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# Safety evaluation of soybean protein isolate oxidized by a hydroxyl radical-generating system

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ABSTRACT

The oxidative modification of soybean protein isolate (SPI) induced by a hydroxyl radical-generating system (HRGS) has a broad range of applications. However, few toxicology studies exist on this material. The safety of HRGS-oxidized SPI was assessed using subchronic and genotoxicity studies. A 30-day subchronic study (250, 500 and 1000 mg/kg·BW) in rats showed no significant adverse effects on food consumption, body weight (BW), mortality, hematology, biochemistry, necropsy, organ weight or histopathology. The result of an Ames test showed that HRGS-oxidized SPI was not mutagenic to the test strains. The results of a bone marrow micronucleus test and mouse sperm abnormality test showed HRGS-oxidized SPI (417.5, 835.0 and 1670.0 mg/kg·BW) did not produce any aberrant effects on bone marrow cells or mouse sperm. Therefore, HRGS-oxidized SPI showed no genotoxicity *in vivo* or *in vitro*. In conclusion, these results support the safe use of HRGS-oxidized SPI as a food and dietary supplement.


## 1. Introduction

Soybean proteins have comprehensive nutritional value and unique health effects (Carbonaro, 2011). The Food and Drug Administration (FDA, 1998) stated that 25 g of soybean protein per day could reduce the risk of cardiovascular and cerebrovascular diseases. Moreover, soybean proteins have various beneficial effects in humans, notably on bone health, weight control, insulin sensitivity, inflammation, cancer prevention, immune cell activity and mineral absorption (Carmignani et al., 2010; Jahan-Mihan et al., 2011; Kashima et al., 2016; Liu et al., 2011; Manuel and Sam, 2007; Nagarajan et al., 2008; Rebello et al., 2014; Sénéchal and Kussmann, 2011). Because of these advantages, soybean protein plays a significant role as a food and dietary supplement.

Soybean protein isolate (SPI) is one of the most common soybean protein products and contains more than 90% of the different proteins and all of the essential amino acids, particularly lysine (Peng et al., 1984). The main protein ingredients are classified into four categories according to their sedimentation coefficients: 2S, 7S, 11S and 15S (Nishinari et al., 2014; Saio et al., 1969). As a popular emulsifier in different food industries, SPI can reduce the interfacial tension in emulsion systems, which results in the ability to adsorb at different interfaces to form a thick layer and stabilizes oil droplets (Keerati-u-rai et al., 2012; Keerati-u-rai and Corredig, 2009). However, SPI is a less efficient emulsifier than whey and casein proteins (Wagner and Gueguen, 1995), which limits the use of SPI in food systems. Therefore, researchers have used different modification methods to change the native structure and improve the functional properties of SPI (Chove et al., 2007; Jambrak et al., 2009; Wan et al., 2013).

Recently, hydroxyl radical-generating system (HRGS)-mediated protein oxidation has received increasing attention. The mechanism of HRGS involves the Fenton reaction, which generates hydroxyl radicals from $H_2O_2$ in the presence of $Fe^{2+}$. Reactions of hydroxyl radicals accelerate the formation of protein cross-links at the highest rate constants (Butterfield and Stadtman, 1997). In our laboratory, we successfully produced HRGS-oxidized SPI, which had improved physicochemical properties and emulsifying activity to act as a better emulsifier and stabilizer in various foodstuffs (Liu et al., 2015). However, the toxicological safety of HRGS-oxidized SPI needs further study. Because SPI has no acute toxicity, the present study was performed to assess the subchronic (30 days) and

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genotoxic effects of HRGS-oxidized SPI. All animals used in this study received care in compliance with the guidelines for the Care and Use of Laboratory Animals of China. All aspects of the studies were carried out under the OECD Guidelines for Testing of Chemicals (Section 471, 1997a; Section 474, 1997b; Section 407, 2008) and the Good Laboratory Practice (GLP) Regulations of the State Food and Drug Administration of China (GB 15193.2–2014).

## 2. Materials and methods

All aspects of these studies were conducted in compliance with the Technical Standards for Testing and Assessment of Health Food published by the Ministry of Health of the People's Republic of China (MOHC, 2003). These regulations conform to the OECD Guidelines for the Testing of Chemicals, and all animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals of China. The test material and methods used are described for each individual study. In all studies, the stated concentrations or doses reflect the amount of HRGS-oxidized SPI administered, and appropriate control groups were used as necessary.

### 2.1. Test materials

HRGS-oxidized SPI was prepared as previously described (Liu et al., 2015). Defatted soy flakes were purchased from Harbin High-Tech Group (Heilongjiang, China). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), butylated hydroxyanisole (BHA), ethylene diamine tetraacetic acid (EDTA), ascorbic acid, 5,50-dithiobis (2-nitrobenzoic acid) (DTNB) and 2,4,6-trinitrobenzenesulfonic acid sol (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All of the chemicals and reagents were of analytical grade. Defatted soy flake powder was dispersed in distilled water (1:10, w/v) and adjusted to pH 8.0. The dispersion was stirred for 2 h to extract protein and then centrifuged at 13,500 × g for 30 min at 4 °C. The supernatant was adjusted to pH 4.5 with 2 M HCl and then centrifuged at 3300 × g for 20 min. The pellet was washed twice with distilled water; each wash was performed by suspension in 5-fold (w/w) distilled water followed by centrifugation at 8000 × g for 10 min. Thereafter, the protein pellet was resuspended in 5-fold (w/w) distilled water and then neutralized to pH 7.0 with 2 M NaOH. The neutral solution was frozen, subsequently lyophilized, and stored at 4 °C until use (Jiang et al., 2009).

Oxidized SPI was prepared according to the method described by Cui et al. (2012). The SPI suspension (30 mg protein/mL) was oxidized at 20 ± 1 °C for 5 h in the HRGS that consisted of 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, and six levels of $H_2O_2$ (0.1, 0.5, 1, 5, 10 and 15 mM). The control sample contained the same buffer as above without the components of the HRGS. Oxidation was terminated by adding BHA/Trolox/EDTA (1 mM each). The oxidized and control samples were subsequently lyophilized. The freeze-dried samples were sealed in polyethylene bags and then stored at 4 °C and 40 % RH until use.

### 2.2. Thirty-day feeding study

Male and female clean-grade Sprague-Dawley rats were obtained from Harbin Medical University (Certificate No. SCXK (Hei) 2013-002). Eighty healthy rats were allocated into four groups with 20 animals in each group (10 males and 10 females). After 7 days of acclimation to the laboratory environment, the rats were stratified by body weight (BW) and randomly assigned to treatment groups. At the start of the study, the rats were approximately 4 weeks old, with males weighing between 100.2 and 117.2 g and females weighing between 75.3 and 95.5 g. The individual BWs of the groups were within ± 20% of the mean for each sex. All rats were housed at five rats per group per sex in stainless steel mesh cages located in rooms with a temperature of 22 ± 3 °C, relative humidity of 50 ± 15 % and light/dark cycle of 12 h.

The HRGS-oxidized SPI was freshly prepared in distilled water and administered by oral gavage at doses of 250, 500 and 1000 mg/kg·BW, whereas the second group received distilled water only by gavage at a constant-volume dose of 10 mL/kg·BW for 30 days. The dose of 1000 mg/kg·BW was selected as the maximum dose based on the results of a previous study. The dose of 250 mg/kg·BW HRGS-oxidized SPI was used as the minimum dose for the 30-day feeding study.

The animals were observed at least twice daily. Their BW and feed consumption were recorded before administration of the test agent and at termination. Their water consumption was recorded during week 4 over a 3-day period for each cage. Blood samples were obtained on day 31 after overnight starvation via an abdominal aorta. Blood samples were collected for hematological and biochemical parameter analyses.

One portion of each blood sample was collected in an EDTA-coated tube for hematological analysis, and the other portion was collected in a dry tube for the separation of serum for biochemical analysis. EDTA was used as an anticoagulant for the hematological measurements. The hematology samples were examined for the following characteristics: white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), platelet count (PLT) and thrombocytocrit (PCT).

The blood samples in dry tubes were centrifuged at 5000 rpm (10 min at 4 °C), and the supernatant (serum) was collected and placed into new tubes. The serum and biochemical parameters were examined for the following characteristics: serum potassium ions ($K^+$), serum sodium ions ($Na^+$), serum chloride ions ($Cl^-$), total protein (TP), total albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin in serum (TBIL), triglyceride (TG), total cholesterol (TCHO), blood urea nitrogen (BUN) and creatinine (CREA).

The heart, liver, kidney, spleen and lung were removed and weighed, and the relative weight (organ weight (g)/100 g·BW) was determined. The liver, kidney and lung were fixed in 10 % formaldehyde for histological analysis.

### 2.3. Genetic toxicity studies

#### 2.3.1. Bacterial reverse mutation assay (Ames test)

A bacterial reverse mutation assay was performed to evaluate the mutagenicity of the HRGS-oxidized SPI, with and without S9, using the following four *Salmonella* strains as prescribed in the OECD Guidelines for Testing of Chemicals (1997a; 1997b) and the Technical Standards for Testing and Assessment of Health Food (MOHC, 2003): TA97, TA98, TA100 and TA102. All strains were provided by the Chinese Center for Type Culture Collection (CCTCC, Wuhan, China) and checked for the maintenance of genetic markers prior to the study. The S9 microsomal fraction of the rat liver homogenate was used as the metabolic activation system (purchased from MOLTOX, Shanghai, China) and was prepared according to previous methods. Four doses, 40, 200, 1000 and 5000 mg/plate, of HRGS-oxidized SPI were tested based on the results of the preliminary toxicity test.

The negative control group, solvent control group (solvent was sterile water) and positive control groups were prepared concurrently with the treatment groups. Standard mutagens were used as