



Assessing the digestibility of genetically modified soybean: Physiologically based *in vitro* digestion and fermentation model

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ABSTRACT

Gastrointestinal digestibility of new proteins inserted in food supply is one of significant parameters for assessing safety of genetically modified (GM) foods on the assumption that digestive stability is undesirable. Digestive stability of GM soybean was evaluated under the physiologically based *in vitro* digestion model. Electrophoresis was used to follow the *in vitro* sequential digestions/fermentation procedure and Western blotting was employed to confirm the new protein in GM soybean.

CP4EPSPS newly expressed in GM soybean was completely digested in 15 s in the gastric fluid in this model. In the assay of fermentation of new protein, CP4EPSPS was also completely degraded and no fermentation products were found during 24 h of colon fermentation. Results from this study suggested that CP4EPSPS in GM soybean does not have a potential allergenicity due to its complete digestion within a very short time in gastric fluid. No formation of colonic protein fermentation products confirms that new protein is not capable of initiating colon cancer.

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1. Introduction

GM soybean used for animal feeds, food ingredient, and food additives is one of the most widely planted genetically modified (GM) plants in the world (Alexander et al., 2004; Kim et al., 2006b; Ocana, Fraser, Patel, Halket, & Bramley, 2007; Poulsen, 2004). These GM soybeans possess a gene that proclaims herbicide or insecticide resistance, such as the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium tumefaciens* CP4 (Ocana et al., 2007). The entering of GM plants to human diet has raised consumer concerns about the possible transfer of new protein from GM plants to human digestive tracts (Kim et al., 2006a, 2006b; Konig et al., 2004; Netherwood et al., 2004). Gastrointestinal digestibility of new proteins inserted in food supply is one of significant parameters for assessing safety of GM foods on the assumption that digestive stability is undesirable (Astwood, Leach, & Fuchs, 1996; Bannon, Fu, Kimber, & Hinton, 2003; Jiang, Qu, Hu, Ni, & Lin, 2007; Kimber & Dearman, 2002; Ladics et al., 2003; Takagi, Teshima, Okunuki, & Sawada, 2003).

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Simulating gastric or intestinal fluid *in vitro* testing has been employed for evaluating digestive stability of new proteins (Astwood et al., 1996; Bannon et al., 2003; Jiang et al., 2007; Jonas, Angignac, Antoine, Classen, & Huggett, 1996). Several studies have reported that the new protein CP4EPSPS was digested in simulating gastric fluid within a short time and its degradation was faster than that in intestinal fluid (Harrison et al., 1996; Kim et al., 2006a, 2006b; Okunuki et al., 2002). *In vitro* models performed in previous studies were, however, evaluated under process of digestion mimic a singular digestive compartment rather than conducted sequentially (Astwood et al., 1996; Fu, Abbott, & Hatzos, 2002; Harrison et al., 1996; Kim et al., 2006a, 2006b; Takagi et al., 2003; Thomas et al., 2004; Yamada et al., 2006). Another limitation of these studies was the lacking of simulating basal conditions on the volume ratio of ingested foods to secreted digestive fluids. Therefore, the objective of our study was to evaluate digestibility of new protein in GM soybean using three phases of the *in vitro* digestion model simulating digestive fluids. Besides the testing allergenic potential, we assessed the formation of colonic new protein fermentation. Newly expressed protein may become indigestible during processing and this leads to increased colonic protein fermentation products, which can initiate colon carcinogenesis (Dona & Arvanitoyannis, 2009; Ferrini, Mannoni, Pontieri, & Pourshaban, 2007; Wilson & Basit, 2005). For testing this objective, the

in vitro colon fermentation model system inoculated with human feces was carried out.

2. Materials and methods

2.1. Materials

A purified high degree of CP4EPSPS protein produced in an *Escherichia coli* expression system was obtained by Dr. Kim at the

Kyung-Hee University (Gjenggi-Do, South Korea). α -Amylase (A1031), pepsin (P 7000), bile acid (B8631), pancreatin (P3292), and protein marker used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Physiologically based *in vitro* digestion model

Simulated salivary, gastric, and intestinal fluid was prepared as described in the United States of Pharmacopoeia (USP [United States Pharmacopoeia], 1995) and Oomen, Tolls, Sips, and Van den Hoop (2003) with some modifications. The volume of fluids ingested, secreted from the gastrointestinal tract was mimic human physiology condition (Guyton, 1991). The ratio of volume ingestion of food to saliva to gastric juice to bile to pancreatic juice was 2.5–1.0–1.5–1.0–1.0. Aliquot of pure protein (CP4EPSPS) were suspended in the 2 mL micro-centrifuge tubes containing 210 μ L of human salivary α -amylase solution (w/v, 0.2 mg/20 mM phosphate buffer, pH 7.0) and incubated at 37 °C for 3 min in a shaking water bath at 55 rpm (Lab companion, Jeio Tech, South Korea). Subsequently, 280 μ L of gastric juice (0.32%, w/v 3.2 mg pepsin/0.1 M HCl) was added to the tubes, followed by acidification to pH 2 with 0.1 M HCl (Analytical grade, Sigma Chemical, Co.). Solutions were incubated at 37 °C for 0, 15 s, 30 s, 1 min, 5 min, 15 min, 30 min, 45 min, and 60 min in a shaking water bath at 55 rpm (Lab companion). After this period, the small intestinal phase was initiated with neutralizing to pH 5.3 by addition of 100 mM sodium bicarbonate solution (2.4 mg/ NaHCO_3). For the last digestion step, 210 μ L of bile acids and 210 μ L of pancreatic juice (0.4 mg/20 mM phosphate buffer) were added. Sample pH was adjusted to 7.0 ± 0.5 using 0.1 M NaOH (Analytical grade, Sigma Chemical Co.), then incubated in a shaking water bath for 2 h at 37 °C, 150 rpm.

GM soybean powder obtained from CJ Food Company (CJ, Seoul, South Korea) was mixed in a blender (FM-681, Hanil, South Korea) and then sieved (testing sieve, aperture 1.00 mm, 710 μ m, Chung-Gye™, South Korea). An aliquot of GM soybean powder (2 g) was mixed with 15 mL of 20 mM phosphate buffer. The ratio of volume ingestion of food to each digestive fluid was adjusted and then di-

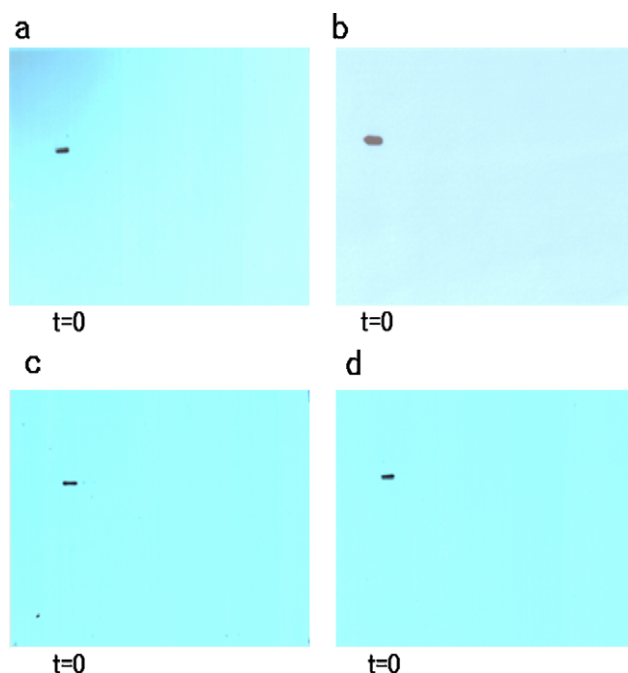


Fig. 1. Recovery of CP4EPSPS spiked into each digestive fluid. (a) CP4EPSPS in buffer only, (b) CP4EPSPS in salivary fluid, (c) CP4EPSPS in gastric fluid, (d) CP4EPSPS in small intestinal fluid.

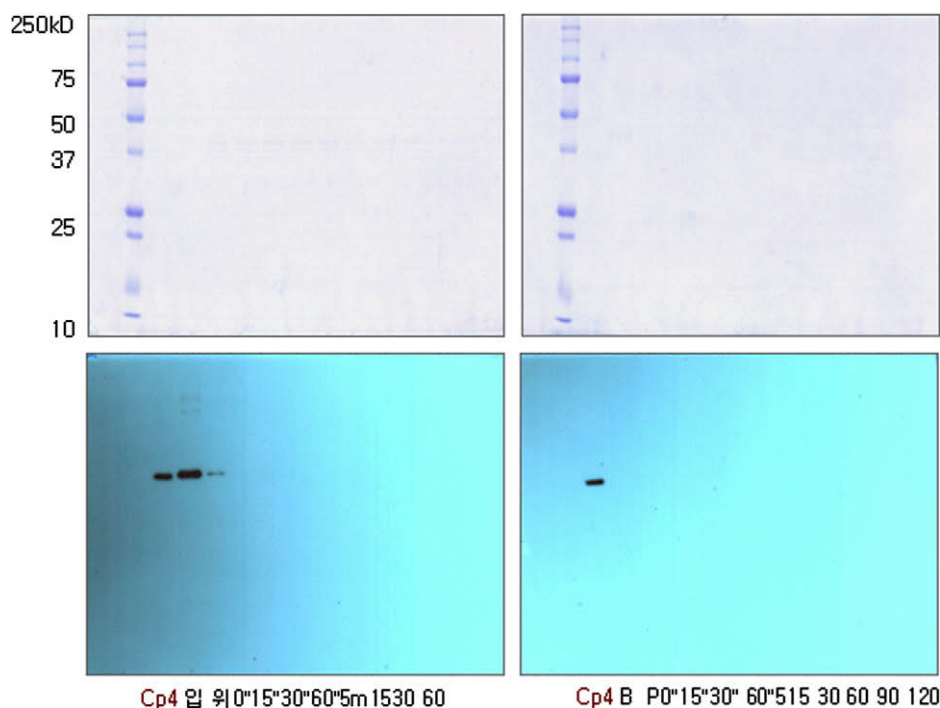


Fig. 2. Digestibility of pure protein (CP4EPSPS) under physiologically based *in vitro* digestion system.

gested as described above. Samples were collected at intervals of 0, 0.5, 5, 15, 30, 60, 120 min and 0.2 M Na_2CO_3 was added to each sample to stop the reaction. An aliquot amount of 2× sample buffer (25 mL 4 × Tris–Cl, 20 mL glycerol [20%, w/v], 4 g SDS [4%, w/v], 2 mL 2-mercaptoethanol, 1 mg bromphenol blue [0.001%, w/v])

was added to each tube containing sample collected each time point, and then reaction was immediately stopped by placing the tube in a boiling water bath for 5 min followed by placing on ice. The samples were then analyzed using the SDS–PAGE procedure described below.

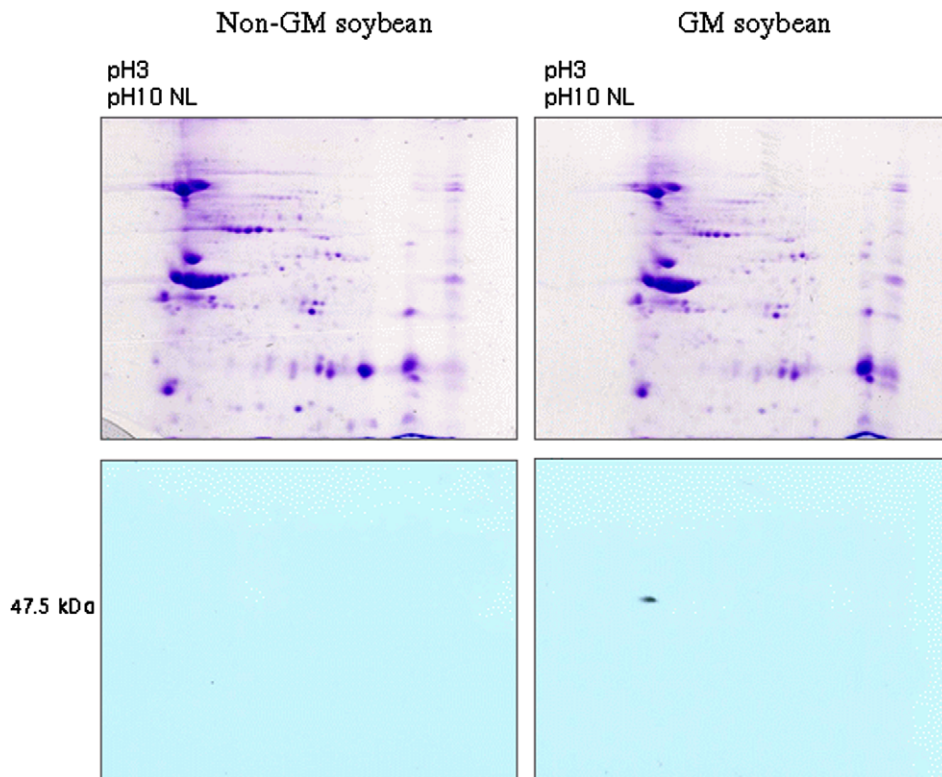


Fig. 3. Two-dimensional gel electrophoresis (top) and Western blotting (bottom) of wild type and GM soybean.

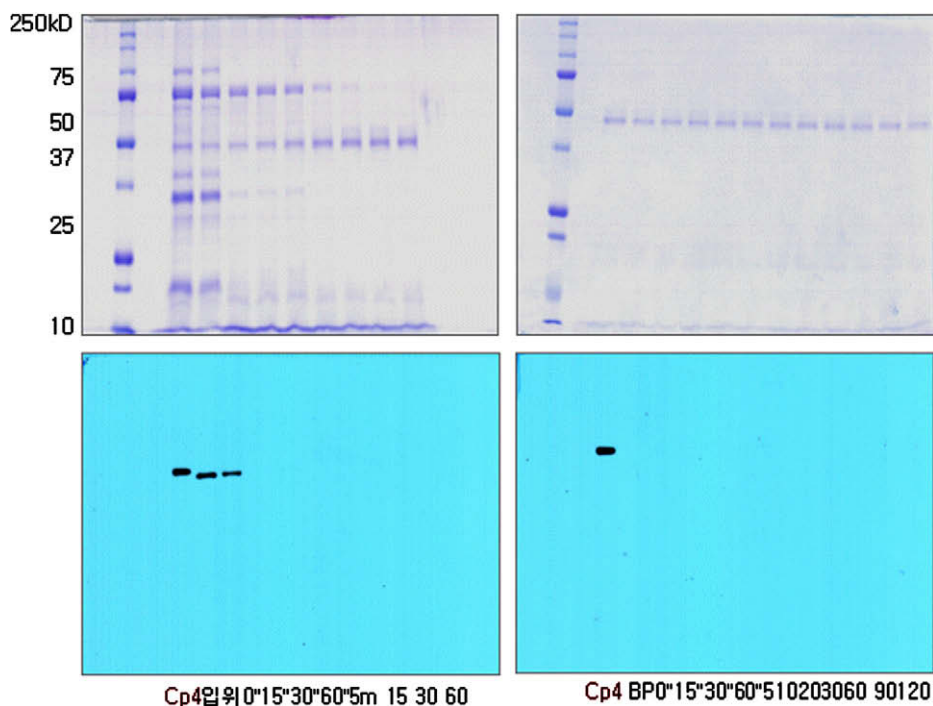


Fig. 4. Digestibility of GM soybean under physiologically based *in vitro* digestion system.

2.3. Fecal fermentation

CP4EPSPS released from GM soybean was also assessed in conditions simulating the human colon. For the preparation of individual fermenter, feces collected from four healthy adults were individually mixed with phosphate buffer (10% w/v, pH 7.2). An aliquot of pure protein (4 μ g) or GM soybean extract (20 μ L) was introduced into individual 5 mL batch culture fermenters. In order to establish anaerobic condition, fermenter filled with positive

nitrogen gas, sealed, and then incubated in a shaking water bath (37 °C, 100 rpm) for 24 h. Samples were collected at 6 and 24 h of incubation for analyzing the profile of protein by SDS–PAGE. CPEPSPS and was detected by Western blotting.

2.4. Protein extraction

An aliquot of sample (40 μ L) was stirred with an extraction buffer (180 μ L of lysis buffer containing 2 μ M thiourea, 7 M urea,

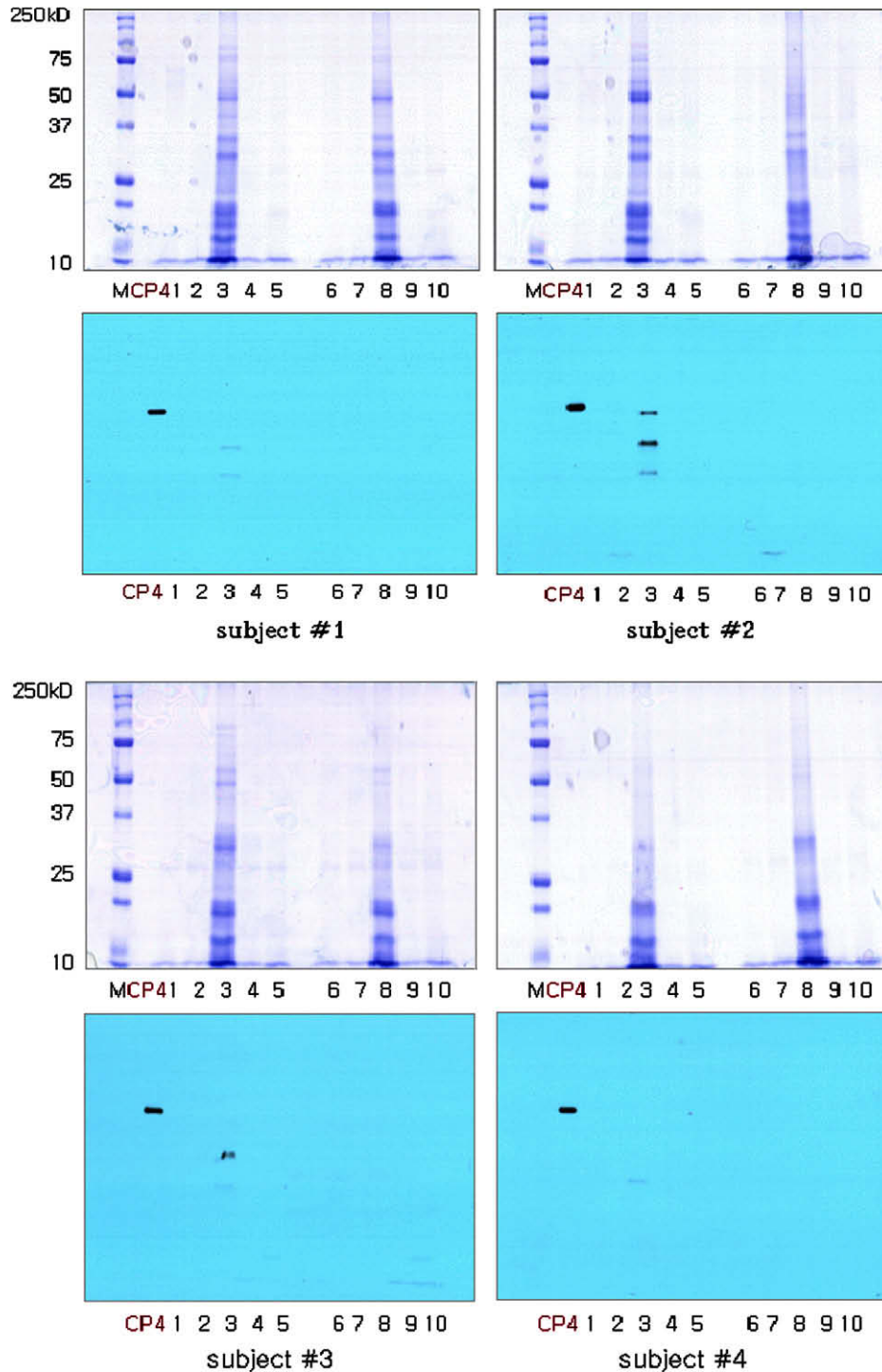


Fig. 5. SDS–PAGE (top) and Western blotting (bottom) analysis of colonic protein fermentation using *in vitro* fermenter system. Lane 1: 6 h-feces, lane 2: 6 h-feces + CP4EPSPS, lane 3: 6 h-feces + GM soybean, lane 4: 6 h-feces + CP4EPSPS digesta, lane 5: 6 h-feces + GM soybean digesta, lane 6: 24 h-feces, lane 7: 24 h-feces + CP4EPSPS, lane 8: 24 h-feces + GM soybean, lane 9: 24 h-feces + CP4EPSPS digesta, lane 10: 24 h-feces + GM soybean digesta.

CHAPS [4%, w/v], 20 μ L of DDT [1%, w/v]) for 3 h and then centrifuged at 12,000g for 30 min. The supernatant was filtered through cheesecloth and then treated with 2 $\times$ sample buffer for analyzed by SDS–PAGE. The protein concentration of the extract was determined by Bio-Rad protein assay (Bio-Rad Laboratories) based on the method of Bradford and the CP4EPSPS content was determined by ELISA (GMO Soya test kit, Stratagenic Diagnostics Inc., USA).

2.5. SDS–PAGE and Western blots

Proteins were separated by SDS–PAGE using a 12% acrylamide gel. SDS–PAGE was performed using a Mini-PROTEAN3 apparatus (BIO-RAD, Hercules, CA, USA) at 100 V for 2 h. Proteins were visualized by Coomassie brilliant blue colloidal staining. Mid-range relative molecular weight protein markers were run with each gel (Promega, Madison, WI). A two-dimensional SDS–PAGE was carried out using 2D Quant kit. Western blotting was performed to detect CP4EPSPS protein from digesta and soybean. Separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk with 0.05% Tween 20 in phosphate buffered saline (PBS) (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl) for 1 h at room temperature and washed six times with PBST (PBS with 0.05% Tween 20). Polyclonal antibody specific for the EPSPS protein was diluted 5000-fold and incubated with the membrane for 1 h at room temperature. The membrane was treated with a 1:10,000 dilution of secondary antibody (Anti-rabbit IgG; Sigma, St. Louis, MO, USA) for 1 h at room temperature. The secondary antibody was then visualized using an ECL kit (Amersham Pharmacia, Bjorkgatan, Sweden).

3. Results and discussion

3.1. Digestive stability of CP4EPSPS protein in simulated digestive fluids

The digestibility of pure protein, CP4EPSPS, was assessed in simulated salivary–gastric–intestinal phase with sequential *in vitro* digestion. The recovery of CP4EPSPS spiked into each digestive fluid was estimated to be approximately 100% by visual comparison of the band intensities of CP4EPSPS in buffer alone and in digestive fluid at $t = 0$ (unincubated) (Fig. 1). Fig. 2 shows the results of SDS–PAGE analysis of the CP4EPSPS digestion products in simulated GI digestive fluids. The band of CP4EPSPS (47.4 kDa) was stable during incubation in salivary fluid for 15 s and in gastric fluid at $t = 0$. However, no CP4EPSPS protein was detected after 15 s of gastric fluid incubation, the earliest time interval evaluated (Fig. 2). The appearance of degraded fragments was not observed. Similar result was found in previous studies that CP4EPSPS protein was rapidly degraded between 15 and 30 s in gastric fluid (Harrison et al., 1996; Kim et al., 2006a; Okunuki et al., 2002). Results from this study imply that CP4EPSPS protein does not cause an allergy based on the relationship between digestibility and allergenicity.

3.2. Effect of food matrices on digestibility of CP4EPSPS

To evaluate the impact of food matrices on the digestion rate of CP4EPSPS, GM soybean powder was evaluated in three phases with sequential *in vitro* digestion. Protein patterns in both non-genetically modified soybean and GM soybean were assessed by 2D gel-electrophoresis and then newly expressed protein, CP4EPSPS, detected by Western blot analysis (Fig. 3). For a valid comparison, the amount of purified CP4EPSPS protein loaded on SDS–PAGE was adjusted to be comparable to the CP4EPSPS present in GM soybean powder. As seen in Fig. 4, CP4EPSPS protein was completely di-

gested within 15 s under gastric fluid incubation. Digestive stability of protein in GM soybean powder was similar to that of purified protein under *in vitro* sequential digestion. This result indicates that the soybean powder food matrix had no effect to digestion of the protein by simulated digestive fluids.

3.3. Fecal fermentation of CP4EPSPS in GM soybean

We explored the formation of inserted protein fermentation products using a batch culture fermentation system simulating for human colon. Fig. 5 shows protein patterns and detection of CP4EPSPS analyzed by SDS–PAGE and Western blotting, respectively. Lanes 2 and 7 in Fig. 5 highlights that pure protein was completely decomposed during colon incubation. Proteins in GM soybean were mostly degraded within 24 h colon incubation (Fig. 5, lanes 3 and 8). The band seen in the Western blotting result from subject #2 and #3 were detected by polyclonal antibody for CP4EPSPS because CP4EPSPS is 26% identical to soybean EPSPS. No fermentation product of CP4EPSPS was detected. This indicates that the bacterial enzymes within the fermenter are completely digesting CP4EPSPS protein. We also found that digesta of GM soybean or pure protein collected from gastro-intestinal digestion did not reached the colon due to its complete digestibility (Fig. 5, lanes 4–5, 9–10). Results from this study imply that the new protein in GM soybean should not lead to any potential colon promotion.

4. Conclusions

In conclusion, CP4EPSPS inserted GM soybean was completely degraded within a very short time under simulating human digestive fluids and produced any colonic protein fermentation products. Results from current study provide data basis for estimating protein digestibility related to safety of new protein with a simple and fast way.

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