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Original Article

Effect of Soy Protein Diet on Mucosa Layer of Murine Small Intestine

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Abstract

Soy and fermented soy are popular and recognized as a health food among Koreans. Since soy proteins are known to be protease resistant, even to pepsin and pancreatin, it is hypothesized that soy proteins may interact with the intestinal tract and trigger certain physiological reactions. To test this hypothesis, mice were fed diets supplemented with soy, Chunkukjang, or casein. The differentially expressed proteins were analyzed using 2-D gels and identified by peptide mass fingerprinting using mass spectrometry. The majority of the differentially expressed proteins could be functionally grouped into metabolic enzymes and calcium-binding proteins. The differential protein expression by the soy-fed groups was also verified based on a representative protein, tropomyosin, using a Western blotting analysis. In addition, the soy-fed groups exhibited a taller villi structure. Therefore, this study suggests that soy proteins can be an effective nutrient and physiological stimulant for the intestines.

Keywords : soy, Chungkukjang, small intestine, villi, proteome

Introduction

The small intestine, which is subdivided into the duodenum, jejunum, and ileum, includes the functions of nutrient absorption, digestion, and secretion. To perform these tasks efficiently, the surface area of the small intestinal lumen is drastically increased by epithelial protrusions, called villi. The structure of the tissue specified for the absorption of virtually all nutrients into the blood contributes approximately 10% of the daily body protein synthesis (Attaix and Arnal 1987; Bermingham et al. 2006). A variety of studies have already shown the effects of specific dietary foods on the small intestine in terms of morphological changes in vivo. Plus, such studies have suggested that the structure of the digestive tract is dynamically changed based on communicating directly with the external environment (Fasano 2000; Patsos and Corfield 2009).

Soybeans are one of the most important staple crops and are grown mainly for edible vegetable oil, high-protein feed supplements, and other phytonutrients (Mattoo et al. 2010). Due to their high protein and lipid content, soybeans and other processed soybean products, such as Chungkukjang and Doenjang, have long been important components of the Korean diet (Lee et al. 2011).

Daily soybean intake can affect the small intestinal mucosa, leading to a change in the expression of certain proteins or the structure of the small intestine. Such pronounced changes in the small intestinal structure can involve an increase in the villus and crypt compartments, as well as an overall increase in the intestinal circumference. Thus, to visualize the influence of dietary soy and Chungkukjang on the small intestine, this study used hematoxylin and eosin staining (H&E staining) of murine small intestinal tissue sections.

A conventional yet powerful and common approach for protein identification, particularly in high-throughput technologies, is two-dimensional(2D) gel separation followed by a mass spectrometric analysis for identification. The ultimate goal of an analysis using 2D gel electrophoresis is to compare the proteomes from more than two different samples to find the changes in the protein expression that manifest differences between the samples being investigated. Peptide mass fingerprinting (PMF) is another essential step for identifying proteins from experimentally collected peptide masses derived from an *in-silico* digest of a sequence database (such as NCBI and Swiss-Prot) (Siepen et al. 2007).

Accordingly, in addition to a structural analysis of the small intestine, this study compared the changes in the protein expression of the small intestinal mucosa when exposed to dietary soy and Chungkukjang or dietary casein as a protein source.

Materials and Methods

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Animals and Diets.

The soy, Daewon offered from Soy Venture, Kyungpook National University, was washed and immersed in water for 18 h. After boiling at 121°C for 30 min, the soy was dried, then one half was ground and lyophilized, Meanwhile, the other half was inoculated with *B. amyloliquifaciencs* 86-1 and incubated at 42°C for 48 h. This fermented soy, Chungkukjang, was then ground, lyophilized, and stored at -20°C.

Twenty-one male BALB/c mice (7 weeks old) were purchased from Bio Korea (Osan, Korea). The mice were kept in a temperature-controlled (22°C) room with a 12 h light/dark cycle and food and water were provided *ad libitum*. Equilibrated according to their weights, the mice were assigned to three groups (n=7) with different diets: Control, Soy, and Chungkukjang, respectively. All the diet formulas were designed to be iso-caloric and iso-nitrogeneous. The experimental diets was started when the mice were 8 weeks old and continued for fourteen days. The animal weights were recorded and the foods stored at 4°C during the course of the study. The diet ingredients are shown in Table 1.

In addition, another twenty-five male BALB/c mice (7 weeks old) were assigned to five groups: Control, Soy, Chungkukjang, Glycerol, and Genistein. The control group was fed commercial AIN93G with casein as the major protein source. As soy contains phytoestrogens, called iso-flavones, primarily genistein and daidzein, these iso-flavones could affect the results. Thus, to eliminate the possible impact of iso-flavones, two more control groups were added: Genistein and Glycerol, since genistein dissolves in glycerol. The Genistein and Glycerol groups were fed the same food as the Control group, yet an intubation process was added, where genistein was dissolved in glycerol and ethanol, and the intubation was conducted using an intubation tube every two days. All the conditions and food formulas during the study were the same as explained above.

Table 1. Compositions of the defined diets

	AIN93G	Chung	Soy
Sample		510	510
Casein	200	0	24.05
Corn Starch	397.486	293.038	267.589
Dextrose	132	132	132
Sucrose	100	100	100
Cellulose	50	0	0
Soybean Oil	70	2.884	0
t-BHQ	0.014	0.014	0.014
Mineral Mix	35	0	0
Vitamine mix	10	10	10
L-Cysten	3	3	3
Choline Bitrartarate	2.5	2.5	2.5
Total(g)	1000	1053.436	1049.153

Intestinal Sampling

The euthanasia was conducted based on exposure to diethyl ether. Thereafter, the small intestinal lumen was washed with ice-cold PBS and cut longitudinally on an ice-cold glass plate. The entire length of the small intestine was scraped, collected, and stored at -80°C until use. To determine the Immunohistochemistry, the PBS-washed tissue was directly transferred into a fixation solution containing 4% (w/v) paraformaldehyde, 0.3% (w/v) glutaraldehyde, and 0.002% (w/v) $CaCl_2$. The scraped mucosa was then ground in liquid nitrogen and the resulting powder (100-150 mg) homogenized in a lysis buffer (200-500 μL) containing 4% (w/v) CHAPS, 10 mM Tris-HCl (pH 8.0), and 60 mM DTT. After sonication at 40 A and 5 pulses for 2 min, the sonicated lysate was centrifuged at 13200 rpm at 4°C for 30 min. The supernatant containing the solubilized proteins was then collected, desalted using a Micro Spin G-25 column from GE Healthcare (Buckinghamshire, UK), precipitated with 10% (w/v) TCA, and the pellet washed with acetone/ethanol and dissolved in a rehydration solution containing 7 M urea, 2 Mthiourea, 4% (w/v) CHAPS, 1 M DTT, and 2% (w/v) ampholyte. All the procedures were performed on ice. The protein concentrations were determined using a protein assay kit from Bio-Rad. While the conditions can vary depending on the sample and the purpose of the study, the above mentioned conditions were the most suitable for this study. Plus, enhancing the resolution on 2-D images was pursued as a result of seeking an optimum way for extracting and harvesting as many proteins as possible.

Two-dimensional Gel Electrophoresis.

The pooled intestinal mucosa proteins were subjected to 2-D PAGE, where the sample volumes were adjusted to analyze the same amount of protein for each set of experiments (800 μg-1mg). An immobilized pH gradient (IPG) strip (17 cm, pH 4-7) was rehydrated for 14 h, followed by iso-electrofocusing (IEF). After equilibration with a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 1% (w/v) DTT, and a solution with 5% (w/v) iodoacetamide, the IPG strip was then placed on top of a polyacrylamide gel (12%, 180×200 mm) and electrophoresed under a constant

current (35 mA) at 20°C until the BPB reached 18 cm. Thereafter, the gels were fixed and the spots detected using 0.1% (wt/vol) Coomassie Brilliant Blue R-250,comprising of 50% methanol and 10% acetic acid, and destained in 15% methanol and 5% acetic acid until the background became clear. The quantitative and qualitative statistical analyses were performed using PDQuest software (Bio-Rad, Hercules, CA, USA).

MALDI-TOF-MS.

Briefly, each spot was cut into roughly 1-mm^3 pieces in a 1.5 mL plastic tube, then washed with deionized water 2 times to remove the ammonium sulfate and destained with 50% acetonitrile (ACN) containing 50 mM ammonium bicarbonate (NH_4HCO_3) until the gel turned opaque. After drying, the gel pieces were re-swollen using a trypsin solution (10 ng/μL sequencing grade modified trypsin, 50 mM NH_4HCO_3) on ice, followed by digestion conducted at 37°C for 16 h. The resulting tryptic fragments were transferred into a 1.5 mL Protein LoBind Tube (Eppendorf, Germany) and extracted using 0.1% (w/v) trifluoroactic acid (TFA) dissolved in 60% (w/v) ACN. The extract was then concentrated to about 10 μL or dissolved in 10 μL of 5% (w/v) formic acid in the case of complete dryness. One μL of a matrix solution (10 mg/ml α-CHCA dissolved in 50% (w/v) ACN) was then mixed with the peptides on the plate and left until co-crystallization. Angiotensin I, bradykinin, and neurotensin were used as the internal standards. The peptide mass measurements were taken using a Voyager DE-STR MALDI-TOF mass spectrometer in a positive ion reflector mode and with a low mass gate of 800. The sample spectra were acquired based on 100 shots with a 337 nm nitrogen laser operated at 20 Hz.

Identification of Proteins by Peptide Mass Fingerprinting

The spectra were calibrated using the m/z values of angiotensin I (1296.5692 Da), bradykinin (1060.5692 Da),and neurotensin (1672.9175 Da), including trypsin autolysis peaks at m/z 2211.10. The calibration was conducted using the m-over-z

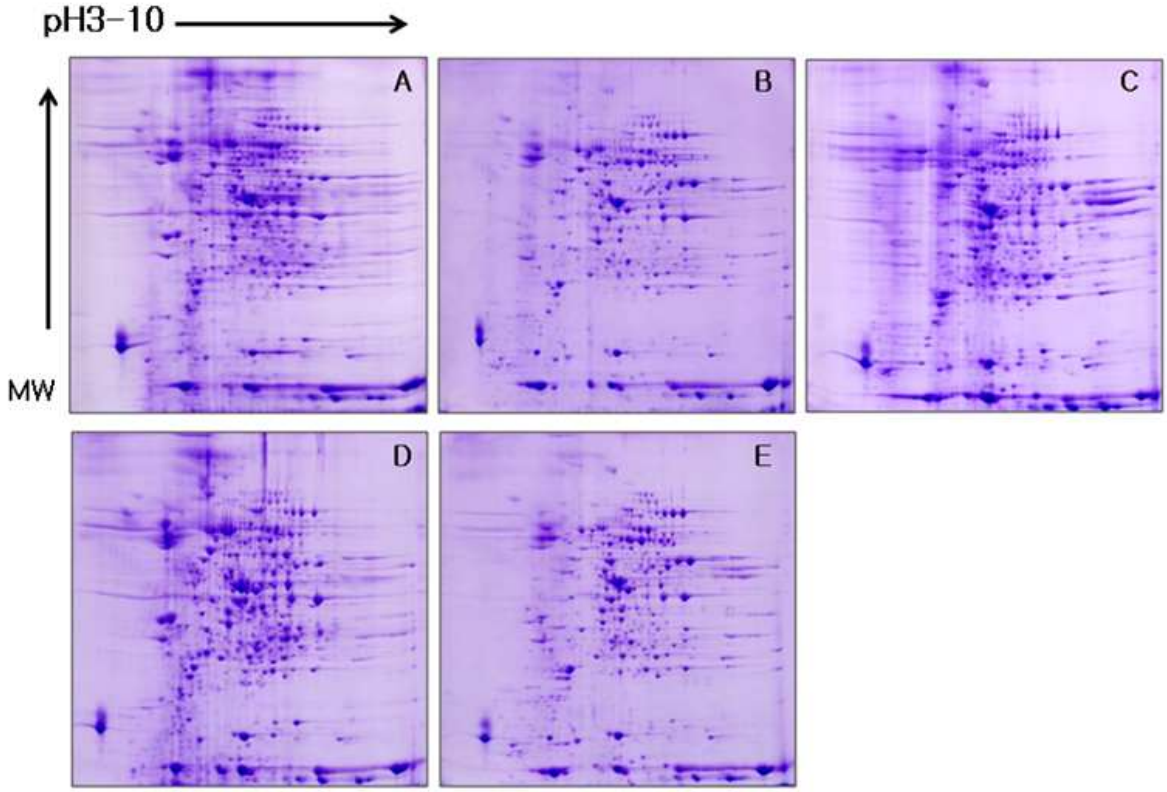

Figure 1. 2-D gel containing intestinal proteins from pool of designated group of small intestinal mucosa samples stained with Coomassie Blue.

IEF was performed on 17-cm length of IPG strip using pH range of 3-10 (linear pH gradient). Second separation was performed using 12% SDS-PAGE gel; A. control, B. soy, C. Chungkukjang, D. glycerol E. genistein.

program (http://bioinformatics.genomicsolutions.com). To identify the proteins, a database search with peptide mass fingerprinting of the analyte was performed against the databases NCBInr (National Center for Biotechnology Information) and SWISS-Prot using the Mascot from Matrix Science version 2.1 (http://www.matrixscience.com) and MS-Fit version 5.3.2 from the Protein Prospector (http://prospector.ucsf.edu/) search engines.

Western Blot blotting.

Fifty milligrams of the ground tissues were lysed in a buffer containing 4% (w/v) CHAPS, 10 mM Tris-HCl (pH 8.0), and 60 mM DTT, incubated on ice for 15 min, and then sonicated, centrifuged, and the supernatant collected. Thereafter, the protein concentrations were determined using a Protein Assay kit (Bio-Rad). Meanwhile, 60 μg of each lysate was eletrophoresed through a 12% SDS-polyacrylamide gel and transferred to a PVDF membrane by electroblotting. Next, the membrane blots were incubated with 5% skim milk to block the non-specific binding sites. The immunodetection of proteins was then revealed using rabbit anti-tropomyosin and goat anti-rabbit IgG horseradish peroxidase-conjugated immunoglobulin at a dilution of 1:500 and 1:1000, respectively. Mouse skeletal muscle tissue and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as the positive controls. The verification of GAPDH binding was performed using the same de-probing method as described

Table 2. Increased proteins of control group (pH 4-7)

Spot No.	Protein MW (Da)/pI	Protein Name
15	57144/4.8	Protein disulfide-isomerase precursor
22	60756/6.1	Gametocyte-specific factor 1
25	52768/5.4	Cytosolic non-specific dipeptidase
27	52247/5.6	Glutathione synthetase
39	48355/6.2	Ornithine aminotransferase, mitochondrial precursor
40	48355/6.2	Ornithine aminotransferase, mitochondrial precursor
43	39992/5.5	Adenosine deaminase
44	39992/5.5	Adenosine deaminase
46	45781/5.9	Aminoacylase-1
52	33196/5.5	3'(2'),5'-bisphosphate nucleotidase 1
54	38879/6.1	Arginase-2, mitochondrial precursor
55	38879/6.1	Arginase-2, mitochondrial precursor
56	42575/5.9	Leukocyte elastase inhibitor A
60	36024/6.3	Delta-aminolevulinic acid dehydratase
83	32751/5.8	Ketohexokinase
84	32277/5.8	Purine nucleoside phosphorylase
99	28405/5.3	Latexin
175	28405/5.3	Purine nucleoside phosphorylase
97	25492/5.5	Proteasome subunit alpha type-3
98	38439/6.5	Proteasome subunit alpha type-3
107	29116/5.5	Cathepsin S precursor
108	29063/6.4	Proteasome subunit beta type-4 precursor
109	24871/5.7	Proteasome subunit beta type-10 precursor
110	27373/6.3	Peroxiredoxin-6
113	22965/6.1	Proteasome subunit alpha type-6
114	32277/5.8	Proteasome subunit beta type-3

above, except the de-probing solution containined 100 mM 2-mercaptoethanol, 2% (w/v) SDS, and 0.5 M Tris-HCl (pH 6.8). The immunodetection was then achieved based on exposure to peroxide and a luminophor/enhancer solution. The detection

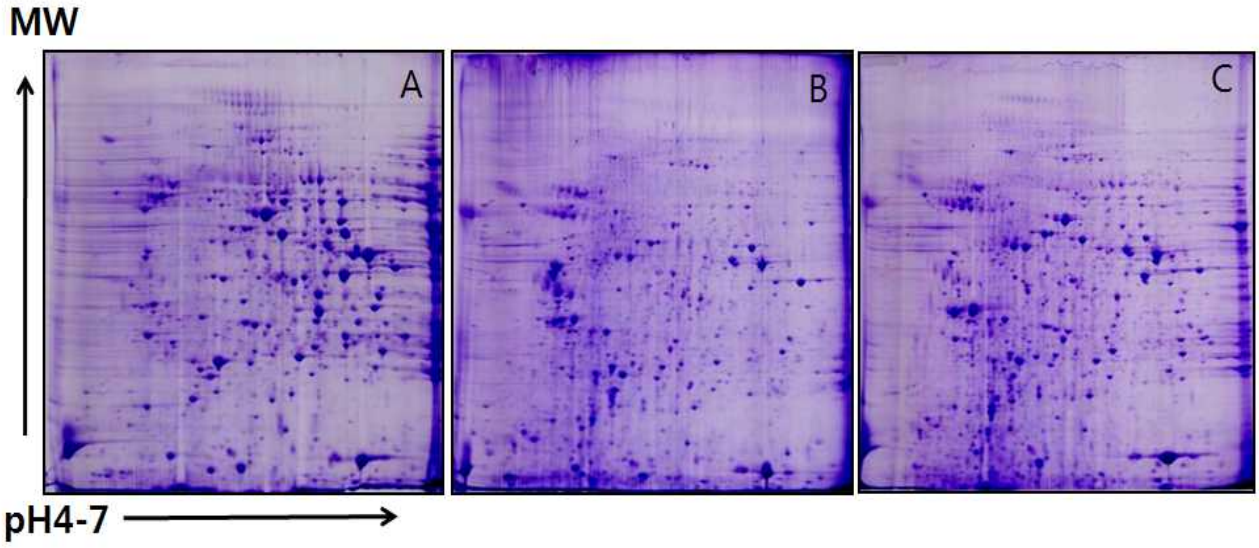

Figure 2. 2-D gel containing intestinal proteins frompool of designated group of small intestinal mucosa samples stained with Coomassie Blue.

IEF was performed on 17-cm IPG strip with pH range of 4-7 (linear pH gradient). Second separation was performed using 12% SDS-PAGE gel; A. control, B. soy, C. Chungkukjang.

Table 3. Increased proteins of soy group (pH4-7)

Sample No.	Protein MW (Da)/pI	Protein Name
soy16	32681/4.7	Tropomyosin alpha-1 chain
soy17	32837/4.7	Tropomyosin beta chain
soy22	32863/4.7	Tropomyosin alpha-3 chain
soy24	35990/5.4	Annexin A4
soy32	23408/5.1	Rho GDP-dissociation inhibitor 1
soy33	23397/5.1	Proteasome subunit beta type-9 precursor
soy34	20810/5.2	Lactoylglutathione lyase
soy39	11651/4.4	60S acidic ribosomal protein P2
soy40	16318/4.8	Cytochrome b5 type B precursor
soy478	23408/5.1	Rho GDP-dissociation inhibitor 1
soy488	20810/5.2	Lactoylglutathione lyase
soy490	20831/5.2	Phosphatidylethanolamine-binding protein 1
soy497	16318/4.8	Cytochrome b5 type B precursor
chung29	32837/4.7	Tropomyosin beta chain
chung208	52768/5.4	Cytosolic non-specific dipeptidase
chung184	42599/5.5	Serpin B6
chung185	42599/5.5	Serpin B6

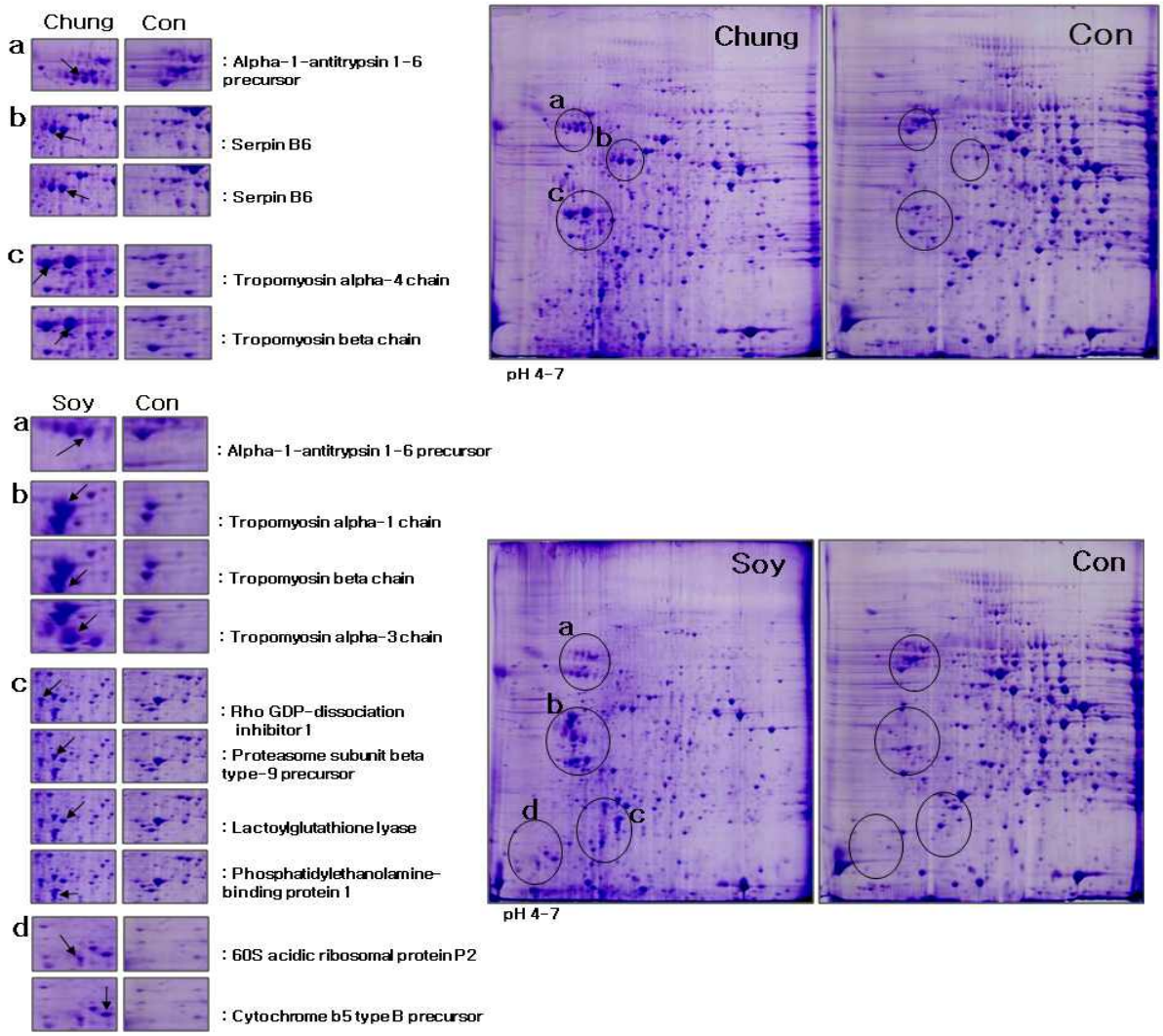

Figure 3. Identities of differentially expressed proteins between groups based on 2-D based MALDI-TOF-MS analysis.

Table 4. Comparative protein expression related to calcium ion binding (pH 4-7)

Spot No.	Protein MW (Da)/pI	Protein Name
con81	36844/5.6	Annexin A8
con125	22432/5.0	Calcium-binding protein p22
con216	53409/5.0	Nucleobindin-1 precursor
soy23	33407/5.2	Regucalcin
soy24	35990/5.4	Annexin A4
soy459	33407/5.2	Regucalcin
soy465	35990/5.4	Annexin A4
chung20	35990/5.4	Annexin A4
chung207	33407/5.2	Regucalcin

was then performed using a chemiluminiscent detector, Las image 4000.

Histology and Immunohistochemistry.

Cryosection strategy: Small tissue samples in a fixative (4% (w/v) paraformaldehyde, 0.3% (w/v) glutaraldehyde, and 0.002% (w/v) $CaCl_2$) were rinsed several times in phosphate buffered saline (PBS, pH 7.4), and then dehydrated in sucrose/PBS using sucrose gradients of 10%, 20%, and 30%. The fixed tissues were then embedded in an OCT compound and kept in a deep freezer. Thereafter, the frozen tissues were sliced (10-15 μm) using a microtome at -28°C, dried, and stored in the freezer until use.

Immunohistochemistry

The tissue samples were permeabilized and blocked in 0.4% (w/v) Triton-X100 and 4% (w/v) normal goat serum (NGS) in PBS at room temperature for 2 h. The primary antibody, rabbit antibody tropomyosin, was diluted 1:100 in a working buffer (0.4% (w/v) Triton-X100, 4% (w/v) normal goat serum in PBS) and incubated with the samples overnight at 4°C. The specimens were then washed several times with PBS before the secondary antibody, goat anti-rabbit labeled with FITC, was applied at 1:500 in the working buffer for 2 h in the dark. Thereafter, DAPI at a concentration of 1 mg/ml was applied to the secondary antibody incubation solution, and the specimens rinsed four times in PBS before being mounted on a fluoromount.

Results

The animals did not show any difference in weight gain among the groups throughout the study.

Investigation with narrow pH and abridgement of supplemented controls

The optimal sample preparation facilitated the successful production of 2-D images, as shown in Figure 1. A semi-quantitative analysis was performed to identify the proteins in the control group. The 2-D based MALDI-TOF-MS analysis resulted in the identification of 95 proteins, most of which play roles in inducing other pathways or bioprocesses (Tables 2 and 3, and Figure 3). Figure 2 shows that most of the protein spots were located near pH 5-6. Although the aggregated parts were not easy to discriminate, an image analysis using PDQuest revealed almost identical 2-D patterns among the groups. Therefore, one-dimensional separation from pH 4-7 was conducted to allow a more thorough observation of the clustered parts and conduct a comparison among the groups. To evaluate the details of the protein expression pattern in the small intestinal mucosa, another semi-quantitative identification of the spots from pH 4-7 was also performed. Figure 3 shows that 126 proteins were identified as a result of the 2-D based MALDI-TOF-MS analysis. Plus, based on the peptide mass fingerprinting process, the proteins identified from pH 4-7 were mostly involved in metabolism, which may reflect the dynamic state of the small intestinal mucosa due to the constant degradation and synthesis reactions. However, on the acidic

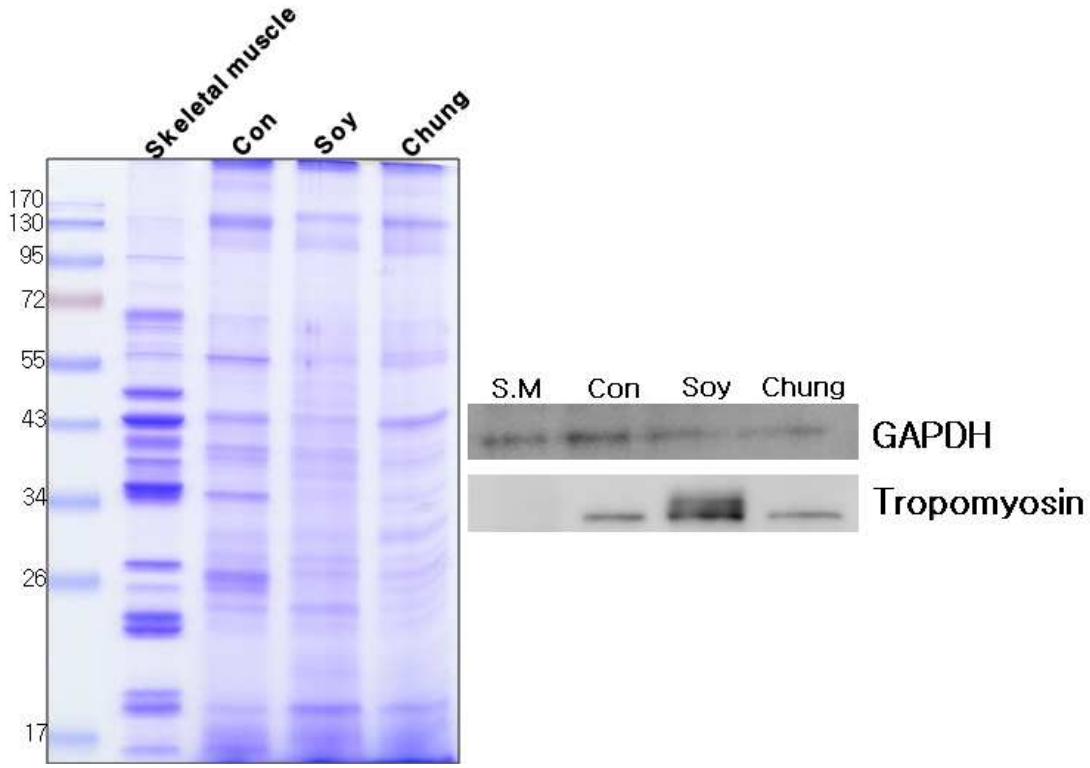

Figure 4. SDS-PAGE and Western blot analysis using rabbit anti-tropomyosin primary antibody.

Tropomyosin bands are shown at 34-36 kDa and GADPH at 37kDa.

side of the gel, higher levels of proteins were expressed by the soy and Chungkukjang diet groups when compared with the control group.

Confirmation of tropomyosin over-expression in soy diet group by immunoblot analysis

To confirm that the selected proteins on the 2-D gels were really over-expressed by the soy-fed groups, one representative protein, tropomyosin, was selected for a Western blotting analysis using the proteins extracted from the small intestinal mucosa. The lysates were analyzed by immunoblotting using an anti-tropomyosin antibody. As the amount of protein loaded in each lane was normalized by the amount of GAPDH, as shown in Figure 4, the small intestine samples from the soy- and Chungkukjang-fed groups expressed more tropomyosin, thereby supporting the idea that the villi structure was more developed in the soy-fed groups (Figure 5).

Tissue staining and immunohistochemistry

For the histological investigation of the small intestinal tissues,

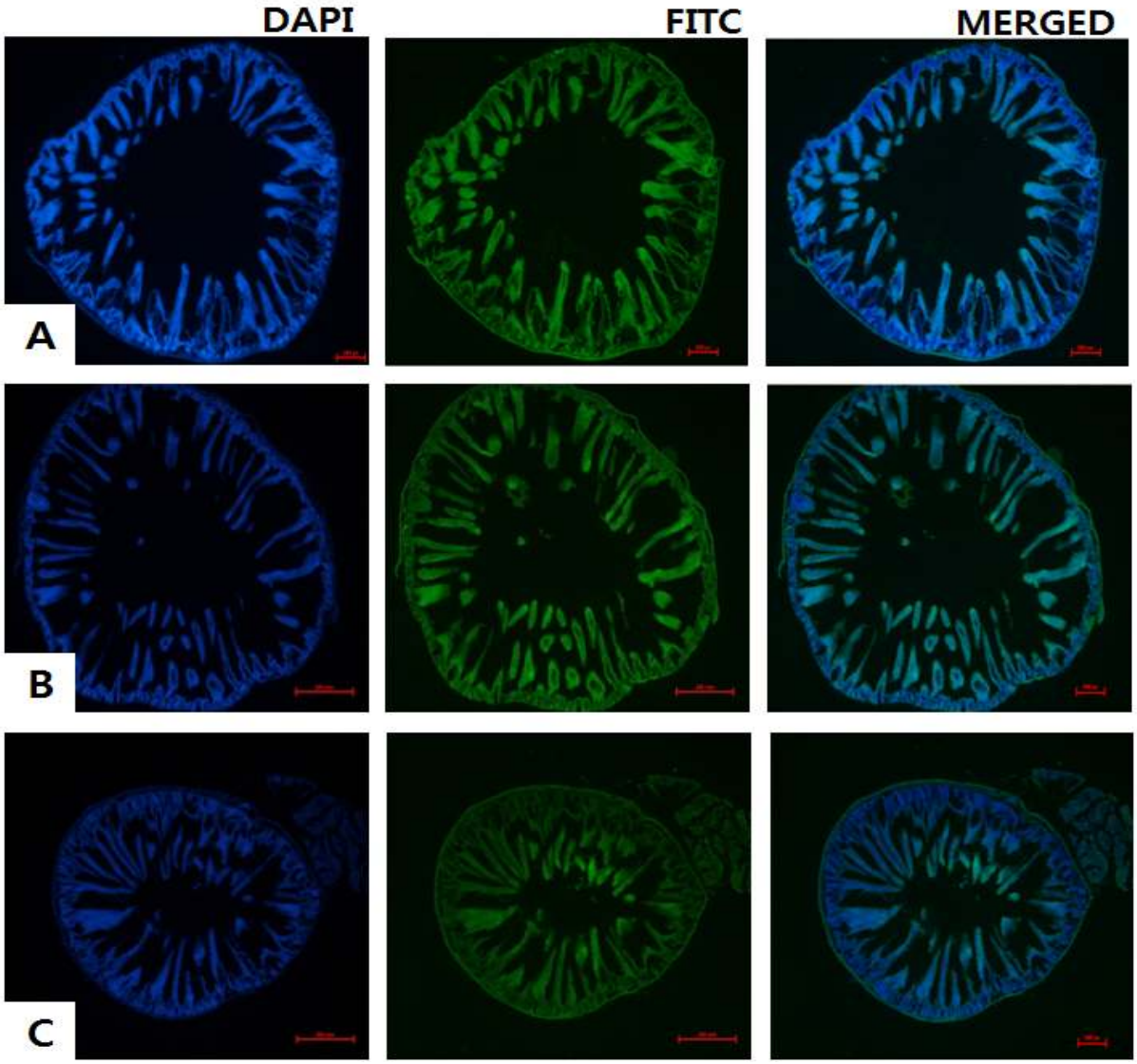

Figure 5. Immunohistochemistry of cryo-sectioned duodenum (15μm in thickness).

Location of primary anti-tropomyosin was detected using binding FITC-conjugated secondary antibody. Non-specific binding was blocked by incubationin buffer containing normal goat serum. Counterstaining was DAPI (1mg/ml); A. control, B, soy, C, Chungkukjang.

the immunohistochemistry staining revealed the location and amounts of tropomyosin in the tissues. The tissues collected from the duodenum were also investigated as regards the villi height and intestinal diameter. As a result, the most developed (tallest) villi structure formation was observed in the soy-fed groups(Figure 5).

Discussion

This study used a proteomic analysis to identify the proteins in the intestinal mucosa of mice fed with Chungkukjang and soy. The small intestine is a flexible organ that expresses and activates specific proteins in response to constant environmental stimuli. A dynamic passage for food, the small intestine mainly produces proteins involved in digestion, food absorption, and protection from xenobiotics. However, the types and amounts of proteins produced by the small intestine are heavily dependent on the foods ingested. Thus, the ingredients and composition of the foods we eat provide the building blocks and homeostasis for the small intestinal mucosa. Therefore, this adaptive system can be a useful source to investigate the effects of particular foods.

Soy has a higher resistance to digestion than casein. As a result, after intestinal digestion, the remaining soy proteins may assist in the development of villi, the absorption structure of the small intestine, by activating the smooth muscle layers. One of the key soy proteins remaining in the small intestinal mucosa is tropomyosin, which is an actin-binding protein that is widely distributed in eukaryotic muscle and non-muscle cells. Actin-myosin interaction during smooth muscle contraction is mediated by tropomyosin. Thus, changes in the heights of the villi and micro villi are relevant to such an adaptive response (Tang et al. 2004). This process also includes the binding of calcium to the proteins (Table 4). Therefore, the digested products of soy proteins or proteins resistant to digestion may be useful for the stimulation and proliferation of the small intestine, where a well-developed small intestine has increased peristalsis, catalysis, and absorption.

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