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Immunomodulatory effects of pepsin-educed soy protein hydrolysate in rats and murine cells

Tolulope Joshua Ashaolu[1], Niracha Yanyiam[2] and Chutha Takahashi Yupanqui[1*]

[1]Interdisciplinary Graduate School of Nutraceutical and Functional Food (IGS-NFF), Prince of Songkla University, Hat-Yai, Songkhla, 90112, Thailand; [2]Department of pharmacology, Faculty of Sciences, Prince of Songkla University, Hat-Yai, Songkhla, 90112, Thailand

Corresponding author: Chutha Takahashi Yupanqui, PhD, Interdisciplinary Graduate School of Nutraceutical and Functional Food (IGS-NFF), LRC Building, Prince of Songkla University, Hat-Yai, Songkhla, 90112 Thailand

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ABSTRACT

Background: There has been an increase in demand for soy and its products, with soy protein having a huge market in particular. Several reports have established how soy protein hydrolysates (SPHs) yield better physiological properties and play essential functional roles than the crude soy protein.

Objective: To investigate the immunomodulatory and acute toxicity of soy protein hydrolysate (SPH) produced with pepsin protease in mice and rats.

Methods: Soy protein hydrolysate was enzymatically produced using pepsin, with E/S (enzyme to substrate ratio) of 0.5% (250 u/mg) and hydrolysis time of 4h. Afterwards, the SPH effects on murine spleen lymphocyte proliferation, and peritoneal macrophage phagocytosis were investigated in vitro. Confirmation studies were explored in rats' sera IgG and IgA, and the acute toxic effect of SPH was observed in mice subjects.

Results: The hydrolysate increased the levels of splenocytes (stimulative index of 10.141 – 10.811) and peritoneal macrophages (phagocytic index of 1.285 – 1.721). Furthermore, the concentrations of sera IgG and IgA obtained from SPH-fed rats ranged from 0.198 – 0.345 mg/ml and 0.0184 – 0.0194 mg/ml, respectively in comparison with the soy protein isolate (SPI) -fed rats (0.208 – 0.322 mg/ml and 0.0188 – 0.0189 mg/ml, respectively). Additionally, 10 mg dose of SPH stably elicited serum IgG in contrast to other doses, while there was a general decrease in the amounts of IgA obtained in the rat subjects. Moreover, there was no acute toxic effect recorded in the mice subjects.

Conclusion: In light of the results, it is possible that SPH prepared with pepsin has the potential of improving the immune system, and may therefore be used as immunomodulatory or functional food product.

Keywords: Soy protein hydrolysates, splenocyte proliferation, immunomodulatory, phagocytosis and pepsin

INTRODUCTION

Soy protein consumption has increased significantly due to the increasing costs of animal protein, vegans' protein option, esoteric supplement requirement, and several functional health benefits, among other reasons. However, consumption of soy-containing food products can cause severe and even fatal allergic reactions such as anaphylactic shock [1], in addition to other immune-related diseases such as asthma and hay fever. Recently, the need to upgrade the functional features of soy protein has led to the development of new technologies, including the genetic modification of soybean, thermal and non-thermal treatments, and enzymatic hydrolysis [1]. Due to market demands for foods produced with the use of less chemical methods, enzyme hydrolysis is an option that has gained more interest. Furthermore, enzymatic hydrolysis is widely applied to reduce loss of biological activity [2].

Soy protein hydrolysates have various physiological activities, including anti-inflammatory [3], anti-adipogenic [4], anti-allergic [5], antihypertensive [6], antioxidant, hypocholesterolemic [7], and ACE inhibitory properties [8]. Soy protein hydrolysates (SPH) have been prepared from soy protein isolate (SPI) with proteases including neutrase [4], corolase [8, 9], pomiferin, cucurbita, pepsin, papain, chymotrypsin [10], novozym, flavourzyme [11], alcalase, flavourzyme, and papain [1, 12]. Alcalase has become predominant in several reports due to its higher degree of hydrolysis values and assessment of the digestibility of proteins [1, 12, 8]. Pepsin has not been used singly in all of the previous reports, being among the paramount digestive enzymes found in mammals including humans, making it an important enzyme for studying the gastrointestinal tract digestion process and clinical nutrition.

The immune system comprises of many biological structures and processes that make resistance to disease possible in an organism. Antibodies play an important role in fighting antigens, including bacteria, viruses, and toxins. The five subclasses of antibodies include Immunoglobulin A (IgA), which is of high concentrations in the mucous membranes; Immunoglobulin G (IgG), the most abundant type of antibody in all body fluids which protects against bacterial and viral infections; Immunoglobulin M (IgM), which is mainly found in the blood and lymph fluid; Immunoglobulin E (IgE), which is mainly associated with allergic reactions; and Immunoglobulin D (IgD), which exists in small amounts in the body. Serum IgA reacts with FC receptor called CD89, which is expressed on immune effector cells, to initiate inflammatory reactions. This causes degranulation of eosinophils and basophils, in addition to the phagocytosis of neutrophils, monocytes, and macrophages [13]. Significant information about the immune system functioning can be accessed when IgA and IgG are measured together.

Immunomodulatory peptides can enhance mucosal immunity in the gastrointestinal tract [14]. They may also enhance immune cell functions, measured as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation [15, 16]. Moreover, immunomodulatory peptides derived from tryptic hydrolysates of rice and soybean proteins act to stimulate superoxide anions (reactive oxygen species [ROS]) which trigger non-specific immune defense systems [17]. These reports indicated that soy protein hydrolysates might play a role in stimulating immunomodulatory activity. Until now, there have been very few reports on the immunomodulatory activity of soy peptides in animal models [18, 19]. Xiang and colleagues [12] prepared soy protein hydrolysates with various proteases besides pepsin and investigated their immunomodulatory potentials in vitro.

In this present study, soy protein hydrolysate was enzymatically prepared with pepsin, and in vitro murine splenocytes proliferation, while peritoneal macrophage phagocytosis was also investigated. The immunomodulatory activity of the hydrolysate was further determined in vivo.

This is the first report of its kind that shows the effects of pepsin-educed SPH on immunomodulation, and thereby has a high prospect of being used as a potential source of low cost immunomodulatory and functional food products.

MATERIALS AND METHODS

Materials

Food grade SPI (protein content 90.2% dry base) was purchased from Wachsen Industry Company, Ltd (Qingdao, China). Pepsin (250 U/mg) was purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals used were of the highest analytical grade commercially available.

Preparation of pepsin-educed soy protein hydrolysate

SPI was dissolved in deionized water in a ratio of 1:8 (w/v). Hydrolysis of SPI with pepsin was carried out at 37^{o}C and pH of 2.0 using 2M HCl, and enzyme-substrate ratio of 0.5%/100 g substrate. The SPI was pre-incubated for 15 min at 37^{o}C prior to hydrolysis process. The hydrolysis time was set to 4h. The enzyme was inactivated using heat treatment at 95°C for 15 min in a thermostat-controlled water bath. Afterwards, the sample was cooled on ice to room temperature and centrifuged at 4^{o}C, 10,000 rpm for 20 min to separate the supernatant from the pellet. Finally, the supernatant (soy protein hydrolysate) was lyophilized and stored at -20^{o}C. Figure 1 shows the experimental flow chart.

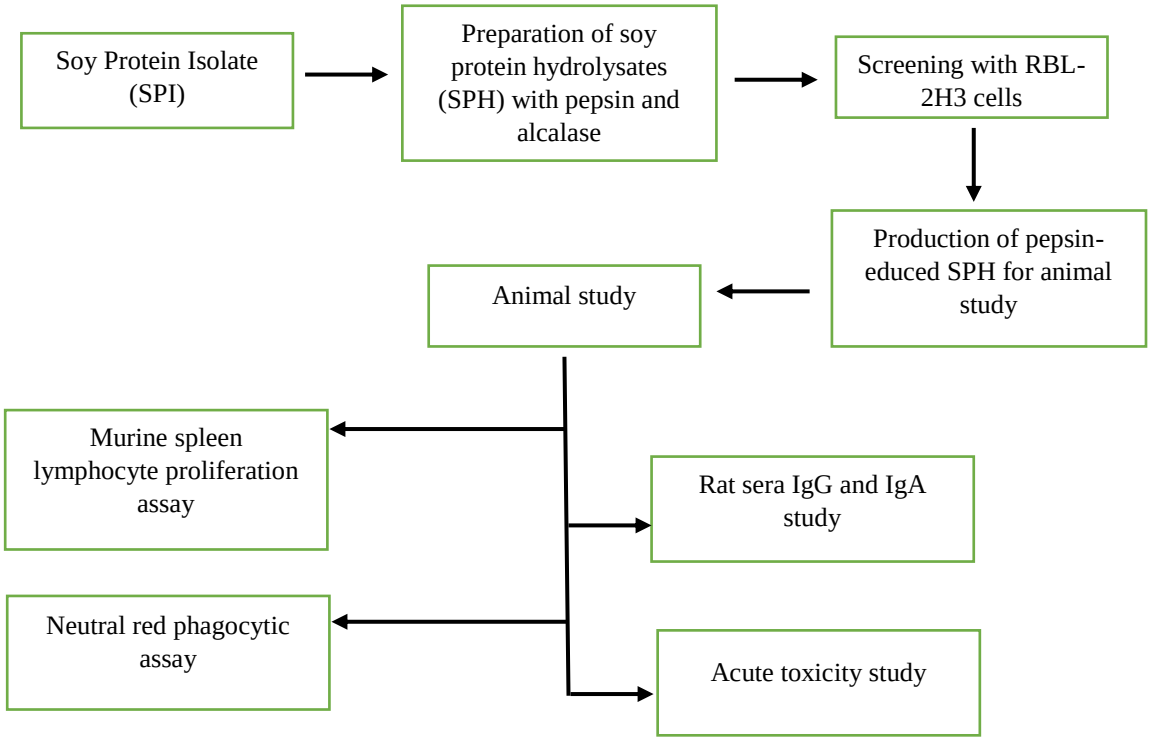

Fig. 1. Experimental study flow

Murine splenocyte proliferation assay

Outbred ICR mice were sacrificed using sodium pentobarbital 100 mg/kg w/w intraperitoneally. The spleen was surgically taken out and washed in a small petri dish containing 2% FBS (fetal bovine serum) in PBS (phosphate buffer saline). Afterwards, the spleen was carefully teased, and the tissue/cell suspension was lysed with ACK lysis buffer (4.145g NH_4Cl, 0.5g $KHCO_3$, 18.6mg EDTA/2Na and 500 ml deionized water) for 10 min. It was centrifuged twice at 6000 rpm for 10 min at 4^{o}C, and the supernatant was removed. The pellet was washed with 2% FBS in PBS twice

at 6000 rpm for 10 min at 4ºC. Finally, the pellet (cells) was adjusted into 5×10^5 cells/ml suspension in RPMI-1640 media supplemented with 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2mM glutamine. Viability of cells was performed using the trypan blue exclusion method and was above 98%. The splenocytes (5×10^5 cells/ml) were incubated with 100 µl of the SPH in volume ratio 1:1, Con A (positive control) or RPMI-1640 medium (negative control) in concentrations of 0.1 mg/ml, 1.0 mg/ml, and 10 mg/ml respectively in a flat-bottom wells (96 wells/plate; Corning, Acton, MA, USA). After incubation at 37ºC, 5% CO_2 and 95% humidity for 72h, the supernatants were removed and 60 µl of fresh RPMI-1640 medium was added to the pellet. This was followed by the addition of 25 µl MTT solution and 2h incubation. Stop solution (100 µl of DMSO) was added and the plate read at 570 nm with a microplate reader. The stimulation index (SI) was calculated as the ratio of absorbances at 570 nm with the SPH to absorbances without the SPH.

Murine peritoneal macrophages phagocytosis assay using neutral red

Outbred ICR mice were injected intraperitoneally with 3 ml of 2% FBS in PBS, and the resulting peritoneal exudates were harvested and washed twice with the same solution after keeping the exudates in ACK lysis buffer for 10 min. The pellet/cells were suspended and adjusted to 1×10^6 cells/ml in a RPMI-1640 medium supplemented with 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2mM glutamine. Viability of cells was performed using the trypan blue exclusion method and was above 95%. Afterwards, 100 µl of 1×10^6 cells/ml of the peritoneal macrophages was incubated with 100 µl of SPHs, Con A (positive control) or just RPMI-1640 medium (negative control) in concentrations of 0.1 mg/ml, 1.0 mg/ml, and 10 mg/ml respectively in a flat-bottom wells (96 wells/plate; Corning, Acton, MA, USA). After incubation at 37ºC, 5% CO_2 and 95% humidity for 24h, the culture soups were removed thoroughly, and 200 µl of 0.075% neutral red was added to each well. The plate was further incubated for 30 min, aspirated, and rinsed twice with PBS. Finally, 200 µl of lysis solution [1M acetic acid: ethanol = 1:1 (v/v)] was added and the plate left overnight at 4ºC for sufficient schizolysis of cells and release of phagotrophic neutral red. The OD was read at 540 nm and the Phagocytosis Index (PI) was calculated as the ratio of absorbances at 540 nm with the SPH to absorbances without the SPH.

Animal model: Feeding and sampling

Male ICR mice and Wistar rats of pathogen-free grade were purchased from Mahidol University (Bangkok, Thailand) and were maintained in an air-conditioned animal facility at a temperature of 25 ± 2 °C and a relative humidity of 50 ± 10%. Body weights of rats at receipt ranged from 85 – 485 g. The animals were acclimatized to the facility for a week with free access to standard rodent chow (CP-082G) and water under a 12-h light/dark cycle. All animal protocols used in this study were approved by the Committee for Animal Experiments of Prince of Songkla University (MOE 0521.11/916). Experimental groups consisted of Young (4 weeks, Y), Mature (7 weeks, M) and Aged (13 weeks, A) of normal control (NC), soy protein isolate (SPI) and soy protein hydrolysate (SPH). The SPH group was fed with 5 – 15 mg of SPH per day, while the SPI group was fed with 5 – 15 mg of SPI by gavage. Throughout the experimental period, the body weights of the animals were measured, and blood samples were collected from the tail vein in order to measure the sera IgG and IgA concentrations.

Measurement of the IgG and IgA concentrations

The rat sera IgG and IgA concentrations were measured by an enzyme-linked immunosorbent assay (ELISA). Briefly, blood samples collected in Eppendorf tubes were stored at 4ºC for 2h in order to allow the formation of blood clots. Afterwards, the sera were separated by centrifugation

at 6,000 rpm for 10 min and stored at –20^{o}C until needed. The IgG and IgA concentrations of the serially diluted sera were measured with rat IgG and IgA ELISA kits (Komabiotech Laboratories, Seoul, Korea) according to the manufacturer's protocol.

Mice study: Acute toxicity

Acute toxicity of pepsin-educed SPH was performed in male mice following the guidelines of the Organization for Economic Co-operation and Development (OECD) for testing of chemicals, TG 425 (adopted -03 October, 2008) with minor modifications. Female mice were avoided due to interactions, which could result from plant oestrogens found in soy with those found in female mice. Experimental animals were divided into two groups, treatment and control. Both were deprived of food, but allowed access to water overnight prior to dosing the treatment group with SPH, and the control group with water. Dosing was started at 550 mg/kg body weight (BW) until 5000 mg/kg BW by gavage with the volume of 0.125 ml/10g BW. They were monitored continuously for 4h afterwards for any signs of toxicity such as reduction in aggressiveness, locomotion, social interactions, and response to stimuli (noise, defensiveness, tail pinch), in addition to diarrheal faeces. General health observations and mortality were monitored for 14 days for any delayed toxicity after treatment. Moreover, the dosing solutions were made every day just before feeding, with the dose concentrations being based on nominal concentrations.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) and are the average of three samples per experiment, except for the rat experiment, which are recorded as an average of five per group. Analyses were performed using SPSS software version 17.0. The experiment was repeated at least thrice, except for the rat experiment to confirm the results. The level of statistical significance, using analysis of variance (ANOVA) with Tukey's test, was set to $p < 0.05$ or 0.01 where stated.

RESULTS AND DISCUSSION

Immunomodulatory activity of SPH

Splenocytes consist of a variety of cell populations such as T and B lymphocytes, dendritic cells, and macrophages, which have different immune functions. Concavalin A (ConA) is a T-cell mitogen often used as the positive control. The effects of the hydrolysate on murine splenocytes proliferation and the phagocytic activity of peritoneal macrophages were evaluated in vitro, in stimulated cells (without mitogen, blank) and stimulated cells (with mitogen, Con A). As shown in Table 1, the hydrolysate stimulated spleen lymphocyte proliferation in all concentrations used (0.1, 1.0, and 10.0 mg/ml). The highest SI value was obtained at 0.1 mg/ml dosage (10.811), suggesting that the soy hydrolysate may act as a mitogen for murine splenocytes. Different proteins and peptides from plants and animals may have immune-boosting potential and reduce risks of cardiovascular disease by limiting inflammation [20]. Xiang and associates [12] employed several proteases to prepare SPH and found alcalase-educed SPH to have the highest SI value of 1.386.

Macrophages are cells responsible for detecting, engulfing, and destroying pathogens and apoptotic cells. Macrophages are recognized as the first line of defence, and are produced through the differentiation of monocytes. The phagocytic activity of peritoneal macrophages cultured with SPH was measured (See Table 1). Like wisely, SPH yielded its highest phagocytic activity (1.721) at the lowest concentration of 0.1 mg/ml. It can be inferred that the hydrolysate induced the release of cytokines able to activate immune cells distant from the site of induction of the immune response [21]. Moreover, other investigators like Dia and colleagues [3] used a combination of pepsin and pancreatin proteases to hydrolyse soy products among other commercially available products, and discovered these were able to stimulate the proliferation of murine peritoneal macrophages.

Table 1. Immune boosting activity of soy protein hydrolysates at different concentrations

Group	Soy Protein Hydrolysate					
	0.1mg/ml		1.0 mg/ml		10.0mg/ml	
	PI	SI	PI	SI	PI	SI
Blank (-)	0.758±0.038	0.130±0.022	0.569±0.064	0.125±0.010	0.558±0.039	0.106±0.022
Con A (+)	1.320±0.009	7.711±0.207	1.758±0.010	7.991±0.065	1.791±0.082	9.402±0.361
SPH	1.721±0.009[a,b]	10.811±0.055[a,b]	1.426±0.035[b]	10.141±0.020[a,b]	1.285±0.012[a]	10.257±0.504[a,b]

PI = Phagocytic index; SI = Stimulation index = ODtest/ODcontrol; Data were shown as mean ± SD (n=3). Samples with a significant difference ($p<0.05$) compared to Con A = [a], Blank= [b] across columns.

Safety of SPH in mice

Acute toxicity tests are generally utilized in the assessment and evaluation of the toxic characteristics of a substance. In this toxicity study, no animal mortality was observed under the doses of 5000 mg/kg BW after administration of the hydrolysate. Moreover, no significant changes in skin, breathing, defecation, postural abnormalities, impairment in food intake, and yellowing or loss of hair of mice were found in the treatment group in comparison with the normal control group during the treatment period. Kobayashi and colleagues [22] reported Shoyu polysaccharides from soy sauce to be safe in vitro. After toxicity study of 600 mg dose for 8 weeks, no toxic effects were found in routine blood tests, hepatic, and renal function tests, in addition to concentrations of proteins and lipid tests [23]. Anadón and colleagues [24] asserted that casein hydrolysates containing RYLGY and AYFYPEL peptides, when orally administered to rats, showed no toxicity at the highest doses investigated in both acute and repeated dose toxicity studies (2000 mg/kg BW and 1000 mg/kg BW, respectively). While conducting a 90-day toxicity study of soy meal and hulls from genetically modified soy DAS-444Ø6-6 in rats, Papineni and colleagues [25] found no evidence of differences in nutritional quality between the treatment and the control groups. They also found the soy to be safe, having no adverse effects in rats, as observed from clinical signs, ophthalmic examinations, functional tests, motor activity, body weights, feed consumption, and other parameters studied.

In the course of this study, it appeared there were no reports on acute toxicity studies of soy protein hydrolysates in mice, especially pepsin-educed soy protein hydrolysates. Further work is recommended in this regard.

Rats' body weights and measurement of sera IgG and IgA levels

The body weights of the animals were measured weekly, and blood samples were collected from the tail vein in order to measure the sera IgG and IgA concentrations. All groups exhibited a general increase in weight, suggesting that the SPH did not confer any loss of weight or any form of abnormality, which may lead to weight loss in the treated rats. No treatment related differences in the mean body weights were detected compared with the control. See Figure 2 below. The young group exhibited less sporadic weight differences compared to the mature and aged groups. This is probably due to the faster rate of metabolic activity in young rats compared to older rats.

Plasma cells producing serum IgA are located predominantly in the bone marrow and to a lesser extent in the spleen [26]. Receptors for the Fc portion of IgA are found on monocytes and granulocytes [27, 28]. Receptor-bound IgA antibodies activate both granulocytes and monocytes and initiate phagocytosis of bacteria and fungi. Many reviews suggest that IgA deficiency and allergy are associated [29, 30, 31]. However, the availability of considerable concentrations of IgG in an organism is a good marker of immune balance.

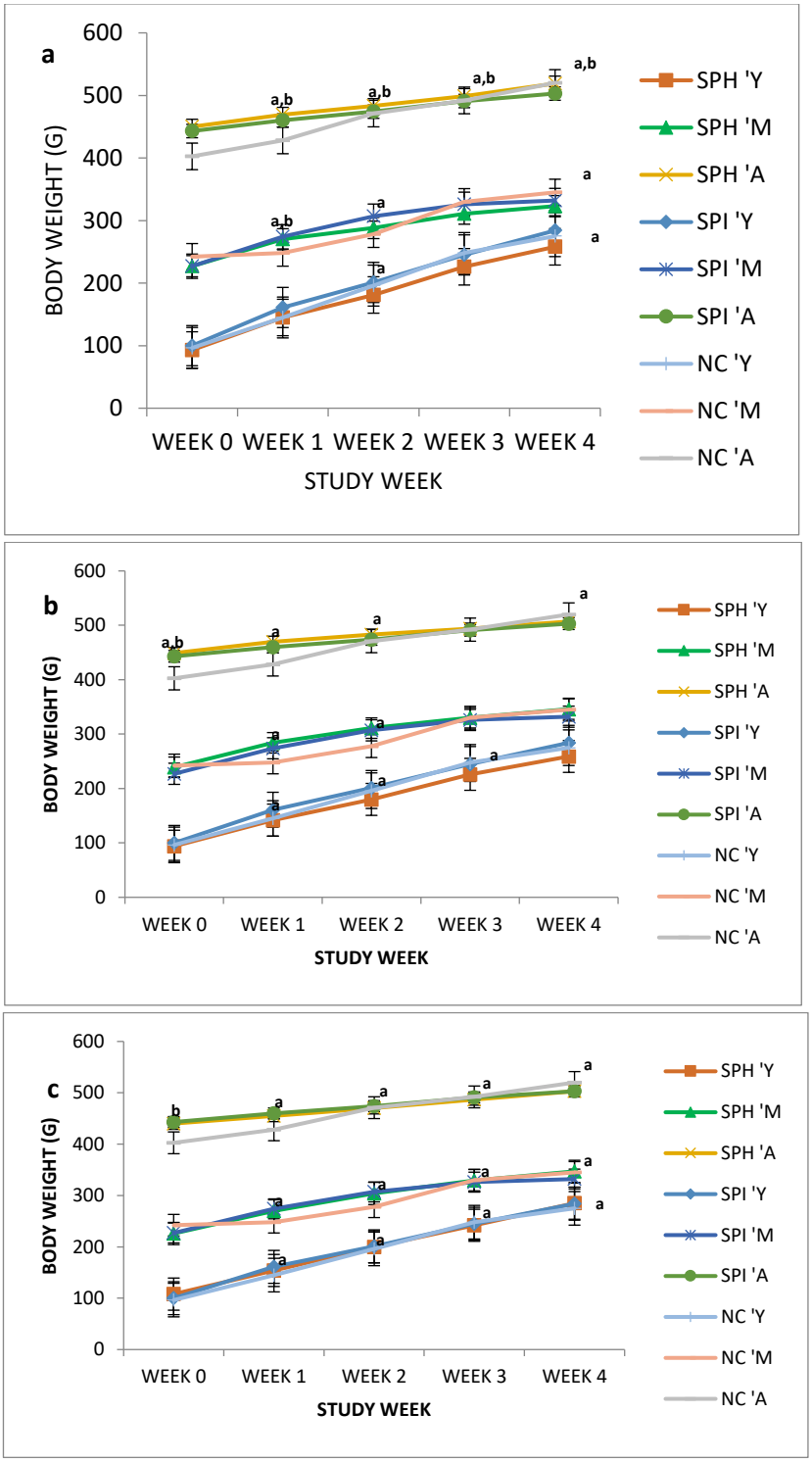

Figure 2. Mean Weekly body weight of animals fed with

a. 5 mg/day SPH, SPI and Sterile water
b. 10 mg/day SPH, SPI and Sterile water
c. 15 mg/day SPH, SPI and Sterile water

SPH 'Y= SPH-Young, SPH 'M=SPH-Mature, SPH 'A=SPH-Aged, SPI 'Y=SPI-Young, SPI 'M=SPI-Mature, SPI 'A=SPI-Aged, NC 'Y= Normal Control-Young, NC 'M= Normal Control-Mature, NC 'A= Normal Control-Aged. Significant differences were detected ($p<0.05$) between [a]SPH and normal control; [b]SPI and normal control.

In this study, Figure 3 showed a relatively general increase in the concentrations of sera IgG in all groups studied. When studying the relationship between cholecystokinin and food, and their effects on the release of immunoglobulin A and immunoglobulin G specific antibodies in the rat intestine, Freier and colleagues [33] employed a hypoallergenic protein hydrolysate formula. The formula was passed through the stomach and duodenum without reaching the perfused segment. This brought about the release of IgA and IgG. Reports on the impacts of soy protein hydrolysates on IgG and IgA concentrations in rats are lacking. However, Bittencourt and colleagues [34] investigated the immunogenicity of the 2S, 7S, and 11S soy protein fractions by evaluating the humoral immune response in mice through the production of IgM and IgG antibodies. There was no production of IgM antibodies for the 2S protein, and IgG antibodies were only detected after mice immunization. The case was different with the 7S soy protein fraction, which had a strong immune response with a high production of IgM and IgG antibodies during, and after mice immunization period. The 11S fraction also induced intense IgG production. There was a similar work previously conducted by another group of researchers [35] who similarly established the intense response of murine IgG antibodies to soy protein.

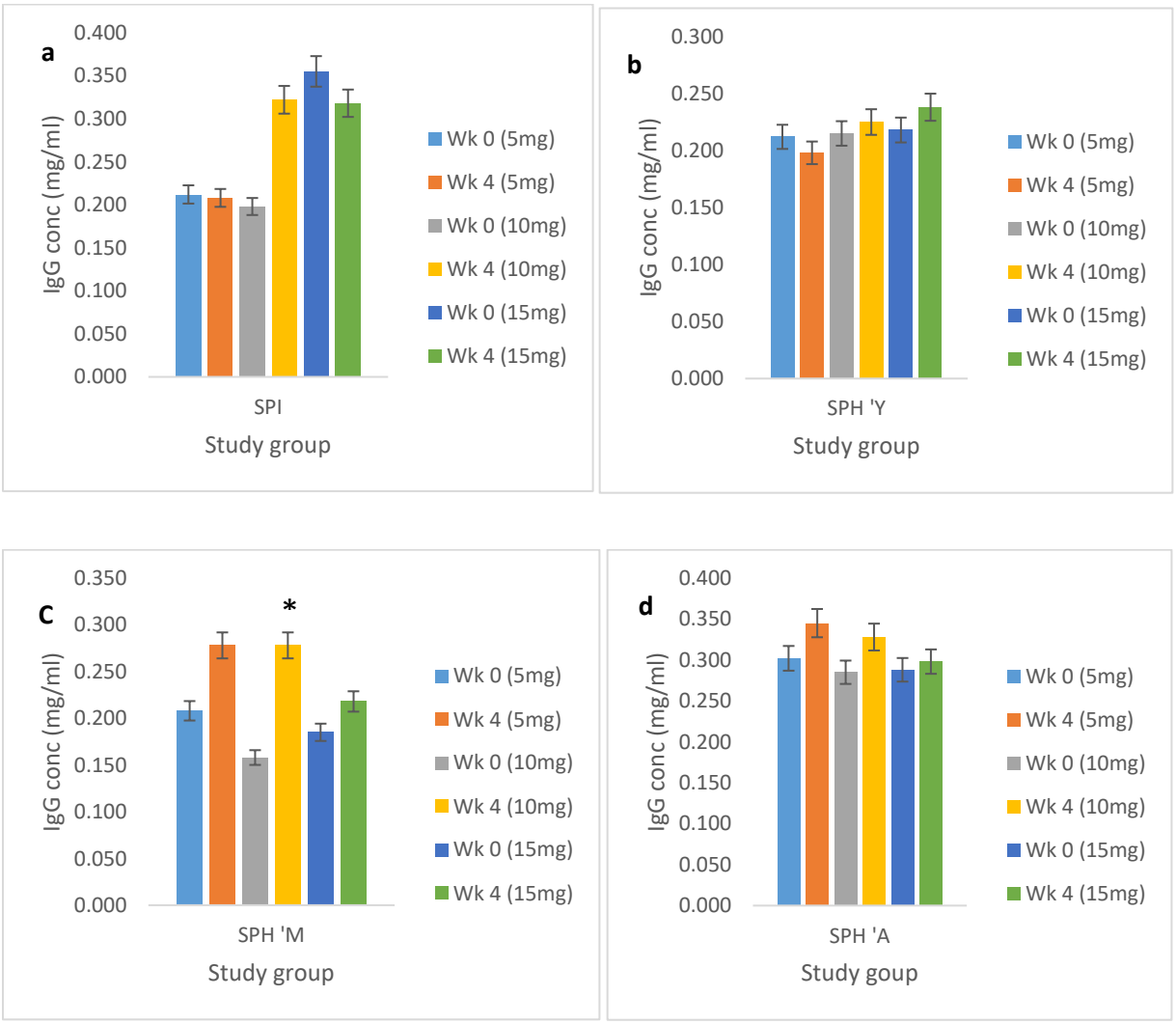

Figure 3. Graphs show IgG concentrations across study groups fed with varying SPH and SPI doses, before and after 4 weeks.

SPH 'Y= SPH-Young, SPH 'M=SPH-Mature, SPH 'A=SPH-Aged, SPI=Soy protein isolate. Values plotted are mean ± SD (n=5). *When compared to the SPI group, only the IgG values in the SPH 'M group were significantly different ($p<0.05$, 0.01) at week 0

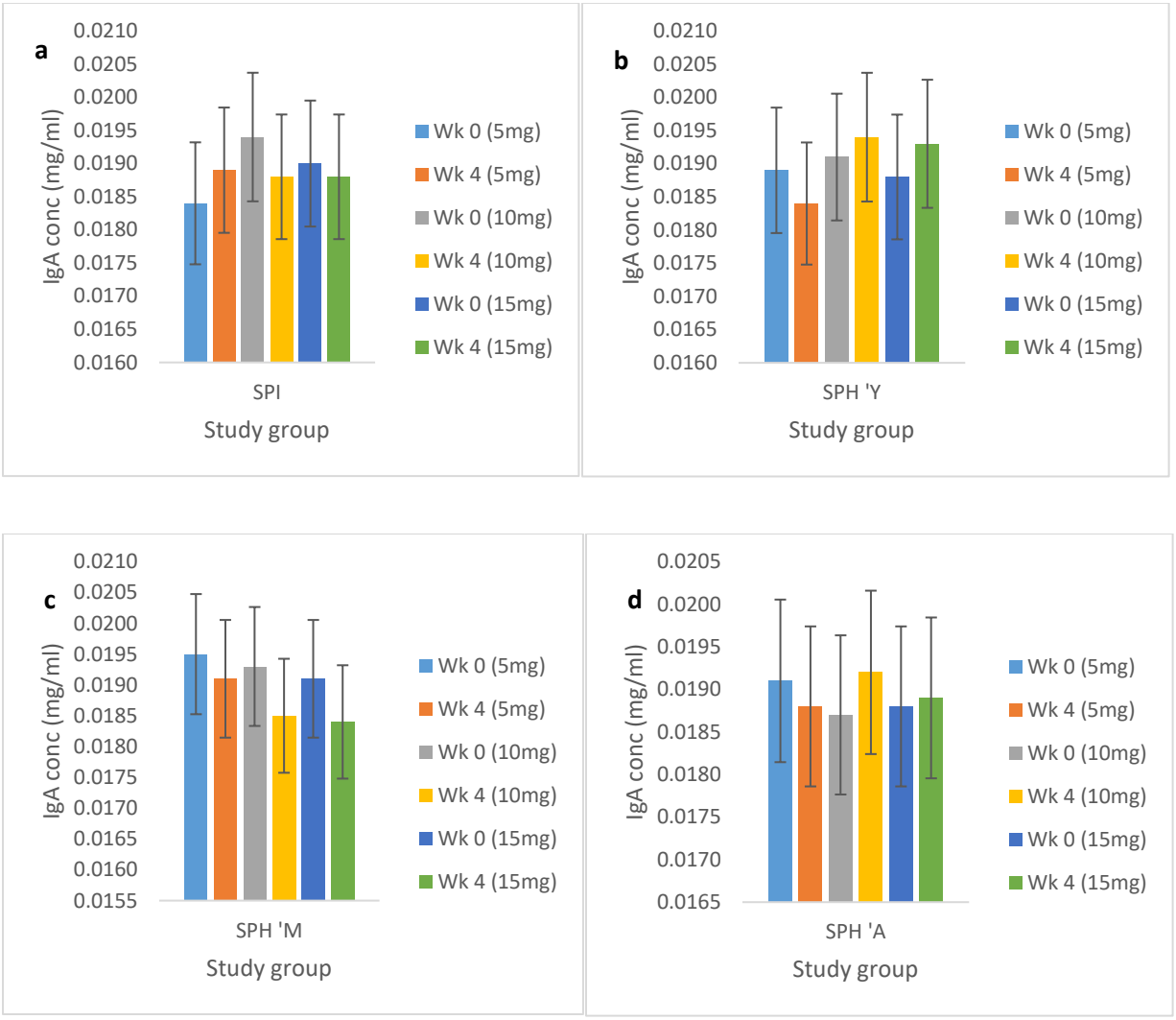

Figure 4. Graphs show IgA concentrations across study groups fed with varying SPH and SPI doses, before and after 4 weeks.

SPH 'Y= SPH-Young, SPH 'M=SPH-Mature, SPH 'A=SPH-Aged, SPI=Soy protein isolate. Values plotted are mean ± SD (n=5). There were no significant difference in all groups ($p<0.05$, 0.01) compared to the SPI group

CONCLUSION

SPH prepared with pepsin had a markedly immunomodulatory impact by stimulating the proliferation of murine spleen cells and exhibiting phagocytic activity on murine peritoneal macrophages. Additionally, the overall levels of sera IgG and IgA concentrations recorded in this study after feeding SPH to the rat subjects confirmed the immune-boosting potential of SPH. Moreover, in the 14-day acute toxicity study in mice, no mortality or signs of toxicity attributable to the administration of the hydrolysate were observed, suggesting the safety of SPH in preclinical trials. Therefore, pepsin-educed SPH may be employed in the development of immunomodulatory or functional food products.

List of Abbreviations: SPI, Soy Protein Isolate; SPH, Soy Protein Hydrolysate; FBS, Fetal Bovine Serum; PBS, Phosphate-buffered saline; MTT, 3-(3,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide.

Competing Interests: The authors hereby declare no competing interests.

Authors' Contributions: Tolulope Joshua Ashaolu, Ph.D. researcher is the main investigator for this study, contributed fundamental conceptualization for the research, wrote the manuscript as well as performed all the experiments. Niracha Yanyiam is an expert on animal welfare, and assisted with the animal studies. Chutha Takahashi Yupanqui, Ph.D., is an expert on cell culture techniques, and she was the supervisor for the study. She provided oversight as well as revision of the written manuscript.

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