

Letter to the Editor



In vivo* Digestive Stability of Soybean β -conglycinin β -subunit in WZS Minipigs

HUANG Qiong^{1,2}, XU Hai Bin^{2,#}, YU Zhou², LIU Shan², and GAO Peng²

By now, the digestive stability experiments provided by most authoritative organizations are *in vitro* tests. Evaluating the protein digestive stability with *in vivo* models should be more objective. The present study aimed to verify the *in vivo* digestibility of soybean β -conglycinin β -subunit in Wuzhishan (WZS) minipigs. Three minipigs were surgically fitted with O-stomach and T-ileum cannulae and fed with soybean meals. According to SDS-PAGE, the 50 kD fraction of soybean β -conglycinin β -subunit persisted in the gastric fluid until 6 h after feeding, which was detected at 3 h and clearly visible at 4-6 h in the intestinal fluid. Western blot with anti- β -conglycinin β -subunit McAb confirmed it.

Potential allergenicity is an important aspect in the safety assessment of genetically modified food (GMF). According to the Codex Alimentarius Commission (CAC), the digestive stability of exogenous gene expressed protein is an essential assessment index in the guideline for the conduct of safety assessment of foods derived from recombinant-deoxyribonucleic acid plants^[1]. The digestive stability is the degree of degradation stability of proteins in human or simulated gastrointestinal digestive system. In 1996, the findings of Astwood et al.^[2] have confirmed that the tolerance to digestive degradation during intestinal absorption (resistance to acid, bile salts, and proteolysis) is required for allergenic proteins before they enter immune system in order to cause allergic reactions. Also, the allergenic proteins or protein fragments can elicit allergy only when sufficient integrity and immunogenicity are maintained. By now, the digestive stability experiments provided by most authoritative organizations including United States Pharmacopoeia, CAC, and the European Food

Safety Authority^[1,3-4] are *in vitro* tests with simulated gastric/intestinal fluid. *In vitro* tests are easily to operate and control, but have shortcomings since the simulated experimental conditions are different from the actual physiological conditions^[5]. Evaluating the protein digestive stability with *in vivo* models should be more objective. However, since ethical and other factors are involved, it may be very difficult to obtain gastric/intestinal fluid samples or conduct experiment directly on human. This promoted the development and application of the animal *in vivo* digestion model. Since it is difficult to conduct parallel sampling at multiple time points in small rodent animals, the large animal models such as pigs or dogs are preferred in studying the digestive stability. These animals possess higher similarity and homology with the human physiological conditions. When compared with *in vitro* experiments, it is advantageous to insert gastric and intestinal cannulas into these large animals for the study of target protein digestion in gastric and intestinal fluids, since *in vivo* experiments are closer to the actual physiological processes. In our earlier researches, a locally domesticated and cultivated experimental inbred Wuzhishan (WZS) minipig were inserted with gastric and intestinal cannulas to collect the gastrointestinal juices at specific time points^[6]. In order to further investigate the digestive stability of soybean protein, anti- β -conglycinin β -subunit monoclonal antibody (McAb) was prepared, and Western Blot analysis was performed to confirm the allergenic proteins in the digestion products. The present study aimed to verify the *in vivo* digestibility of soybean β -conglycinin β -subunit in Wuzhishan (WZS) minipigs.

A total of 3 mature castrated male WZS minipigs (weight: 22-30 kg) were offered by Institute of

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1. Guangdong Provincial Center for Disease Control and Prevention, Guangzhou 511430, Guangdong China; 2. Key Laboratory of Food Safety Risk Assessment of Ministry of Health, China National Center for Food Safety Risk Assessment, Beijing 100022, China

Animal Sciences, Chinese Academy of Agricultural Sciences (Beijing, China) [animal license number: SYXK (Beijing) 2008-0007]. The minipigs were single-housed with free access to water. They were fed twice per day at the amount of 3% body weight for 3 to 4 d before the experiment. All the minipigs were treated according to the guidelines for minipig care approved by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee.

The minipigs were fasted for 48-72 h to empty the intestinal contents. Water deprivation was initiated for the animals at 12 h before surgery. The animals were premedicated with an intramuscular injection of atropine sulfate (Lingrui Pharmaceutical, 0.05 mg/kg) and Zoletil® 100 (Virbac, 5 mg/kg) for the induction of anesthesia. Enflurane (Jiupai Pharmaceutical) was used for the maintenance of anesthesia and mask inhaled to effect. A pre-sterilized T-ileum cannula (Yideyihua Technology) was placed and fixed by a purse-string suture. Another 1-2 cm incision was cut at the greater curvature of the stomach to insert an O-stomach cannula (Yideyihua Technology). The minipigs were fed with liquid food and then gradually advanced to solid food. The digestive stability experiment was preformed 14 d after the surgery. All the minipigs were fasted for 16 h before the experiment. The animals were orally fed with the test substance (100 g of Hebei nf327 soybean powder with 250 mL of water). The gastric juice (1.5-2 mL) was collected through the O-stomach cannula at 0, 15 m, 30 m, 45 m, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after feeding; and the intestinal fluid (0.5-1.5 mL) was collected by T-ileum cannula at 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after feeding. The samples were then centrifuged, pH value of the supernatants was measured with accurate pH test paper.

Proteins content in the digestive juices were quantified using the Bradford method. Protein gel electrophoresis was conducted after adjusting the loading amount of proteins to be less than 10 µg. Coomassie blue G250 was used to stain the protein, which was followed by conventional decolorizing. Bio-Rad image analysis system was used to compare the digestive stability of soybean protein in the digestive juices at different time points. BALB/c mice were injected with Freund's complete and incomplete adjuvant mixed recombinant β-conglycinin β-subunits for 6 times, and the indirect ELISA and Western Blot analysis were conducted to detect the successfully fused cells. The positive hybridoma cells were cloned. Ascites IgG McAb was

purified by a Protein G1 column.

Proteins from the gastric and intestinal juices at different time points were separated by SDS-PAGE and wet transferred onto polyvinylidene difluoride (PVDF) membranes. The primary antibody (anti-β-conglycinin β-subunit McAb prepared with the protein concentration at 0.095 mg/mL) was added and incubated for 20 min at room temperature. The secondary antibody (HRP-conjugated goat-anti-mice IgG secondary antibody diluted at the rate of 1:1000) was added and incubated for 10 min at room temperature. The membrane was taken out and carried out DAB colorization to detect the protein blots.

The same results were got from all three animals, but only one of them was presented.

The pH value of gastric juice reached to the lowest after 16 h of fasting and varied from 1.5-2. After the minipigs were fed with soybean powder, the pH value increased rapidly at first and dropped after 4-6 h due to the gastric acid secretion. The gastric acid was neutralized after 0.25 h of soybean feeding, with the peak pH value being 6.0; and the peak secretion of gastric acid occurred at 5 h after soybean feeding, with the pH value decreased to 2.

As shown in Figure 1, 40 kD protein fraction remained in the gastric fluid at 1 h after soybean feeding. At 2 h after soybean feeding, the protein remained in the gastric fluid included the following: 150 kD, 80 kD, 71 kD, 67 kD, and 20 kD fractions. At 3 h after soybean feeding, 15 kD fractions remained in the gastric fluid; while 50 kD, 34 kD, 17 kD, and 13 kD fractions remained in the gastric fluid until 6 h after soybean feeding. At 4-6 h after soybean feeding, 50 kD, 34 kD, 20 kD, and 17 kD fractions remained in the intestinal fluid. The 34 kD and 20 kD fractions, which decreased in the gastric juice after 3 h of soybean feeding, appeared in the intestinal fluid after 4 h of soybean feeding. The 50 kD, 20 kD, and 17 kD fractions could be detected in the intestinal fluid; while other proteins including 150 kD, 80 kD, 71 kD, 67 kD, 40 kD, 15 kD, and 13 kD fractions could not be detected in the intestinal fluid due to the digestion of gastric acid. Based on the results of SDS-PAGE and the list of soybean antigenic proteins reported by Wilson^[7], it may be inferred that the four digestive stable proteins from soybean could be β-conglycinin β-subunit (50 kD), immunodominant allergen (34 kD), 2S globulin subunit (17 kD), and actin (13 kD).

Soybean is a common and major cause of allergy, and the primary allergens include soybean glycinin

and β -conglycinin. According to the research of Zhao et al.^[8], β -conglycinin has a higher immunogenicity in cecum and colon when compared with glycinin. FU et al.^[9] has confirmed the *in vitro* digestive stability of β -conglycinin α subunit. As shown in Figure 2, the results of Western blot indicated that the prepared ascites McAb prepared could recognize the 50 kD fraction of soybean β -conglycinin β -subunit and

another fraction weighed about 34 kD. This protein subunit could be detected in the digestion products (as shown in Figure 3), indicating that it was also a soybean-derived antigen protein.

As shown in Figure 3, the results of Western blot indicated that the 50 kD fraction of β -conglycinin remained in the gastric fluid until 6 h after soybean feeding, while the 34 kD fraction remained in

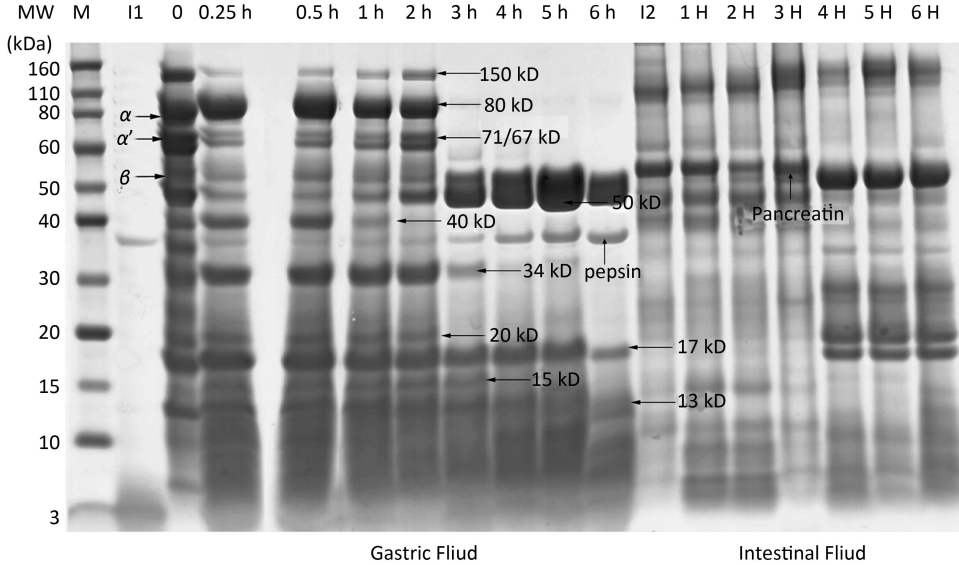


Figure 1. SDS-PAGE of gastric and intestinal fluids at different time points after soybean feeding. M: Marker; I1: Initial gastric fluid before soybean feeding; 0-6 h: gastric fluid sampling time points; I2: Initial intestinal fluid before soybean feeding; 1-6H: intestinal fluid sampling time points; α : α subunit of β -conglycinin; α' : α' subunit of β -conglycinin; β : β subunit of β -conglycinin.

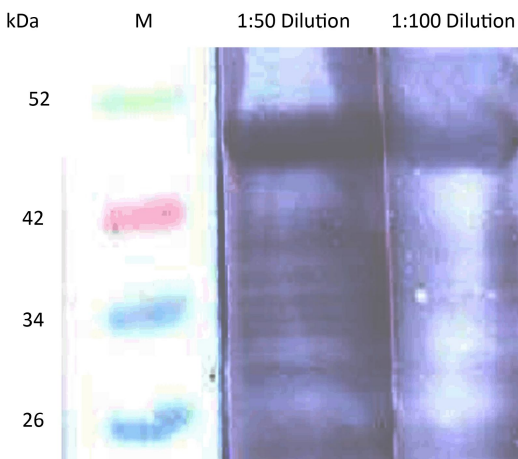


Figure 2. Western blot detection of soybean recombinant β -conglycinin β -subunit using McAb ascites. M: Marker; 1:50 dilution: Ascites diluted at 1:50; 1:100 dilution: Ascites diluted at 1:100.

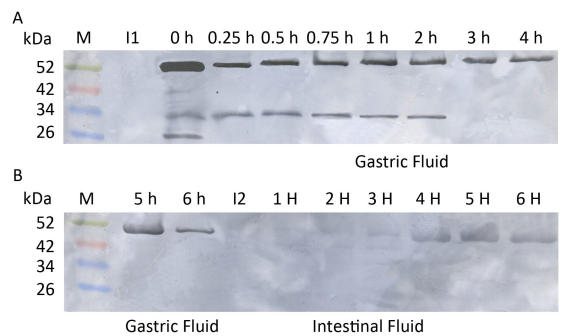


Figure 3. Western blot detection of digestion products using anti- β -conglycinin β -subunit McAb. A: M: Marker; I1: Initial gastric fluid before soybean feeding; 0-4 h: gastric fluid sampling time points. B: M: Marker; 5-6 h: gastric fluid sampling time points; I2: Initial intestinal fluid before soybean feeding; 1-6H: intestinal fluid sampling time points.

the gastric fluid until 2 h after soybean feeding. These results were consistent with that of the protein electrophoresis. The 28 kD fraction could be detected in the initial gastric fluid, while no proteins could be detected in the initial intestinal fluid in the first 2 h after feeding.

Western blot is preferred to detect proteins in digestive stability experiments using the serum or McAb from patients that are allergic to specific types of food. Since the purity of β -conglycinin β -subunit directly extracted from soybean might not be ideal (normally between 70%-80%), the anti-recombinant β -conglycinin β -subunit McAb was prepared. According to SDS-PAGE, and western blot analysis, the 50 kD fraction of soybean β -conglycinin β -subunit persisted in the gastric fluid until 6 h after feeding. However, the 28 kD and 34 kD fractions having common epitopes with β -conglycinin β -subunit were degraded by the gastric juice immediately and after 2 h, respectively. Since the 50 kD fraction of β -conglycinin β -subunit has extreme digestive stability in WZS minipigs, even more stable than α and α' subunit^[10], it is suitable to be a positive control protein for *in vivo* digestive stability model in large animals.

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[#]Correspondence should be addressed to: XU Hai Bin, Tel: 86-10-52165548, Fax: 86-10-52165549, E-mail: hbxu1231602@vip.sina.com

Biographical note of the first author: HUANG Qiong, MD, female, born in 1976, major in food Safety and food toxicology.

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