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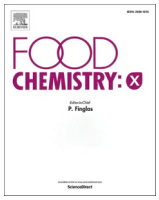

Ultra-high-pressure passivation of soybean agglutinin and safety evaluations

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ABSTRACT

Soybean agglutinin (SBA) is a heat-sensitive anti-nutritional factor (ANF). It affects nutrient absorption and causes organism poisoning. This study explored the SBA passivation ability and mechanism by ultra-high pressure (HHP), a non-thermal food processing technology. The results indicated that more than 500 MPa HHP treatment reduced the SBA activity by destroying its secondary and tertiary structures. Also, the cell and animal experiments showed that HHP treatment reduced the cytotoxicity of SBA, improved the mice's body weight, and alleviated liver, kidney, and digestive tract damage *in Vivo*. These results demonstrated that HHP had a high passivation efficiency against the SBA, thereby HHP promoting the safety of soybean products. This study provided supporting evidence for ultra-high-pressure treatment applications in soybean processing.

Introduction

Soybean is a food crop that originated in China and is widely planted globally. It is one of the world's most economically valuable agricultural products. Soybean is the main source of plant protein, oil, and bioactive components in food and livestock feed (Chan, Qi, Li, Wong, & Lam, 2012; Bouchenak & Lamri-Senhadji, 2013; Singh, Yadav, & Vij, 2019). Because of its nutritional and functional properties, it has been used as a food and dietary supplement. Also, soybean consumption has been reported to reduce the risk of cancer, diabetes, obesity, and cardiovascular disease (Cederroth, Zimmermann, & Nef, 2012; Chen, Erh, Su, Liu, Chou, & Cheng, 2012; Bassani, Valquiria, Linck, Teixeira, Helder, Ferreira, et al., 2015). Furthermore, soybean is a good source of functional substances. For example, some of its proteins, such as soybean β-Conglycinin, postpone the development of fatty-liver disease (Shan, Yu, Lyu, & Fu, 2021). In addition, soybean saponins ameliorate the cholesterol content in plasma and inhibit the development of some diseases (Ascencio, Torres, Isoard-Acosta, Gómez-Pérez, Hernández-Pando, & Tovar, 2004; Nakashima, Okubo, Honda, Tamura, Matsuda, & Yamamoto, 1989). Soybean isoflavones and some other substances, such as soybean saponin, also have healthcare effects (Kang, Han, Sung, Yoo, Kim, Kim, et al., 2008; Fjh & Jqcb, 2013). As a result of the nutritional and functional benefits of soybean and their processed foods, the global demand for soybean is increasing. However, the anti-nutritional factors (ANFs) within the soybean affect the absorption and utilization of protein and other nutrients. Also, ANFs cause adverse reactions such as body poisoning. Anti-nutritional factors were defined as substances produced by plant metabolism that has anti-nutritional effects on animals through different mechanisms ("USDA DataBase"). According to their heat resistance ability, ANFs can be divided into thermal-stability and thermal-instability anti-nutritional factors. The thermal stability anti-nutritional factors include soybean globulin, beta-conglycinin, phytic acid, saponin, and tannin; The thermal instability anti-nutritional factors include trypsin inhibitor, urease, soybean lectin, etc. (Molla & Mandal, 2013).

Soybean agglutinin (SBA) is the main anti-nutritional substance in soybean. The content of SBA in mature soybean seeds is about 10% of the total protein. It is an oligomeric β- Sheet protein that can form a tetramer with a 30 kDa subunit and an isoelectric point of 5.81 and belongs to the legume lectin family (Halder, Surolia, & Mukhopadhyay,

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2016). Studies have shown that SBA is resistant to digestive enzymes in the gastrointestinal tract (B. Zhao, Che, Adams, Guo, Han, Zhang, et al., 2019). Although it can be slowly degraded by pepsin, it cannot be enzymolysis by trypsin. By binding with specific receptors on the surface of small intestinal epithelial cells, SBA destroys the mucosal structure of the intestinal wall, causing damage to intestinal tissue and resistance, inducing anti-nutritional effects, and even causing acute gastroenteritis (Fasina, Classen, Garlich, Black, Ferket, Uni, et al., 2006; Z. Li, Li, & Qiao, 2003; Molla & Mandal, 2013). The research results of Yuan Zhao et al. (2011) showed that SBA could destroy cell permeability, inhibit cell viability, reduce the mechanical barrier function of the intestine, affect the intestinal structure and function of animals, and thus reduce the utilization efficiency of soybean protein by the body (Y. Zhao, Qin, Sun, Che, Bao, & Zhang, 2011; Lis & Sharon, 1998). Also, SBA impacted the metabolism and growth regulation of animals, leading to growth inhibition and even poisoning (Tang, Li, Qiao, Piao, & Zang, 2006).

Therefore, effective measures should be taken to inactivate anti-nutritional factors in soybean processing. With the continuous progress of science and technology, anti-nutritional factor passivation technology has been developed. At present, the emerging food processing technology mainly focuses on non-thermal physical processing technology such as ultra-high pressure, ultrasonic wave, and pulse electric field, which can passivate the harmful substances in food to the maximum, destroy the original nutrients in food to the minimum, and maintain the biological and sensory characteristics of food (Jadhav, Annapure, & Deshmukh, 2021). Ultra-high-pressure (UHP) is a gradually widely used technology in the food industry. Ultra-high-pressure food processing technology refers to a pressure of more than 100 MPa to inactivate enzymes, proteins, and other substances in food under low-temperature conditions, as well as kill pathogenic microorganisms such as bacteria in food. Compared with traditional heat treatment technology, HHP can not only maintains the texture, shape, and color of food but also preserve its flavor and nutritional characteristics (van der Ven, Matser, & van den Berg, 2005; Bingyu, Zhimin, Linlin, Mingshou, Xiuqing, Huan, et al., 2022). Garcia Mora et al. reported that when treating cucurbit bean protein with HHP, pressurization (100–300 MPa) improved the protein's hydrolysis efficiency, and the enzymatic hydrolysates can also promote the digestion and absorption of proteins after ultrahigh-pressure (Linsberger-Martin, Weiglhofer, Thi Phuong, & Berghofer, 2013). Van et al. used a high-pressure homogenizer with a flow rate of 120 L/h, at 70-90℃, and under the pressure of 525–750 MPa. They found that 90% of the trypsin activity can be inactivated in 2 min (Vanga, Singh, & Raghavan, 2018).

Nowadays, the effect of HHP on soybean processing remains a research gap. Although few pieces of literature have reported the effect of HHP on anti-nutritional factors such as lipoxygenase, urease, and trypsin inhibitor in soybean milk, its property on SBA and protein conformation change is unknown. In this study, HHP (350, 400, 450, 500, 550 MP, 15 min) was used to treat soybeans to explore its influence on the inactivation of soybean lectin and determine whether the degradation products are safe. The research outcomes provided a theoretical basis for non-thermal technology in soybean processing.

Materials and methods

2.1. Materials and chemicals

Soybean (variety: Dongnong 252, water content 8.3%, crude protein content 42.87%, crude fat content 20.06%) was purchased from Dongxiaonong black potato workshop in Harbin, Heilongjiang Province. Lectin protein was purchased from Shanghai Yuanye Biotechnology Co., Ltd; Absolute ethanol was purchased from Beijing Chemical Plant Co., Ltd. All other chemicals used in this study were of reagent grade.

2.2. Maintenance of animals

The 4-week-old SPF grade CD-1 male mice purchased from Beijing weitonglihua Experimental Animal Technology Co., Ltd were selected as the experimental animals. This research was approved by the *animal welfare and animal experiment ethics review committee* of China Agricultural University. The quality certificate number is SCXK (Beijing) 2016–0006, and the animal experimental facility certificate number is SYXK (Beijing) 2020–0052. The maintenance feed was purchased from Beijing keaoxieli Feed Co., Ltd. (experimental animal feed license No.: SCXK (Beijing) 2015–0013).

The experimental animals were kept in the specific pathogen free (SPF) animal room (temperature 22-24℃, humidity 50–65%) of the agricultural product quality supervision, inspection, and testing center of the Ministry of Agriculture (Beijing). The mice were fed with standard mouse feed, and the air was exchanged 15 times per hour. The dark and light times were 12 h each day, respectively. We observed the animals every day to ensure their body surface characteristics, limb behavior, and mental state were normal. Also, we weighed the mice once every two days to observe the weight change. The bedding and cages for mice were cleaned once a week.

The experiment started one week after the mice had adapted to the environment. Five mice were divided into each group, and each group was kept in one cage. The drug was administered by gavage. During the experimental period, the mice ate and drank freely. The groups were as follows:

Control group: Sterile water was given to this group by gastric irrigation according to body weight of 10 ml/kg (b.w.) at regular intervals every day, diet and drinking water were given freely;

SBA group: Pure SBA protein solution at a concentration of 10 mg/ml was given by gastric irrigation according to body weight of 10 ml/kg (b.w.) at regular intervals every day, diet and drinking water were given freely;

HHP-treated SBA group: HHP-treated SBA protein solution at a concentration of 10 mg/ml was given by gastric irrigation at regular intervals according to the body weight of 10 ml/kg (b.w.) every day, diet and drinking water were given freely.

At the end of the experiment, mice in each group were fasted for 12h after the last drug administration, the blood samples were collected by enucleating eyeball and all animals were sacrificed by cervical dislocation to harvest other organs. The blood was collected from the the inner canthus vein. Blood samples were collected and kept at room temperature for 30 min before centrifugation at 4℃ and 12,000 rpm for 10 min to obtain the serum sample. All fecal and tissue samples were collected and stored at − 80℃ until analysis.

2.3. Pretreatment

Soybeans (50g) were washed three times and soaked in deionized water for 16 h at a ratio of 1:10. Then, the beans' skins were removed and dried in a 45℃ oven (PH-070a. Yiheng Technology Co., Ltd., Shanghai, China) after the soaking water was drained. After drying, the soybeans were grounded with a grinder for 90 s (M150A. Tianxi kitchen appliance Co., Ltd., Anqing, China). The grinder was stopped every 30 s to avoid the beans from overheating. After grounding, the soybean flour was sieved and stored at − 20℃ until analysis.

2.4. Ultra-high-pressure treatment

The soaked soybeans are pre-packed into vacuum screw bags and sealed with a vacuum sealing machine (LQL-08. Yueqing Shangxuan Electronic Technology Co., Ltd.). The samples were put into the ultra-high pressure processing chamber (CQC30l-600. Beijing Suyuan Zhongtian Technology Co., Ltd.), and the samples were treated at 350 MPa, 400 MPa, 450 MPa, 500 MPa, and 550 MPa for 15 min respectively. After processing, the samples were taken out and placed in a glass

dish. After drying in an oven at 45 °C, the samples were transferred to a small crusher for crushing for 90 s. To avoid overheating during crushing, crusher paused every 30 s. After crushing, the obtained soybean powder was sieved using an 80 meshes aluminum and then stored at −20 °C for analysis.

2.5. Extraction of soybean lectin

Soybean lectin was extracted according to the method of Zhang Bolin et al. (Bolin, Ning, Shuagshuang, Zhaoyan, & Zhen, 2019). 0.1 g of soybean powder was fully dissolved in 1 ml of physiological saline (0.01 M phosphate buffered saline containing 0.001% Tween-20 and 0.1%, pH 7.4) and then placed in a refrigerator at 4℃ overnight. The solution was shaken several times to ensure complete extraction and then centrifuged at 9000 R / min for 20 min under 4 ℃ (TDL-5-A. Anting Scientific Instrument Factory, Shanghai, China). The supernatant was collected for determination.

2.6. Sandwich ELISA assay

Soybean agglutinin ELISA Kit (Nanjing enzyme-linked Biotechnology Co., Ltd. Nanjing, China) was used to determine the activity of soybean agglutinin. The soybean agglutinin antibody was coated in 96 well plates in advance, and the sample, standard sample, and HRP-labeled detection antibody was sequentially added. After incubation at 37℃ for 30 min, it was washed. The substrate TMB is used for color development. It is converted into blue under the catalysis of peroxidase and finally into yellow under the action of acid. There was a positive correlation between color deepening and soybean agglutinin in samples. The samples' concentrations were calculated by measuring the absorbance at 450 nm with a microplate reader (UV-2102PC, Unico Co., Ltd., Shanghai, China). Except that no HRP antibody was added to the blank control, the other operations were carried out in parallel as above, and the specific steps were carried out according to the operating instructions of the kit.

2.7. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze standard SBA protein and soybean powder samples. Using a vertical gel electrophoresis device (Mini protein II; Bio-Rad Laboratories, Richmond, CA), the samples were mixed with the loading buffer (0.35 m Tris / HCl, pH 6.8, 10% SDS, 30% glycerol, 5% MCE, 0.01% bromophenol blue) at 1:4 (V/V), and heated in a metal bath at 80℃ for 10 min. The gel was loaded for 10 μL.

2.8. UV absorption analysis

The lectin structure after HHP treatment was characterized by UV spectrum. SBA was prepared into 1 mg/ml solution with PBS buffer as solvent. After HHP treatment, the samples were placed in a 10 mm quartz cuvette for determination. The UV scanning wavelength was 240 nm ~ 320 nm (VaR flash 3001. Thermo Fisher Scientific, Inc., MA, USA).

2.9. Intrinsic fluorescence spectroscopy

The structure of HHP-treated lectin was characterized by fluorescence spectroscopy. 1 mg/ml SBA solution with PBS buffer as solvent was prepared. After HHP treatment, the samples were placed in a 10 mm quartz cuvette with a light transmission on all sides. The fluorescence change of intrinsic tryptophan fluorescence of lectin after microwave treatment was measured by fluorescence spectrophotometer at 280 nm excitation wavelength (930F. Unico Co., Ltd., Shanghai, China). The fluorescence emission spectrum was monitored under 290 ~ 400 nm, and the gap width was 5 nm.

2.10. Circular dichroism (CD) spectroscopy

The secondary structure of lectin after HHP treatment was determined with a far ultraviolet circular dichroic spectrometer (J-1500. JASCO, Japan). The concentration of SBA was 0.5 mg/ml. After HHP treatment, the samples were scanned by far UV CD spectroscopy under 190 ~ 260 nm. The scanning temperature was 25 ℃± 0.5 ℃, the scanning speed was 120 nm/min, the response time was 2 s, the resolution was 0.5 mm, and the sensitivity and bandwidth were set to 1 nm.

2.11. Cell culture

Human colorectal cancer cells (Caco-2) purchased from the institute of basic medicine, Chinese Academy of Medical Sciences were cultured in MEM medium (Solebo Technology Co., Ltd., Beijing). 20% fetal bovine serum (FBS) (Gbico, USA) and 1% double antibody (penicillin and streptomycin) (100u/ml) (Thermo Fisher Scientific, USA) were added to the medium. The culturing temperature was 37 ℃, and the carbon dioxide concentration was 5%. SBA was dissolved in dimethyl sulfoxide (DMSO) (cell culture grade) (Solebo Technology Co., Ltd., Beijing) at a concentration of 100 mm and stored at −20℃ before use. The concentration of DMSO in the culture medium was 0.1% in the treatment and solvent control groups.

2.12. Cell viability assay

Cell viability was detected using the enhanced cell counting kit-8 (C0042. Biyuntian Biotechnology Co., Ltd., Shanghai). Cells were incubated into 96-well plates to a density of 2.0×10^4, 100 μL cell suspension was added to each well, and the drug was administered after 24 h of cell culture. The cells were incubated with a series of SBA concentrations (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, or 10 mg/ml) for 24 h. Then, the medium was replaced with an SBA-free medium, and 10 μL CCK-8 reagent was added to each well. After incubation for 1h, the absorbance value was measured at 450 nm using an enzyme reader (UV-2102PC. Unico Co., Ltd., Shanghai, China).

In this experiment, the effect of lectin at different concentrations (0.00001–10 mg/ml) on the viability of Caco-2 cells was measured first, and a concentration causing significant cell death was selected. At this concentration, SBA was dissolved in PBS and passivated under different conditions to investigate the efficiency of these treatments in enhancing cell viability.

2.13. Determination of blood biochemical indexes

Blood was collected from the eyes of the mice after 12 h of overnight fasting before the mice were killed by cervical dislocation. The blood was collected and centrifuged at 12,000 R/min for 10 min after the blood stood still for 0.5h. The clear supernatant without hemolysis was removed into new 1.5 ml centrifuge tubes. An automatic biochemical analyzer was used for blood biochemical detection (BS-420. Mindray Biomedical Electronics Co., Ltd., Shenzhen). The measured blood biochemical indices were alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE), and serum urea nitrogen (urea).

2.14. Calculation of organ coefficient

The changes in organs were observed, and the liver, spleen, kidney, and testis were weighed to calculate the organ coefficient of body weight.

Organ coefficient = organ weight/fasting body weight * 100%.

2.15. Histopathological observation

The liver, spleen, kidney, pancreas, stomach, duodenum, jejunum,

and ileum were obtained and fixed with 40% paraformaldehyde solution for histopathological observation. After fixation over 48h, the organs were rinsed with running water, dehydrated, transparent, waxed, embedded, sectioned, and stained with H&E dyes. The pathological changes in the organs were observed by microscopic examination.

2.16. Statistical analysis

At least three experimental replicates were performed in each test. One-way analysis of variance (ANOVA) and a *t*-test were used to analyze the data. The results were expressed as mean ± SD, and the significant difference between groups was calculated by the student's *t*-test. $P < 0.05$ and $p < 0.01$, which were considered to have significant differences, were labeled by "*" and "* *" in the figures, respectively; $p < 0.001$ was considered to have an extremely significant difference and was labeled by "* * *" in figures. The GraphPad Prism 5 software package was used for generating graphs.

Results

3.1. Effect of ultra-high-pressure treatment on the activity of soybean lectin

To explore the effect of HHP treatment on SBA activity under different pressure, the SBA activity was analyzed by sandwich ELISA after being treated under 350, 400, 450, 500, and 550 MPa for 15 min, respectively. The results are shown in Fig. 1A. Compared with the untreated SBA, the effect of different HHP pressure on SBA inactivation can be divided into two categories. When the pressure was<500 MPa, the SBA activity rate was higher than 50%; At 500 MPa and 550 MPa, the residual rate of SBA reduced to 40.21% and 36.74%, respectively. It is predicted that the decrease in SBA activity may be caused by the ultrahigh pressure and oxidation-leaded protein structure changes (Tolouie, Mohammadifar, Ghomi, & Hashemi, 2018). However, the time gradient had little effect on SBA inactivation. As shown in Fig. 1B, under the condition of 450 MPa, the inactivation rate of SBA remained when the treatment time increased from 5 min to 25 min. Hence, we chose the treatment time of 15min to explore the influence of different pressure on the activity and structure of SBA.

SDS-PAGE was used to verify whether HHP treatment impacts soybean lectin protein. Generally, the SBA sub-soybean exists as tetramer glycoprotein, and the molecular weight of each subunit is 30 kDa (Ma & Wang, 2010). As shown in Fig. 1C, 400–550 MPa treatment gradually reduced the band strength of SBA protein at 30 kDa, which indicated that ultra-high-pressure treatment damaged the tertiary and quaternary structures of SBA protein. The protein structure destruction was more significant with the HHP pressure increased to 550 MPa.

3.2. Effect of ultra-high-pressure treatment on the structure of soybean lectin protein

3.2.1. UV spectrum

To further clarify the effect of ultra-high pressure on the structure of SBA protein, the structure and chemical changes of SBA protein are identified by UV spectral scanning. The protein molecule of some treated SBA showed the light of a specific wavelength in the ultraviolet region and generated the ultraviolet absorption spectrum. Therefore the UV spectrum can be used to represent the content of certain groups in the protein can be expressed by. These results showed that, the π - π * transition of the aromatic ring in the amino acid and the tyrosine residue in the polypeptide chain show the protein's strong absorbance in the ultraviolet region near 280 nm (Wang, Luo, Zhong, Cai, Jiang, & Zheng, 2017). The results indicated that the interaction of the amino acids in the protein resulted in ultraviolet absorption.

It can be observed from Fig. 2A that with the increase of HHP treatment pressure, the maximum absorption peak of SBA did not move

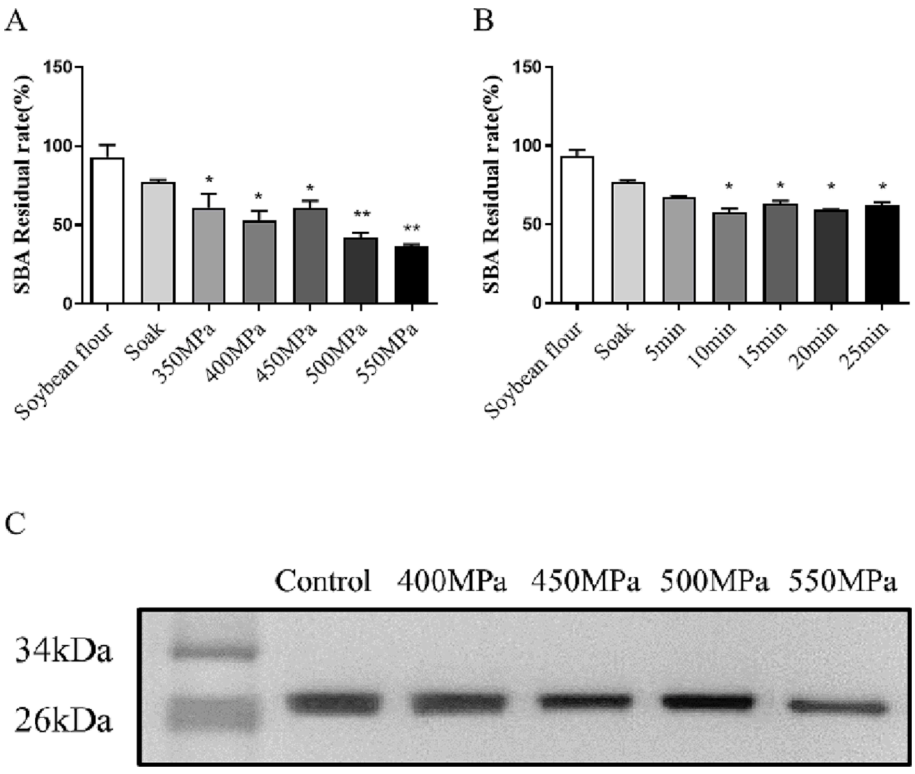

Fig. 1. The effect of ultra-high-pressure time and pressure on the activity of SBA. (A) HHP 350 W ~ 550 W treatment for 15 min; (B) HHP 450 W treatment for 5 ~ 25 min; (C) HHP 400 W ~ 550 W treatment for 15 min. (*$p < 0.05$ or **$p < 0.01$ indicates the difference is significant compared with the untreated soybean flour; ***$p < 0.001$ means the difference is extremely significant compared with the untreated soybean flour).

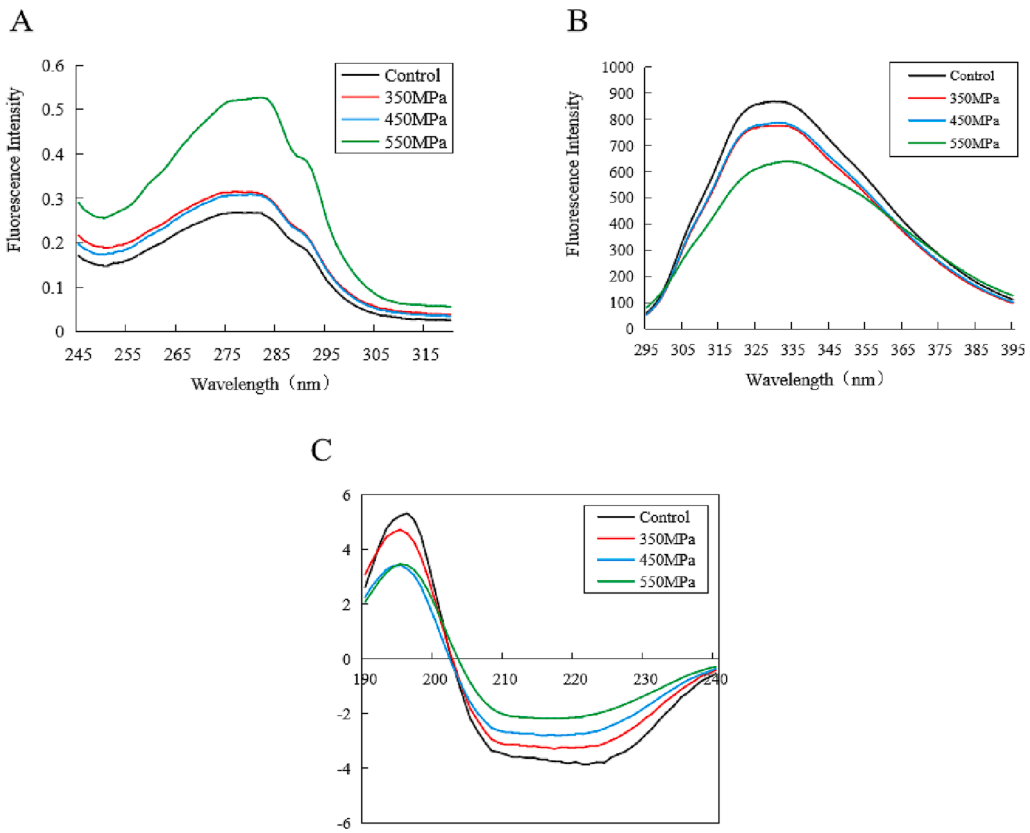

Fig. 2. The influence of HHP treatment pressure on SBA circular dichroism(A), UV spectrum(B), and fluorescence spectrum(C). (Ultra-high pressure 350 W ~ 550 W treatment for 15 min).

significantly and remained at 281 nm, but its maximum absorption value gradually decreased. Under the pressure of 350 MPa and 450 MPa, the absorption values of SBA at 281 nm slightly shifted from 0.308 to 0.315; When treated at 550 MPa, the UV absorption value obviously increased to 0.537. The increase in the UV absorption value of the protein indicated that the amino acid residues such as tryptophan and tyrosine were exposed, the protein structure expanded, exposing the amino acid residues originally embedded in the molecule, and the protein structure became loose.

3.2.2. *Fluorescence spectrum*

The intrinsic fluorescence emission spectra of proteins can provide accurate information about the microenvironment of aromatic amino acids, mainly from tryptophan (TRP), and thus can provide information about protein conformation, intermolecular interactions, and kinetic properties (Khan, Chaturvedi, & Khan, 2013). Tryptophan is an aromatic and hydrophobic amino acid, which is more likely to be embedded in the protein's hydrophobic core (Ghisaidoobe & Chung, 2014). In addition, tryptophan residues are the main intrinsic fluorophores of proteins to evaluate the microenvironment changes of proteins. When tryptophan residues are exposed to highly polar water, their fluorescence emission will be quenched. Tryptophan fluorescence is strongly influenced by the polarity of the surrounding microenvironment, hydrogen bonds, and other noncovalent interactions and shows a redshift when exposed to a more polar environment (Li, Li, Dai, Hu, Niu, Liu, et al., 2020).

The endogenous fluorescence intensity of lectin protein after being treated with different HHP pressure is shown in Fig. 2. Increasing the pressure of HHP treatment decreased its endogenous fluorescence intensity. As shown in Fig. 2B, the fluorescence intensity of untreated SBA was 867.58; When the pressure was 350 MPa and 450 MPa, the fluorescence intensity decreased to 775.28 and 785.92, respectively; However, when the pressure was increased to 550 MPa, the fluorescence intensity decreased significantly to 639.87, indicating that the pressure greater than 500 MPa had a great impact on the tertiary structure of SBA.

Also, with the increase of the treatment pressure, a redshift of the maximum fluorescence wavelength was observed (indicated by the red arrow). The maximum wavelength shifted from 331 nm (control) to 332 nm (450 MPa) and then 333 nm (550 MPa). The redshift of the maximum fluorescence wavelength indicated that the conformational rearrangement of SBA occurred, resulting in more hydrophobic amino acids (tyrosine, tryptophan, phenylalanine) located in the protein core exposed to the surface of SBA, and the tertiary structure of SBA changed.

3.2.3. *Circular dichroism*

Far ultraviolet circular dichroism (CD) is an effective spectral technique for characterizing the secondary structure of proteins, in which the peptide bonds act as chromophores. The characteristic far UV CD spectrum ranges from 190 nm to 250 nm. In this range, the typical negative peaking at 208 nm and 222 nm, together with the positive peaking at 190 nm, typically present the α- helix; β- sheet has a negative peak near 215 nm and a positive peak near 195 nm; β- turn has a positive peak at 205 nm, and the protein with dominant random coil has a negative peak near 200 nm (Do, Deeth, & Besley, 2013). The secondary structure of SBA after microwave treatment can be characterized by circular dichroism.

It can be observed from Fig. 2C that with the increase of HHP treatment pressure, the CD spectral shape of SBA and α- helix and β- sheet characteristic peaks remained. However, the α- helix content gradually decreased, β- sheet and irregular curl content increased. It can be seen from Table 1 that when the SBA secondary structure was treated under the pressure conditions of 350 MPa and 450 MPa, the α- helices in the secondary structure of SBA slightly decreased from 43.74% to 40.16%; However, when treated at 550 MPa, the content significantly

Table 1
The influence of different HHP pressure on the secondary structure content of SBA.

Treatment Conditions	α-helix	β-sheet	β-turn	random coil
Control	44.51%	9.14%	21.77%	24.68%
350 MPa	43.74%	10.37%	21.53%	26.35%
450 MPa	40.16%	11.33%	20.11%	28.30%
550 MPa	35.03%	20.25%	15.53%	30.29%

reduced to 35.03% and accompanied by the increasing of β- sheet and random coil.

3.3. Cytotoxicity of soybean lectin and its degradation products

The concentration of SBA was diluted with culture medium to 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10 mg/ml, respectively. The cytotoxicity was detected by the CCK-8 method. As shown in Fig. 3A, when the concentration of SBA was<0.001 mg/ml, there was no obvious toxicity to Caco-2 cells. However, when the SBA concentration increased to 0.01 mg/ml, the viability of Caco-2 cells decreased to 70% ($p < 0.05$). When the concentration was 0.1, 1, and 10 mg/ml, the cell survival rate was only 55%, 40%, and 25% ($p < 0.001$), respectively. The cell viability decreased with the increase in SBA concentrations.

The concentration of 0.1 mg/ml was selected to explore the toxicity of HHP-treated SBA. As shown in Fig. 3B, the results showed that after HHP treatment, the cytotoxicity of degraded SBA was reduced, and the cell viability reached 78%, significantly higher than the control (65%).

3.4. Animal safety evaluation of soybean agglutinin and its degradation products

The impact of SBA with and without HHP treatment on the body weight of mice is shown in Fig. 4A. After administration of untreated SBA, the body weight gain of mice began to decrease continuously, and their body weight was lower than the initial body weight on days 6 and 8. After 10 days of SBA administration, the body weight of the mice remained stable. After HHP treatment, the weight gain of mice was significantly restored compared with the untreated group. These results showed that SBA in soybean could inhibit the growth and development of mice. After HHP treatment, the body weight reduction was effectively improved.

Fig. 4B shows the effect of SBA on the weight-to-body ratio of the liver, spleen, kidney, and testis of mice. In the SBA positive control group, it was observed that the ratio of the liver to the body was decreased compared with the blank control group, and the ratio of the testis to the body was increased, but there was no significant difference (p greater than 0.05). The ratio of organs in the HHP treatment group was slightly restored.

The effects of anti-nutritional factors and their degradation products on mice can be judged from the blood-biochemical indexes. As shown in Fig. 4C-E, the contents of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and glutamic oxaloacetic transaminase (AST) in serum were indicators to evaluate the liver function of mice. The contents of urea nitrogen (Urea) and creatinine (CRE) were used as indicators to evaluate the renal function of mice. As shown in Fig. 4G, after the administration of untreated SBA, the CRE content in serum significantly increased ($p < 0.001$), indicating that SBA damaged the renal function of mice. However, the CRE content in mice oral-injected with HHP-treated SBA did not decrease as expected.

3.5. Effects of soybean agglutinin and its degradation products on tissue lesions in mice

As shown in Fig. 5A, after administration of untreated SBA, a small area of hepatocyte necrosis was seen locally in the liver tissue, accompanied by a small amount of inflammatory cell infiltration, as shown by the yellow arrow. The spleen tissue structure was moderately abnormal, the spleen nodule structure was scattered, the red and white pulp boundary was not clear, and no necrosis of lymphocytes was observed; A small number of neutrophils was seen in the red pulp, as shown by the yellow arrow; Red arrows indicated splenic trabeculae. Individual renal tubular epithelial cells shed in the kidney tissue, as shown by the yellow arrow; Some renal tubular epithelial cells were slightly edematous and the cytoplasm was lightly stained, as shown by the red arrow.

After administration of SBA treated with ultra-high pressure (HHP-treated group), no lesions were observed in the liver tissue except for a small amount of inflammatory cell infiltration; The tissue structures of the spleen and kidney were normal, and there were no lesions.

As shown in Fig. 5B, after administration of SBA, the gastric tissue of the mice developed pathological changes, the structure of the tissue was disordered, the structure was unclear, and the gaps between the cells were large, as shown by the yellow arrow. Local mucosal epithelial cells were seen shedding in the duodenum, as shown by the yellow arrow. A small number of inflammatory cells were seen locally in the jejunum, as shown by the yellow arrow. Necrosis of individual epithelial cells was seen in the ileum, as shown by yellow arrows; The local crypt structure of the mucosal layer disappeared, and a large number of inflammatory cells infiltrated, as shown by the red arrow.

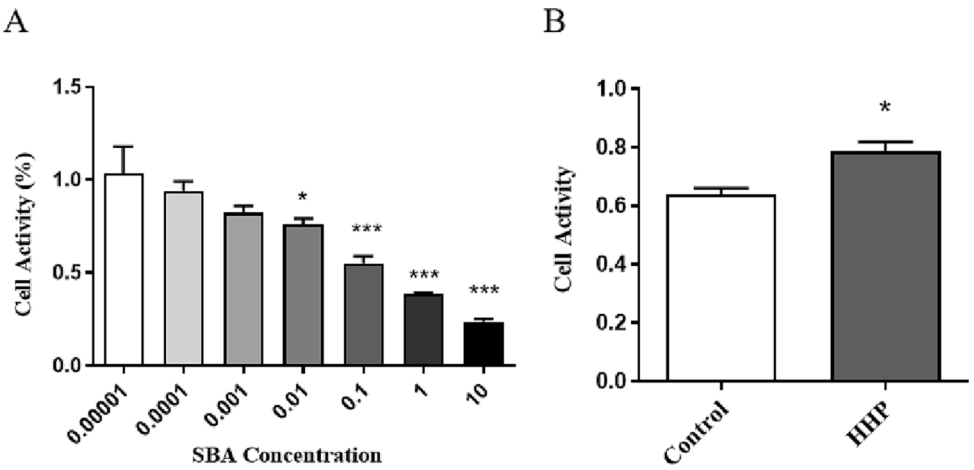

Fig. 3. Cytotoxicity of soybean lectin and its degradation products. (A) The influence of SBA on cell viability; (B) The influence of SBA degradation products on cell viability.*$p < 0.05$ or **$p < 0.01$ indicates a significant difference compared with the control; ***$p < 0.001$ indicates a very significant difference compared with the control.

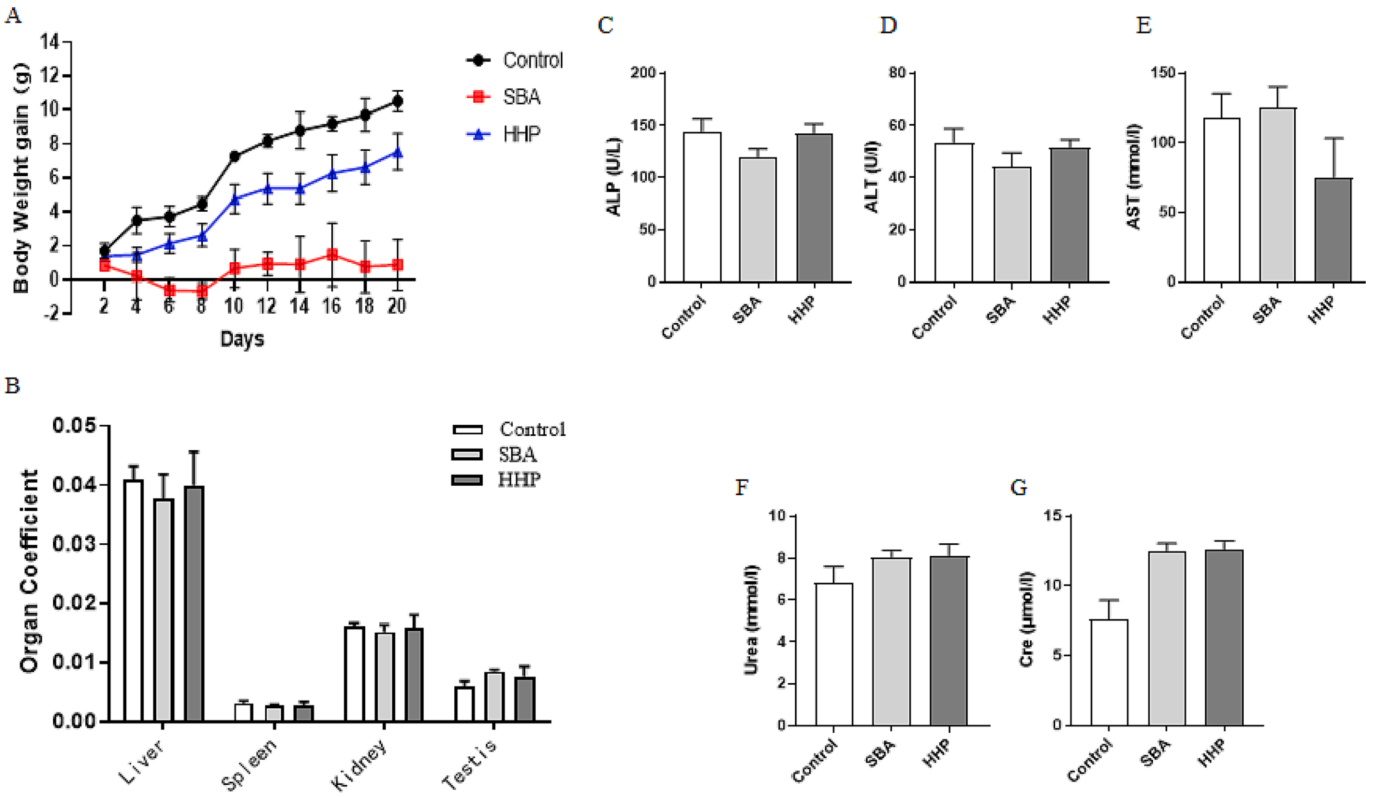

Fig. 4. The impact of HHP-treated and untreated SBA on the mice's body weight gain, organ coefficients and blood biochemical indexes in mice. (A) Body weight gain of the mice; (B) Organ coefficient; (C-E) Liver function index; (F-G) Renal function index.*$p < 0.05$ or **$p < 0.01$ indicates a significant difference compared with the control; ***$p < 0.001$ indicates a very significant difference compared with the control.

However, the mice administered with HHP-treated SBA did not develop lesions in the gastric tissue except that the mucosal epithelial cells were not clear and partially shed (yellow arrow). Also, only a few inflammatory cells were found in the ileum, and no other lesions were observed.

Discussion & conclusion

Heat treatment brings a series of adverse effects on food, including affecting the sensory quality of food such as color and flavor, and reducing the active ingredients and utilization of some nutrients in food. For soybeans, ultra-heat treatment cause a Maillard reaction between carbohydrate and basic amino acids, such as lysine, thus reducing the content of free amino acids, resulting in decreased protein digestibility and loss of protein nutritional value. Therefore, the non-thermal processing of food has become a research hotspot. Ultrasonic treatment, ultra-high pressure (HHP), and dielectric barrier discharge (DBD) cold plasma are novel non-thermal processing methods. In this paper, soybean was treated with HHP, and the activity of SBA was measured. The inactivation mechanism was investigated by UV spectrum, fluorescence spectrum, and circular dichroism spectrum. The residual rate of active-SBA was about 36% when the soybean was treated at the pressure of 550 MPa for 15 min. The significant inactivation occurred only under the pressure of more than 500 MPa. Also, the secondary and tertiary structures showed the same trend, indicating that the reduction of SBA activity by HHP treatment is related to its destruction of secondary and tertiary structures. Our findings indicated that the higher the pressure, the greater the change in the secondary structure. However, due to the limitation of equipment, research on higher pressure was not conducted. Also, there is a certain limit to the structural damage of protein caused by pressure, and SBA can still maintain its original structural characteristics to a certain extent.

In addition, it can be observed in Fig. 3A that with the increase of HHP treatment pressure, the maximum absorption peak of SBA did not move significantly, still at 281 nm, that is, with the change of treatment conditions, the UV absorption spectrum of SBA protein did not show red shift or blue shift, indicating that the polarity of the microenvironment where tyrosine is located did not change significantly, the peptide bond was not damaged and the primary structure did not change. Moreover, there were no changes in the primary structure in the fluorescence spectra and circular dichroism results. The decrease in fluorescence was mainly related to changes in hydrogen bonds and other non-covalent interactions in the SBA molecule, which may be interfered by ultra-high pressure induced SBA oxidation, such as NH -/NH2– oxidation of peptide bonds. As for the change of chemical bond in other primary structures and the influence of other forces, other detection methods need to be used to evaluate. There was a certain limit to the structural damage of proteins caused by HHP, and SBA can still maintain its original primary structural characteristics.

After exploring the physical passivation technology of HHP soybean anti-nutritional factor, the toxicity of lectin, a protein anti-nutritional factor, and the safety of degradation products after non-thermal processing are still unknown, and there are few reports on its toxicology. Therefore, a toxicological evaluation combining cell and animal experiments was used in this paper to evaluate the toxicity of soybean lectin and the safety of its degradation products after HHP treatment. SBA is toxic to Caco-2 cells and is positively correlated with concentration. Also, it can inhibit the growth and development of mice and cause lesions in the liver, spleen, kidney, stomach, and small intestine of mice. After HHP treatment, the cytotoxicity of SBA was reduced, and the adverse effects of SBA on the weight gain of mice and the damage to the liver, kidney, and gastrointestinal tract were significantly alleviated. Liener et al. fed rats with raw soybeans with selective removal of SBA, and the results showed that SBA accounted for 50% of the growth

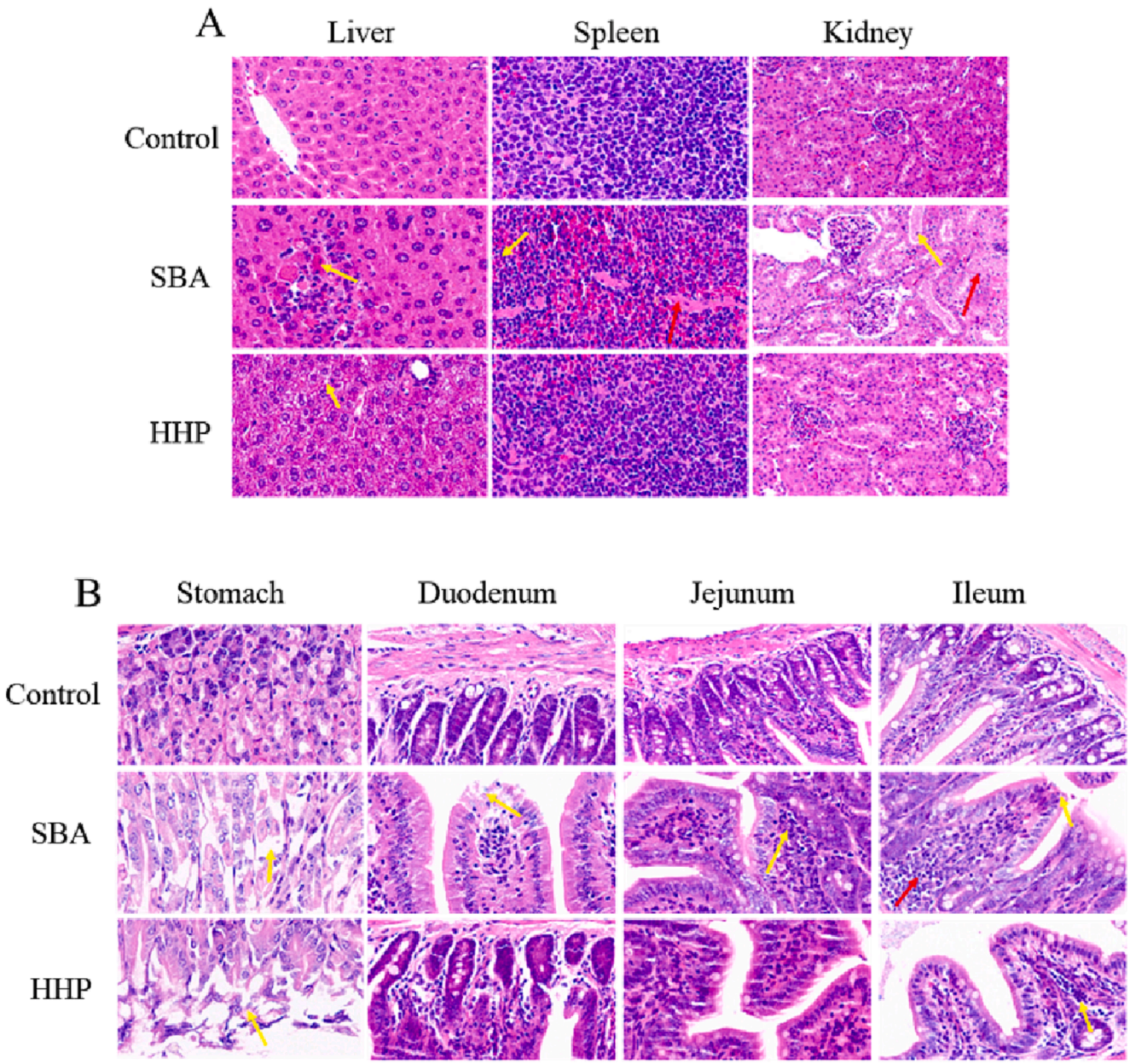

Fig. 5. The effect of SBA degradation products on the pathological changes of organs and tissues in mice. (A) H&E staining of liver, spleen, and kidney; (B) H&E staining of stomach, duodenum, jejunum and ileum.

inhibitory effect of raw soybeans on rats (Liener, 1994).

In addition, SBA destroys the mucosal structure of the gastrointestinal wall by binding with specific receptors on the surface of intestinal epithelial cells, causing the loss of epithelial resistance, leading to intestinal tissue damage, inducing anti-nutritional effects, and even leading to acute gastroenteritis (Fasina, et al., 2006). The research results of Yuan Zhao et al. showed that SBA destroyed cell permeability, inhibited cell viability, reduced the mechanical barrier function of the intestine, affected the intestinal structure and function of animals, and thus reduced the utilization efficiency of soybean protein by the body (Y. Zhao, Qin, Sun, Che, Bao, & Zhang, 2011). In addition, SBA impact on the metabolism and regulation of the body, leading to growth inhibition and even poisoning (Tang, Li, Qiao, Piao, & Zang, 2006). This is consistent with our findings. Within 20 days of SBA intake, the body weight of mice did not increase, indicating that the toxic effect of SBA inhibited the growth and development of the body. Also, lesions were observed on gastrointestinal tissue. Furthermore, the local structure of gastric tissue was disordered, the structure was unclear, and the gaps between cells were large. Local mucosal epithelial cells were exfoliated in duodenum; A small number of inflammatory cells were seen locally in the jejunum; In the ileum, some epithelial cells were necrotic, the local crypt structure of the mucosal layer disappeared, and many inflammatory cells infiltrated. However, the ultra-high-pressure treatment group alleviated these pathological changes to a certain extent.

The traditional processing technology of soybean milk is heat treatment. After the soybean is processed into soybean milk, there are still residues of trypsin inhibitor, urease, soybean agglutinin and other protein anti nutritional factors. This study confirmed that HHP can effectively reduce the content of anti-nutritional factors in soybeans, especially in low temperature and short time. Compared with traditional hot working, ultra-high pressure has less impact on the physical and chemical properties, rheological properties, color and luster of soybean milk, passivates harmful substances in food to the greatest extent, destroys the original nutrients of food to the minimum, and maintains the

biological and sensory properties of food. This method has good feasibility in soybean milk processing, and provides a theoretical basis for the industrial application of non -thermal process methods in soybean milk processing or other fields, such as sterilization and protein modification in the fields of alcoholic products, meat products, dairy products, and fruit and vegetable beverage processing.

In conclusion, our experimental results showed that the damage of ultra-high-pressure treatment on the structure of soybean lectin protein is significant and has great application potential. It can be tried to apply to the passivation of other anti-nutritional factors in soybean when the impact of ultra-high-pressure treatment on the nutritional and functional characteristics of soybean products is further studied.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

Xiao Han: Methodology, Investigation, Validation, Writing – original draft. **Yu Sun:** Methodology, Investigation, Validation, Writing – original draft, Methodology, Validation, Visualization. **Bingxin Huangfu:** . **Xiaoyun He:** Writing – review & editing. **Kunlun Huang:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Ascencio, C., Torres, N., Isoard-Acosta, F., Gómez-Pérez, F. J., Hernández-Pando, R., & Tovar, A. R. (2004). Soy protein affects serum insulin and hepatic SREBP-1 mRNA and reduces fatty liver in rats. *The Journal of Nutrition, 134*(3), 522–529.

Bassani, V., Linck, T., Helder, F., Moraes, R., & Cougo, & von.. (2015). Bioactive soy isoflavones: Extraction and purification procedures, potential dermal use and nanotechnology-based delivery systems. *Phytochemistry reviews: Proceedings of the Phytochemical Society of Europe, 14*(5), 849–869.

Bingyu, S., Zhimin, L., Linlin, L., Mingshou, L., Xiuqing, Z., Huan, W., & Zhiyuan, S. (2022). Research Progress on Effects of High Pressure Homogenization on Structure and Fermentation Characteristics of Soy Protein. *Science and Technology of Food Industry, 43*(13), 425–433.

Bolin, Z., Ning, L., Shuagshuang, W., Zhaoyan, L., & Zhen, H. (2019). Study on purification and agglutination activity of soybean lectin. *Jiangsu Agricultural Sciences, 47*(12), 221–224.

Bouchenak, M., & Lamri-Senhadji, M. (2013). Nutritional Quality of Legumes, and Their Role in Cardiometabolic Risk Prevention: A Review. *Journal of Medicinal Food, 16*(3), 185–198.

Cederroth, C. R., Zimmermann, C., & Nef, S. (2012). Soy, phytoestrogens and their impact on reproductive health. *Molecular and Cellular Endocrinology, 355*(2), 192–200.

Chan, C., Qi, X., Li, M.-W., Wong, F.-L., & Lam, H.-M. (2012). Recent Developments of Genomic Research in Soybean. *Journal of Genetics and Genomics, 39*(7), 317–324.

Chen, K.-I., Erh, M.-H., Su, N.-W., Liu, W.-H., Chou, C.-C., & Cheng, K.-C. (2012). Soyfoods and soybean products: From traditional use to modern applications. *Applied Microbiology and Biotechnology, 96*(1), 9–22.

Do, H., Deeth, R. J., & Besley, N. A. (2013). Computational Study of the Structure and Electronic Circular Dichroism Spectroscopy of Blue Copper Proteins. *The Journal of Physical Chemistry B, 117*(27), 8105–8112.

Fasina, Y. O., Classen, H. L., Garlich, J. D., Black, B. L., Ferket, P. R., Uni, Z., & Olkowski, A. A. (2006). Response of Turkey Poults to Soybean Lectin Levels Typically Encountered in Commercial Diets. 2. Effect on Intestinal Development and Lymphoid Organs. *Poultry Science, 85*(5), 870–877.

Fjh, A., & Jqcb, C. (2013). Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms - ScienceDirect. *Food Science and Human Wellness, 2*(3–4), 146–161.

Ghisaidoobe, A. B. T., & Chung, S. J. (2014). Intrinsic Tryptophan Fluorescence in the Detection and Analysis of Proteins: A Focus on Förster Resonance Energy Transfer Techniques. In. *International Journal of Molecular Sciences, 15*, 22518–22538.

Halder, S., Surolia, A., & Mukhopadhyay, C. (2016). Dynamics simulation of soybean agglutinin (SBA) dimer reveals the impact of glycosylation on its enhanced structural stability. *Carbohydrate Research, 428*, 8–17.

Jadhav, H. B., Annapure, U. S., & Deshmukh, R. R. (2021). Non-thermal Technologies for Food Processing. *Frontiers. Nutrition, 8*.

Kang, J.-H., Han, I.-H., Sung, M.-K., Yoo, H., Kim, Y.-G., Kim, J.-S., … Yu, R. (2008). Soybean saponin inhibits tumor cell metastasis by modulating expressions of MMP-2, MMP-9 and TIMP- 2. *Cancer Letters, 261*(1), 84–92.

Khan, J. M., Chaturvedi, S. K., & Khan, R. H. (2013). Elucidating the mode of action of urea on mammalian serum albumins and protective effect of sodium dodecyl sulfate. *Biochemical and Biophysical Research Communications, 441*(3), 681–688.

Li, T., Li, X., Dai, T., Hu, P., Niu, X., Liu, C., & Chen, J. (2020). Binding mechanism and antioxidant capacity of selected phenolic acid - β-casein complexes. *Food Research International, 129*, Article 108802.

Li, Z., Li, D., & Qiao, S. (2003). Effects of soybean agglutinin on nitrogen metabolism and on characteristics of intestinal tissues and pancreas in rats. *Archives of Animal Nutrition, 57*(5), 369–380.

Liener, I. E. (1994). Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition, 34*(1), 31–67.

Linsberger-Martin, G., Weiglhofer, K., Thi Phuong, T. P., & Berghofer, E. (2013). High hydrostatic pressure influences antinutritional factors and in vitro protein digestibility of split peas and whole white beans. *LWT - Food Science and Technology, 51*(1), 331–336.

Lis, H., & Sharon, N. (1998). Lectins: Carbohydrate-Specific Proteins That Mediate Cellular Recognition. *Chemical Reviews, 98*(2), 637–674.

Ma, Y., & Wang, T. (2010). Deactivation of Soybean Agglutinin by Enzymatic and Other Physical Treatments. *Journal of Agricultural and Food Chemistry, 58*(21), 11413–11419.

Molla, A. R., & Mandal, D. K. (2013). Trifluoroethanol-induced conformational change of tetrameric and monomeric soybean agglutinin: Role of structural organization and implication for protein folding and stability. *Biochimie, 95*(2), 204–214.

Nakashima, H., Okubo, K., Honda, Y., Tamura, T., Matsuda, S., & Yamamoto, N. (1989). Inhibitory effect of glycosides like saponin from soybean on the infectivity of HIV in vitro. *AIDS, 3*(10).

Shan, D., Yu, H., Lyu, B., & Fu, H. (2021). Soybean β-Conglycinin: Structure Characteristic, Allergenicity, Plasma Lipid-Controlling, Prevention of Obesity and Non-alcoholic Fatty Liver Disease. *Current Protein & Peptide Science, 22*(12), 831–847.

Singh, B. P., Yadav, D., & Vij, S. (2019). Soybean Bioactive Molecules: Current Trend and Future Prospective. In J.-.-M. Mérillon, & K. G. Ramawat (Eds.), *Bioactive Molecules in Food* (pp. 267–294). Cham: Springer International Publishing.

Tang, S., Li, D., Qiao, S., Piao, X., & Zang, J. (2006). Effects of purified soybean agglutinin on growth and immune function in rats. *Archives of Animal Nutrition, 60*(5), 418–426.

Tolouie, H., Mohammadifar, M. A., Ghomi, H., & Hashemi, M. (2018). Cold atmospheric plasma manipulation of proteins in food systems. *Critical Reviews in Food Science and Nutrition, 58*(15), 2583–2597.

USDA DataBase. In., vol. 28 9 2022.

van der Ven, C., Matser, A. M., & van den Berg, R. W. (2005). Inactivation of Soybean Trypsin Inhibitors and Lipoxygenase by High-Pressure Processing. *Journal of Agricultural and Food Chemistry, 53*(4), 1087–1092.

Vanga, S. K., Singh, A., & Raghavan, V. (2018). Changes in soybean trypsin inhibitor by varying pressure and temperature of processing: A molecular modeling study. *Innovative Food Science & Emerging Technologies, 49*, 31–40.

Wang, K.-Q., Luo, S.-Z., Zhong, X.-Y., Cai, J., Jiang, S.-T., & Zheng, Z. (2017). Changes in chemical interactions and protein conformation during heat-induced wheat gluten gel formation. *Food Chemistry, 214*, 393–399.

Zhao, B., Che, D., Adams, S., Guo, N., Han, R., Zhang, C., … Jiang, H. (2019). N-Acetyl-d-galactosamine prevents soya bean agglutinin-induced intestinal barrier dysfunction in intestinal porcine epithelial cells. *Journal of Animal Physiology and Animal Nutrition, 103*(4), 1198–1206.

Zhao, Y., Qin, G., Sun, Z., Che, D., Bao, N., & Zhang, X. (2011). Effects of Soybean Agglutinin on Intestinal Barrier Permeability and Tight Junction Protein Expression in Weaned Piglets. In. *International Journal of Molecular Sciences, 12*, 8502–8512.