caco-2 cells. Fresh antibiotic-free medium of 190 μL was added to the upper chamber, and 1000 μL of fresh antibiotic-free medium was added to the lower chamber. Equivalent numbers of the non-enteroinvasive *E. coli* DH5α and enteroinvasive *S. typhimurium* were added to the monolayers. *E. coli* was used to confirm the integrity of the monolayers because this strain is noninvasive and will not pass through confluent monolayers unless they are damaged. The cell monolayers were gently agitated for 10 min before they were incubated for 3 h. In this

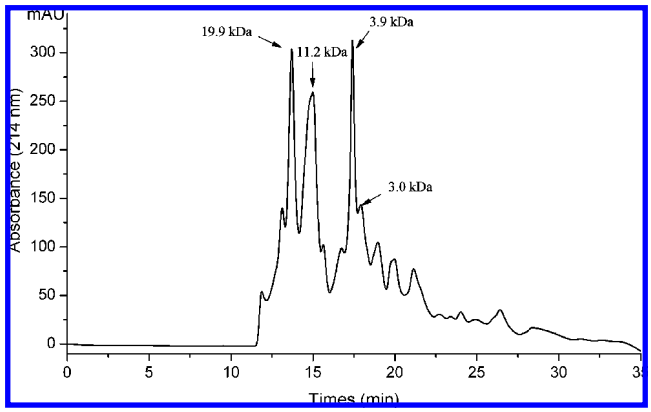

Figure 1. SE-HPLC chromatogram of β-conglycinin hydrolysates. Arrows indicate the molecular masses of the peptides at their corresponding retention times.

experiment, six transwell inserts were assigned to each treatment group. At 3 h, medium was withdrawn from the lower chambers of the transwell units and plated onto HE agar plates either directly or after 10-fold serial dilution in sterile PBS. Plates were incubated at 37 °C for 24 h, at which time colony-forming units were counted. *S. typhimurium* appeared as greenish colonies with black centers; *E. coli* appeared as bright orange colonies.

TER Measurements. The TER value of Caco-2 monolayers grown in transwell units was measured with a Millicell-ERS apparatus (Milipore), according to the manufacturer's instructions. Three transwell inserts alone with DMEM medium were used as blank. The readings were corrected for the surface area of the monolayer, and the data were expressed as $\Omega \cdot cm^2$.

Transmission Electron Microscope (TEM). Caco-2 monolayers were washed three times with 0.1 mol/L sterile PBS and fixed in cold (4 °C) 2.5% glutaraldrhyde and 0.1 mol/L PBS (pH 7.2−7.4) overnight; samples were postfixed in cold 1% OsO_4 in 0.1 mol/L phosphate buffer for 60 min and then rinsed twice with 0.1 mol/L PBS. Samples were dehydrated in a series of alcohol concentrations and embedded in epoxy resin SPURR. Samples were sectioned and stained with uranyl acetate and lead citrate and examined with a JEOL-1230 electron microscope.

Statistical Analysis. The means and standard deviations for each treatment group were calculated in all experiments. The significance of differences between the means was evaluated by ANOVA using the OriginPro 7.5 software package (OriginLab Corp.). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

We have isolated β-conglycinin according to the method of Nagano et al. (*9*). Maximum hydrolysis of the β-conglycinin with alcalase was reached after 6 h, and the DH was determined to be 21.7%. **Figure 1** shows the molecular mass profile for the hydrolysates as determined with SE-HPLC. As observed in **Figure 1**, 11 peaks showing a range of retention time from 11.8 to 21.1 min were obtained for the protein hydrolysates. The areas of the peaks of the peptide fractions present on the chromatogram are indicated. The major peaks for this study were noted at retention times of 13.7, 14.9, 17.4, and 17.9 min. The relative percentages of the different molecular mass fractions at these times were 11.8% of 19.9 kDa, 17.8% of 11.2 kDa, 11.2% of 3.9 kDa, and 6.6% of 3.0 kDa, respectively. On the basis of the profile of the SE-HPLC obtained above, the hydrolysates were fractionated into <10 kDa (SP1), 10−20 kDa (SP2), and >20 kDa (SP3) via an ultrafiltration process. The recovery yields (w/w) of SP1, SP2, and SP3 were 58.2, 38.4, and 1.9%, respectively. The molecular mass of the peptides present in each of the three fractions was further analyzed by SE-HPLC. The results in **Table 1** showed that SP1 contained

Table 1. Biochemical Characterization of the Protein and Carbohydrate Composition in β-Conglycinin Hydrolysates[a]

				av mol mass		
hydrolysate	recovery yield	protein	carbohydrate	>20000	20000−10000	<10000
SP1	58.2	97.3	1.4	10	21	69
SP2	38.4	90.6	8.5	12	50	38
SP3	1.9	88.6	5.8	51	16	33
hydrolysate	100	91.7	4.7	9	35	56

[a] Recovery yield for each class of hydrolysates is expressed in w/w (%) relative to the total β-conglycinin hydrolysates.

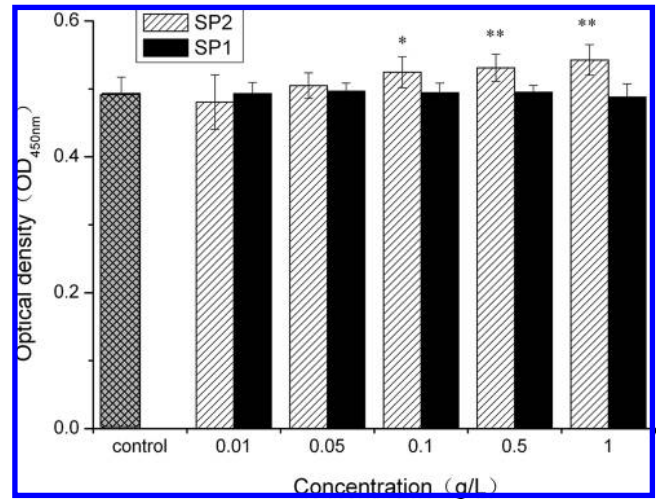

Figure 2. Effects of SP1 and SP2 on the growth of Caco-2 cells. Values are means ± SE, $n = 6$, significantly different from control at **, $p < 0.01$; *, $p < 0.05$.

70% of peptides that were <10 kDa; SP2 contained 38% of <10 kDa fraction as well as 50% of 10−20 kDa peptides. Moreover, SP2 had a higher soluble sugar content than the SP1 fraction. This could mean that 4−5% of the sugar content present within the crude protein hydrolysates of the β-conglycinin was largely present within the SP2 fraction. SP3 contained 51% of peptides that were >20 kDa. The carbohydrate content of it was 5.8%, which was consistent with β-conglycinin (*14*). Therefore, it could be concluded that SP3 was mainly composed of β-conglycinin subunit with a lower DH and was not considered in subsequent experiments.

The intestines are an important organ responsible for nutrient absorption, metabolism, and recognition of food signals. Studies indicated that the intestines are one of the targets for food functional peptides. These peptides express their functions in the intestinal tract and/or modulate intestinal epithelial cell functions. The intestine also acts as a physical and biological barrier against harmful substances including food pathogens and environmental chemicals (*15*). Intestinal dysfunctions that alter the intestinal epithelial permeability could lead to increased susceptibility to bacterial infection and translocation. In addition, bacterial translocation can also lead to the development of systemic infection in the human body. The study of the effects of β-conglycinin hydrolysates through in vitro growth studies in Caco-2 cells has shown that Caco-2 cells that were cultured with a range of concentrations >0.1 g/L of SP2 showed a significantly higher optical density than the control ($p < 0.05$) (**Figure 2**). However, Caco-2 cells that were grown with a similar range of concentrations of SP1 showed no significant differences from control. Kuhn et al. (*16*) have found that glutamine present in dietary proteins and peptide-based enteral preparations help to maintain intestinal mucosal integrity, as

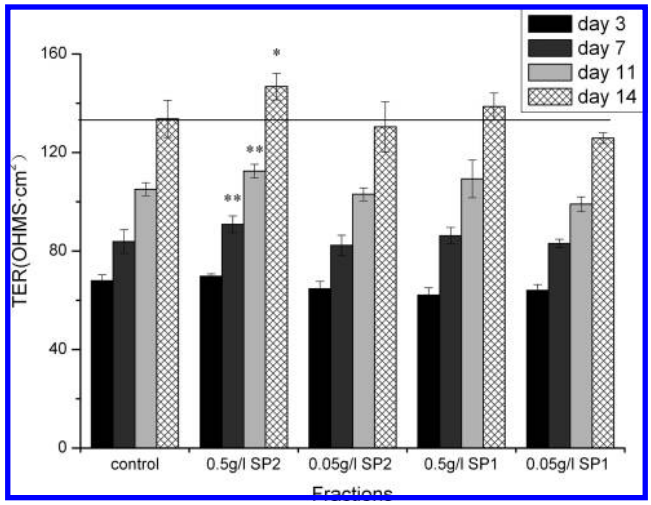

Figure 3. Relationship of TER and different concentrations of SP1 and SP2 at different growth stages of Caco-2 cells. Values are means ± SE, $n = 6$, significantly different from control at **, $p < 0.01$; *, $p < 0.05$.

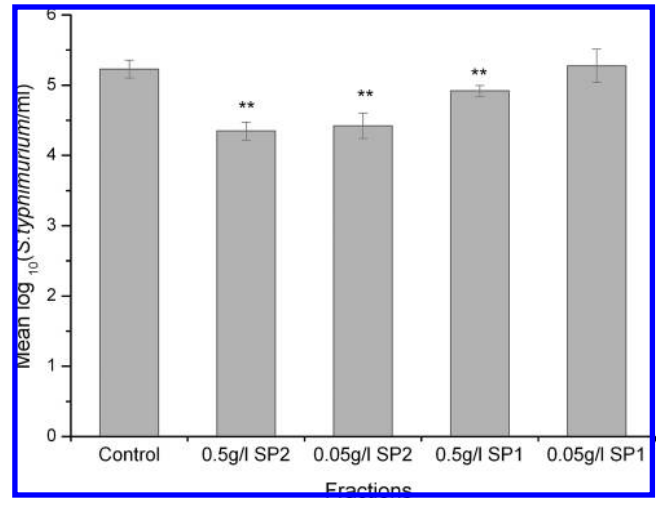

Figure 4. Penetration of *S. typhimurium* through Caco-2 monolayer. Values are means ± SE, $n = 6$, significantly different from control at **, $p < 0.01$.

well as activating enterocytes growth and enhancing immune response. Our examination of the amino acid composition of the β-conglycinin hydrolysates had found that SP2 contains a relatively high proportion (28.9%) of glutamine and glutamic acids. In contrast, SP1 contained 16.3% of glutamine and glutamic acids content. Because upon total hydrolysis by hydrochloric acid the original glutamine residue cannot be distinguished from the original glutamic acid residue, we can make only a guess, at present, that a high content of glutamine is responsible for the highly beneficial effect of SP2.

Caco-2 cells cultured to confluence undergo a spontaneous differentiation process that exhibits characteristic phenotypic features such as microvilli, tight junctions, and desmosomes that are observed in mature intestinal epithelial cells (*17*). The integrity and permeability of the monolayer could be evaluated by TER values. The in vitro culture of Caco-2 cell monolayers on transwell insert was performed for 14 days. Culture of Caco-2 cells grown in a medium with 0.5 g/L SP2 showed significantly higher TER values for days 7, 11, and 14 than control. For example, TER at day 14 was 146.8 ± 5.4 Ω·cm^2, and this was significantly ($p < 0.01$) greater than the control value of 133.8 ± 7.6 Ω·cm^2 (**Figure 3**). No significant differences between control TER values were observed for Caco-2 cells cultured with SP1. Cruz et al. (*7*) have determined cellular confluence and tight junction integrity of the Caco-2 monolayers by measurements of dextran permeability and TER values.The optimal culture period is approximately 14 days, because by this time the Caco-2 cells have formed an intact monolayer that is highly impermeable to dextran blue. Claude (*18*) had shown that the number of reticulating strands present at the intercellular junctions of epithelial cells was positively correlated with the "tightness" of the cells. This association could also be translated to mean that the TER value was also positively correlated with the number of strands present. Therefore, from the results above, it has been shown that the β-conglycinin hydrolysates were effective both in promoting growth of Caco-2 cells and in increasing the intercellular tight junction. Furthermore, results have shown that the high molecular weight (MW) fraction of the β-conglycinin hydrolysates—SP2—was more effective in raising the impermeability of the Caco-2 cells. This may suggest a plausible role for SP2 in the treatment of intestinal barrier failure type dysfunctions as well as in preventing bacterial infections and translocations through the intestinal system.

This study has investigated the action of *S. typhimurium* in the infection and translocation process of the differentiated Caco-2 monolayers. After inoculating 10^7 CFU of *S. typhimurium* onto the apical side of the 14 day Caco-2 cell cultures, bacterial count at the basolateral side of the monolayer was measured after 3 h of treatment. The results showed that the log mean count for the control was 5.23 ± 0.13 CFU/mL (**Figure 4**). In contrast, the log mean count of bacteria was found to be significantly lower ($p < 0.01$) in Caco-2 cells that had been cultured with 0.05 and 0.5 g/L of SP2 (4.42 ± 0.18 and 4.35 ± 0.13 CFU/mL, respectively) (**Figure 4**). Moreover, Caco-2 cells that were cultured with 0.5 g/L of SP1 (4.92 ± 0.07 CFU/mL) also showed a significantly lower bacterial count than control ($p < 0.01$). As an internal control to check for monolayer integrity, equal amounts of noninvasive *E. coli* DH5α were added at the same time. The results showed that *E. coli* DH5α did not appear at the basolateral side of the monolayer at 3 h (data not shown). According to the reports, *E. coli* does not normally penetrate these monolayers unless the tight junctions are disrupted or the epithelial cells are physically damaged (*13*). These indicated that the integrity of monolayers was not disrupted at 3 h in the present experiments.

The bacteria were inoculuted into the upper chamber for 3 h, and the TER values were determined. The results showed that the TER values decreased in all of the groups tested (**Table 2**). The TER values of control decreased 20 ± 6.8%. In contrast, the change of TER values (%TER) was found to be significantly lower ($p < 0.01$) in Caco-2 cells that had been cultured with 0.5 and 0.05 g/L of SP2 (6.6 ± 0.9 and 4.6 ± 2.5%, respectively). Also, the difference between TER values of monolayer pre-inoculum and post-inoculum was of no significance when cultured with SP2. Moreover, Caco-2 cell monolayers that were cultured with SP1 showed a significantly lower TER values than pre-inoculum ($p < 0.05$) (**Table 2**). Study indicated that paracellular permeability is regulated primarily by the most apical epithelial intercellular junction—the tight junction (*19*). The paracellular pathway of Caco-2 monolayers can be monitored by serial measurements of TER values in the transwell system (*7*). These results suggested SP2 fractions could improve tight junctions, which modulate the paracellular pathway between adjacent epithelial cells, and might play an important role in the inhibition of *S. typhimurium* penetration into the monolayers.

Table 2. Changes in TER Values ($\Omega \cdot cm^2$) of Caco-2 Cell Monolayers Infected Apically with *S. typhimurium* for 3 h[a]

		SP2 (g/L)		SP1 (g/L)	
	control	0.5	0.05	0.5	0.05
pre-inoculum	133.8 ± 7. 6	146.8 ± 5.4#	130.4 ± 10.3	138.7 ± 5.5	125.8 ± 2.2
3 h post-inoculum	107.3 ± 14.4∗	137.1 ± 6.1##	124.2 ± 6.7	120.0 ± 8.1∗∗	110.6 ± 11.2∗
% TER	20 ± 6.8	6.6 ± 0.9##	4.6 ± 2.5##	13.5 ± 3.6	12.1 ± 7.7

[a] %TER = 1-(3 h post-inoculum/pre-inoculum) × 100. Significance relative to pre-inoculum with the same treatment: ∗, $p < 0.05$; ∗∗, $p < 0.01$. Significance relative to control, same time: #, $p < 0.05$; ##, $p < 0.01$.

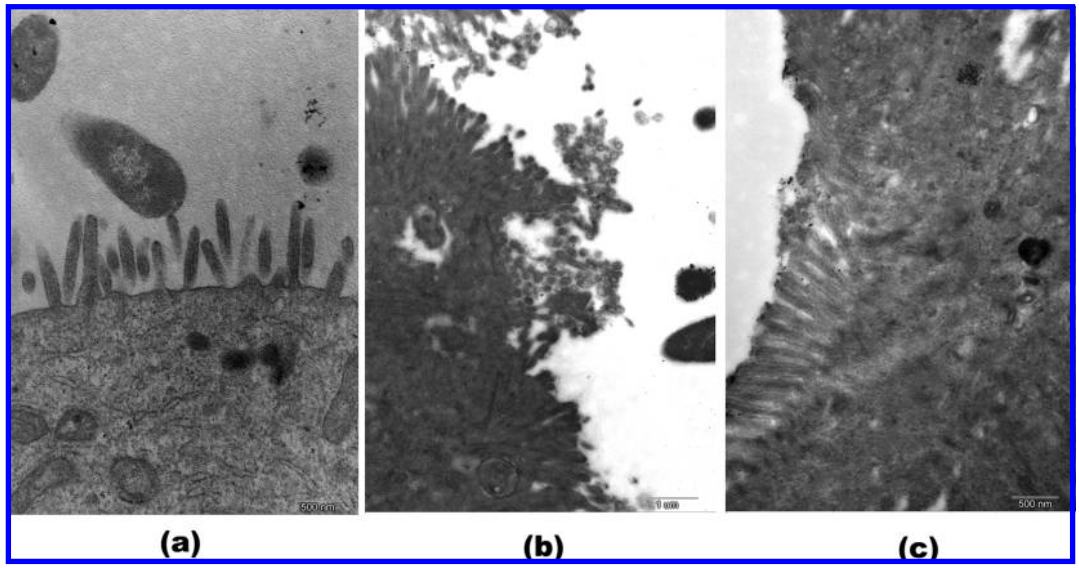

Figure 5. Transmission electron micrographs showing Caco-2 epithelial cells under infection by *S. typhimurium*: (**a**) adhesion between *Salmonella* and the microvillus cell surface (bar marker = 500 nm); (**b**) disruption of microvilli as *Salmonella* enters Caco-2 cells (bar marker = 1.0 μm); (**c**) Caco-2 cells cultured with SP2, showing the tight junctions (bar marker = 500 nm).

Further examination of the process of *S. typhimurium* infection was performed using TEM. As observed in **Figure 5a**, uninfected cells of Caco-2 monolayer were shown to be entire and well-defined with numerous intact microvilli. In addition, contact between the *Salmonella* and the microvilli surface could be observed (**Figure 5a**), and this eventually led to an effective adhesion between the two cell surfaces. Finlay and Falkow (*13*) had found that this adhesion stage was the initial sign of infection and was attributable to an interaction between the surface receptors present on the epithelial cells and the fimbriae of Salmonellae. Moreover, it has been demonstrated that *S. typhimurium* was able to cause the rearrangement of cytoskeletal components in the vicinity of adherent/invading bacteria to facilitate its penetration into the epithelial cell (*20*). The initial process of penetration into epithelial cells by *Salmonella* can be observed during the infection process. Consequently, more microvilli structures are disrupted, which allowed more penetration of *Salmonella* to occur. As indicated in **Figure 5b**, the microvilli on the cell surface were structurally damaged and disintegrated as *Salmonella* penetrated the cell. Moreover, the structural integrity of the Caco-2 monolayers was also disrupted. In addition, internalization of bacteria (**Figure 5b**) can be observed. However, this loss of structural integrity was not observed in Caco-2 monolayers that were cultured in SP2. Intercellular junctions such as tight junctions and desmosomes could still be observed between cells (**Figure 5c**). These results further support the role of SP2 in increasing the impermeability of epithelial cells and hence prevent the invasion by *S. typhimurium*.

Bacterial adhesion to host cells is considered to be an important initial event in bacterial infection and pathogenesis (*21*). In many cases, the adhesion is mediated by interaction between specific adhesins on the microbial surface and complementary carbohydrate (glycoproteins or glycolipids) receptors on the mucosal surface of the host (*22*). Sharon (*23*) had pointed out that addition of suitable glycoprotein molecules could mimic the carbohydrate chains of host cell surface and, hence, results in less binding of the bacterial lectin chains to the host cell. Examples could be observed in Rhoades et al. (*24*), who had noted the effectiveness of caseinoglycomacropeptide in blocking the adhesion of pathogenic *E. coli* strains to cultured human cells. In addition, ovomucin glycopeptides were able to bind with *E. coli* O157:H7 and thus prevented the bacteria from binding to its host cell surface (*25*). *S. typhimurium* expresses type 1 fimbriae adhesin on its cell surface, binding it to mannose receptors on the intestinal epithelium (*26*). The hydrolysates utilized in the experiment were isolated from β-conglycinin. Kimura et al. (*27*) had found that 96.6% of the N-glycans of the storage glycoprotein in soybean were composed of the high-mannose type of sugar residues. In this study, the majority of the carbohydrate content in the β-conglycinin hydrolysates was shown to be distributed within the SP2 fraction (8.5%). Therefore, it was assumed that the mannose substructure present on SP2 recognized by *S. typhimurium* blocks the adhesion of bacteria to Caco-2 cells and, therefore, reduces their effective penetration of epithelial cells.

In conclusion, this study has examined the effects of β-conglycinin hydrolysates on (1) the growth and development of in vitro Caco-2 epithelial cells, (2) the formation of intercellular tight junctions, and (3) the resistance against *S. typhimurium* in the penetration of epithelial cells. The results in this study have clearly determined that the high molecular weight (MW) fraction of the hydrolysates—SP2—is able to maintain the structural integrity and increase the impermeability

of epithelial cells during the infection by *S. typhimurium*. Moreover, stronger effects by SP2 than the lower MW fraction SP1 in all treatments were observed. The present study is concerned primarily with the influences of the molecular weight profile of the β-conglycinin hydrolysates on the effects metioned above. However, to understand how SP2 promotes intestinal defense response, future studies will be needed to examine the mechanism of how SP2 interacts with intestinal epithelial cells and *S. typhimurium*.

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