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Suppressive activity of enzymatically-educed soy protein hydrolysates on degranulation in IgE-antigen complex-stimulated RBL-2H3 cells

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ABSTRACT

Background: Soy protein isolate (SPI) is increasingly used in foods because it is a high quality non-dairy protein with excellent functional properties. However, soy allergy is one of the world's major eight food allergies.

Objective: To investigate the anti-allergic activity of soy protein hydrolysates (SPHs) produced with alcalase and pepsin proteases.

Methods: SPI was enzymatically hydrolysed using the proteases, while evaluating the reaction conditions which include E/S (enzyme to substrate ratio) of 0.5%, 1.0% and 1.5% (250 u/mg and 5 u/g of pepsin and alcalase respectively); and hydrolysis time (0 min, 30 min, 1h, 2h, 4h and 8h). Afterwards, rat basophilic leukaemia (RBL)-2H3 cells activated by the IgE-antigen complex were used to assess mast cell degranulation inhibitory activity of the SPHs by the release of *β*-hexosaminidase. RBL-2H3 cells were sensitized with monoclonal anti-dinitrophenol (DNP) specific IgE and challenged with the antigen DNP-bovine serum albumin in the presence or absence of SPHs.

Results: It was observed that 0.1 mg/mL concentration of the 0.5% E/S SPHs prepared in the first 4h significantly ($P < 0.05$) inhibited *β*-hexosaminidase release in an IgE-antigen complex-stimulated RBL-2H3 cells compared to those produced at other time intervals, E/S, and concentrations.

Conclusion: This is the first report of its kind that shows the ability of SPHs to suppress degranulation of RBL-2H3 cells. Consequently, SPHs have good prospects to be used as potential sources of low cost hypo or anti-allergic protein.

Keywords: Soy Protein Isolate, Soy Protein Hydrolysates, RBL-2H3 Cells, *β*-Hexosaminidase, Anti-allergy

INTRODUCTION

It is estimated that in the industrialized world every one in three people have allergies [1]. A food allergy is an immunological response to food, whether IgE-mediated or not. The mechanisms involved in allergy includes the exposure of linear protein to Th2 cells by the antigen-presenting cells (APC), thereby stimulating B cells to release antigen-specific IgE to bind the target antigen surface. Afterwards, mast cells bind to the receptors of IgEs produce and become degranulated in order to release histamines and other mediators such as cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), leukotrienes, heparin, and many proteases into the environment [2]. These chemical mediators cause the characteristic symptoms of allergies. Due to the entrapment of *β*-hexosaminidase in the granules, its release is used as a suitable marker for the degree of degranulation [3]. Food allergens are generally proteins mediated by IgE. Based on the primary structure of proteins, which may be linear or sequential, the portion of the protein recognized by IgE is called the epitope [4]. IgEs, which bind to epitopes that have sequential amino acids, are associated with persistence of allergy compared to epitopes dependent on folding conformations [5]. However, the biological properties of proteins are significantly dependent on the three dimensional (3D) or tertiary structure, which can be disrupted by certain food processing methods such as chemical hydrolysis or thermal treatments. Enzymatic hydrolysis of food proteins is preferred in order to avoid the decimation of certain amino acids like cysteine, tryptophan, and serine, which may cause subsequent loss of biological activity [6].

Soy protein isolate (SPI) is increasingly used in foods as functional and nutritional ingredients and as a substitute for animal-derived proteins from milk, meat, and eggs because it is a high quality non-diary protein with excellent functional properties. It is a vegetable protein which can be employed in food products due to its good water and fat holding capacity. However, soy allergy is one of the major food allergies posing global concerns. The Food Allergy Research and Education institute [7] has reported that soy allergy is one of the eight most common food allergies worldwide of which children are mostly affected. 0.4% of children are allergic to the soy protein used in their nutrition formula [8], with many vegetarians considering soy as their alternative to meat. At least 16 IgE-binding soy proteins with molecular masses ranging from 7.5 to 97 kDa have been characterized as being involved in clinical allergy with storage proteins Gly m5 (b-conglycinin) and Gly m6 (glycinin) identified as the major allergen-containing components [9, 10]. Therefore, protein structural modification techniques that can help eliminate soy protein allergenicity are of paramount importance [11]. These techniques often involve acidic, alkaline, and enzymatic treatments among others. Enzymatic hydrolysis of SPI involves proteolytic enzymes such as pomiferin, cucurbita, pepsin, papain, chymotrypsin [12], novozym, flavourzyme [13], alcalase, neutrase, and corolase [14]. These enzymes have been used synergistically to attain limited or extended degrees of hydrolysis. Alcalase has become predominant in several reports due to its higher degree of hydrolysis values [15-17] and assessment of the digestibility of proteins. Pepsin has either been rarely or singly used in all the previous reports, and is among some of the most important digestive enzymes found in mammals including humans, thereby making it an important enzyme for studying the gastro-intestinal tract digestion process. Enzymatic hydrolysis of proteins results in smaller molecular mass with less secondary structure and in some cases

improved functional properties compared to the intact protein [18]. Moreover, the hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective due to the increase of solubility [19].

As a result, soy protein hydrolysates (SPHs) have been reported to exhibit various physiological activities which include hypolipidaemic and hypocholesterolemic properties [20], antioxidant activity [21], reduction of blood pressure [22], improvement in both arterial compliance and endothelial function [23], insulin resistance, and weight loss in obesity [24]. SPHs have also been used as clinical products, such as infant formulas and hospital diets for patients in addition to food substitutes [25]. However, the anti-allergic activity of soy protein hydrolysates prepared with one or several proteases, and evaluated with RBL-2H3 cells have not been investigated systematically until now. In this study, we were prompted to investigate the anti-allergic activity of alcalase and pepsin-educed SPHs, taking into consideration different enzyme to substrate ratio concentrations (E/S) of 0.5%, 1.0%, and 1.5%; and hydrolysis time intervals of 0 min, 30 min, 1 h, 2h, 4h, and 8h respectively. SPI was enzymatically hydrolysed using commercially available proteases, alcalase, and pepsin, while evaluating these reaction conditions. Thereafter, mast cell degranulation inhibitory activity of the SPHs by the release of β-hexosaminidase from RBL-2H3 cells was investigated at equal concentrations of 0.1 mg/mL, 1.0 mg/mL, and 10 mg/mL in order to ascertain the effective doses of the prepared SPHs. This is the first report of its kind that shows the ability of SPHs to suppress degranulation of RBL-2H3 cells, and thereby has a high prospect to be used as a potential source of low cost hypo or anti-allergic protein.

MATERIALS AND METHODS

Materials

Food grade SPI (protein content 90.2% dry base) was purchased from Wachsen Industry Company, Ltd (Qingdao, China). Alcalase (5 U/g) and pepsin (250 U/mg) respectively were purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals used were of the highest analytical grade commercially available.

Enzymatic hydrolysis

Hydrolysis with alcalase and pepsin was performed at optimum pH and temperature according to previous literature reviews [15, 26]. See Table 1.

Table 1. Parameters used for the enzymatic hydrolysis of soy protein isolate

Parameters	**Conditions**	**Conditions**
Enzymes	Alcalase	Pepsin
Enzyme concentration	0.5, 1.0 and 1.5%	0.5, 1.0 and 1.5%
Reaction time	0.5, 1, 2, 4, 8 hours	0.5, 1, 2, 4, 8 hours
pH	8.0	2.0
Temperature	50 ^{o}C	37 ^{o}C

Preparation of soy protein hydrolysates

SPI was dissolved in deionized water in the ratio of 1:8 (w/v) for both alcalase-treated and pepsin-treated samples. Hydrolysis of SPI with alcalase and pepsin was achieved at 50 ^{o}C and 37 ^{o}C, using 2M NaOH and 2M HCl at the pH of 8.0 and 2.0 respectively. Enzyme-substrate ratios used were

0.5%, 1.0%, and 1.5%/100 g substrate. The SPI was pre-incubated for 15 min at respective optimum enzymatic temperatures prior to hydrolysis process. The hydrolysis time was set up to 8h. The hydrolysate samples were taken at 0 min, 30 min, 1h, 2h, 4h, and 8h intervals, for future analysis. Each enzyme was inactivated using heat treatment at 95 °C for 15 min in a thermostat-controlled water bath. Afterwards, the samples were 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 hydrolysates) was lyophilized and stored at -20 ^{o}C. Fig. 1 shows the production flow chart.

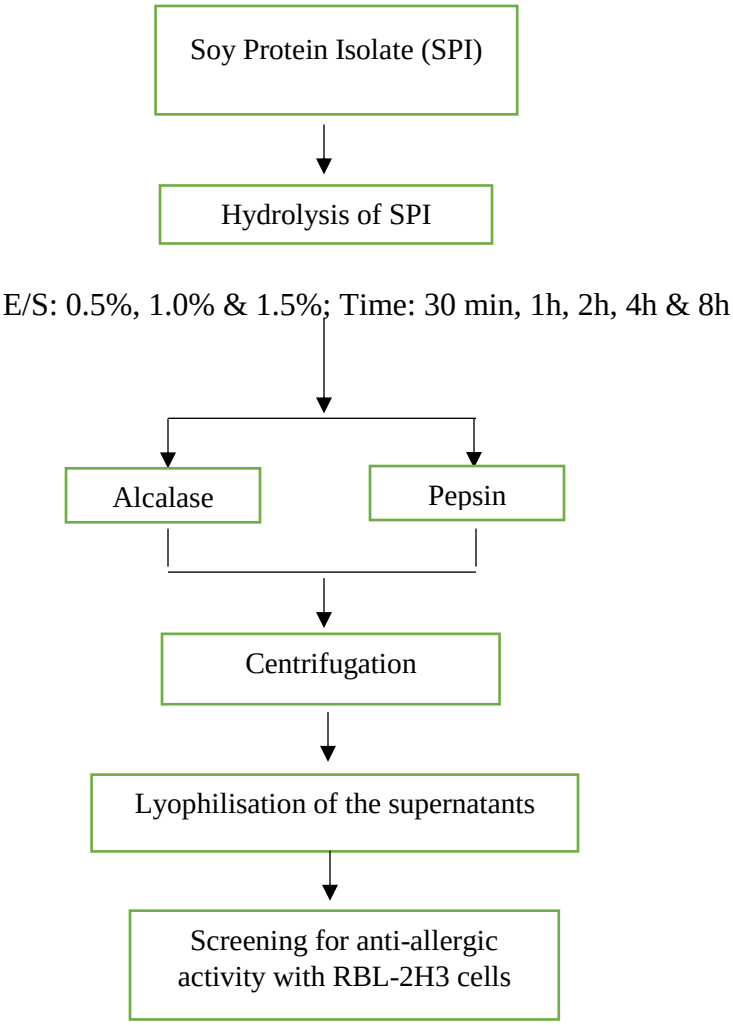

Figure 1. Flow chart showing the production of soy protein hydrolysates using alcalase and pepsin

Degree of hydrolysis (DH)

The DH of hydrolysates samples obtained was analyzed based on the availability of free amino group upon hydrolysis, with OPA (*o*-phthaldialdehyde) reagent. The degree of hydrolysis was carried out according to the method of Nielsen and their colleagues [27]. 400 µL of SPHs was added to 3 mL of freshly prepared OPA solution. The solution was immediately mixed for 5 seconds and incubated for 2 min at room temperature. The absorbance was measured at 340 nm using UV-vis spectrophotometer (Genesys 10 series., Madison, WI, USA). The amount of free amino acid groups in the SPHs was calculated as serine -NH_2 using L-serine as a standard. The total number of α-amino nitrogen in the sample was determined by acid hydrolysis (complete hydrolysis) of sample in 6 N HCl, at 110 ^{o}C for 24 hr. The percentage degree of hydrolysis (% DH) was calculated using the following equation:

$$\% \text{ DH} = \frac{([NH_2]_{Tx}-[NH_2]_{T0}) \times 100}{([NH_2]_{Total}-[NH_2]_{T0}}$$

Where:

$[NH_2]_{T0}$ = amount of free -NH_2 groups at 0 min of hydrolysis (mg/mL)

$[NH_2]_{Tx}$ = amount of free -NH_2 groups in the supernatant after × min of point protease hydrolysis for each experiment (mg/mL)

$[NH_2]_{Total}$ = amount of -NH_2 groups resulting from acid hydrolysis after 24 h (mg/mL)

Protein recovery of SPHs

Protein recovery protocol of the Kjeldahl method [28] was implemented, and was calculated as the amount of protein (%N × 6.25) present in the hydrolysates relative to the initial amount of protein present in the reaction mixture [15].

β-hexosaminidase release assay

Modified method of Matsuda and colleagues [29] was used. Anti-DNP IgE, DNP-BSA and *ρ*-nitrophenyl N-acetyl-*β*-D-glucosaminide (PNAG) were purchased from Sigma (USA). The rat basophilic leukemia RBL-2H3 cells were purchased from American Type Culture Collection (ATCC CRL-2256, VA, USA). The cells were cultured in Eagle's minimum essential medium (E-MEM) supplemented with 15% (v/v) fetal bovine serum (FBS) and 1.25% Pen-Strep (a mixture of 10,000 U/mL penicillin and 10,000 µg/mL streptomycin; purchased from Gibco Co. (Grand Island, NY, USA) at 37 ºC in a humidified, CO_2-controlled (5%) incubator. Briefly, RBL-2H3 cells was cultured in 24-well plates at a concentration of 2×10^5 cells/well using Eagle's Minimal Essential Medium containing FBS (15%), penicillin (100 units/mL), streptomycin (100 units/mL), and 40 µL anti-DNP IgE (50 µg/mL). The plate was incubated overnight at 37 ºC in 5% CO_2 for sensitization of the cells. The cells were washed twice with 200 µL of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N, N'*-bis (2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albulmin (BSA), and 40 mM NaOH, pH 7.2] and then incubated in 160 µL of Siraganian buffer for an additional 10 min at 37 ºC. Afterwards, 20 µL of 0.1 mg/mL, 1.0 mg/mL, and 10 mg/mL of the test sample (SPHs solutions prepared with PBS buffer) were added to each well and incubated for 20 min, followed by the addition of 20 µL of antigen (DNP-BSA, final concentration was 10 µg/mL) at 37 ºC for 20 min. This step stimulates the cells to degranulate. The supernatant was transferred into 96-well plate and incubated with 50 µL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 ºC for 2h. The reaction was stopped by adding 200 µL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm within 30 min. A similar experiment was also set up for Ketotifen fumarate, as the standard. The inhibition of *β*-hexosaminidase release (%) by both the test and standard samples was calculated by the following equation:

$$\% \text{ Inhibition} = [1- (T-B) / (C-B)] \times 100.$$

Where:

Control (C) = DNP-BSA (+), Test sample (-)
Test (T) = DNP-BSA (+), Test sample (+)
Blank (B) = DNP-BSA (-), Test sample (+)
Normal (N) = DNP-BSA (-), Test sample (-)

Cell proliferation assay

The activity of SPHs on cell survival was evaluated by 3-(3,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. RBL-2H3 cells (5 × 105/well) were cultured in 24-well plates. After growth in E-MEM containing 15% FBS at 37 ºC and 5% CO2 overnight, 0.1 mg/ml, 1.0 mg/ml, and 10 mg/ml concentrations of the test samples (SPHs) were added to the plates, and incubated for 4h with 200 µL MTT (5 mg/mL). After incubation, the supernatant was removed and the resultant precipitate was dissolved with 200 µL DMSO. The absorbance was measured at 570 nm. The cell viability (%) was calculated by the following equation:

$$\% \text{ Viability} = 100 - [(C\text{-}T/C) \times 100]$$

Where:

Control (C) = Control; DNP-BSA (+)
Test (T) = Test; DNP-BSA (+), Test sample (+)

Amino acid profile of SPHs

Soy protein hydrolysates with the most potent anti-allergic activity was sent to Central laboratory Thai (Songkla, Thailand) for amino acid composition analysis. The amino acid profile was determined by hydrolyzing 1 mg of the SPH sample with 6 mol/L HCl at 110 oC for 20 h under vacuum. Afterwards, the hydrolysate was evaporated to dryness under vacuum. The dried sample was dissolved in 200 µL of borax buffer and centrifuged. 100 µL of the resultant supernatant was loaded onto an Agilent 1100 LC/MSD Model G1946D with Phenomenex (C18) 250 × 2 mm column (Agilent Technologies, Palo Alto, CA, USA) at 40 oC with o-phthalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate pre-column derivatization and diode array detector.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD), being the average of three samples per experiment. Analyses were performed using SPSS software version 17.0. The experiment was repeated at least thrice to confirm the results. The level of statistical significance, using analysis of variance (ANOVA) with Tukey's test, was set to $p < 0.05$.

RESULTS AND DISCUSSION

Enzymatic hydrolysis of SPI

Protein hydrolysates are meant to provide nutritional benefits to different classes of consumers and patients in contrast to crude proteins. The production of extensive protein hydrolysates by varying proportions of the enzyme in use is invariably an effective way to obtain protein hydrolysates with defined characteristics. DH is a measure of the extent of hydrolytic degradation of a protein, and is the most widely used indicator for comparison among different proteolysis process [15]. Fig. 2 (a) shows the hydrolysis curves obtained by hydrolysing SPI with alcalase under hydrolysis conditions of E/S = 0.5, 1.0, and 1.5%; pH 8.0, T = 50 oC and t = 0 min, 30 min, 1h, 2h, 4h, and 8h.

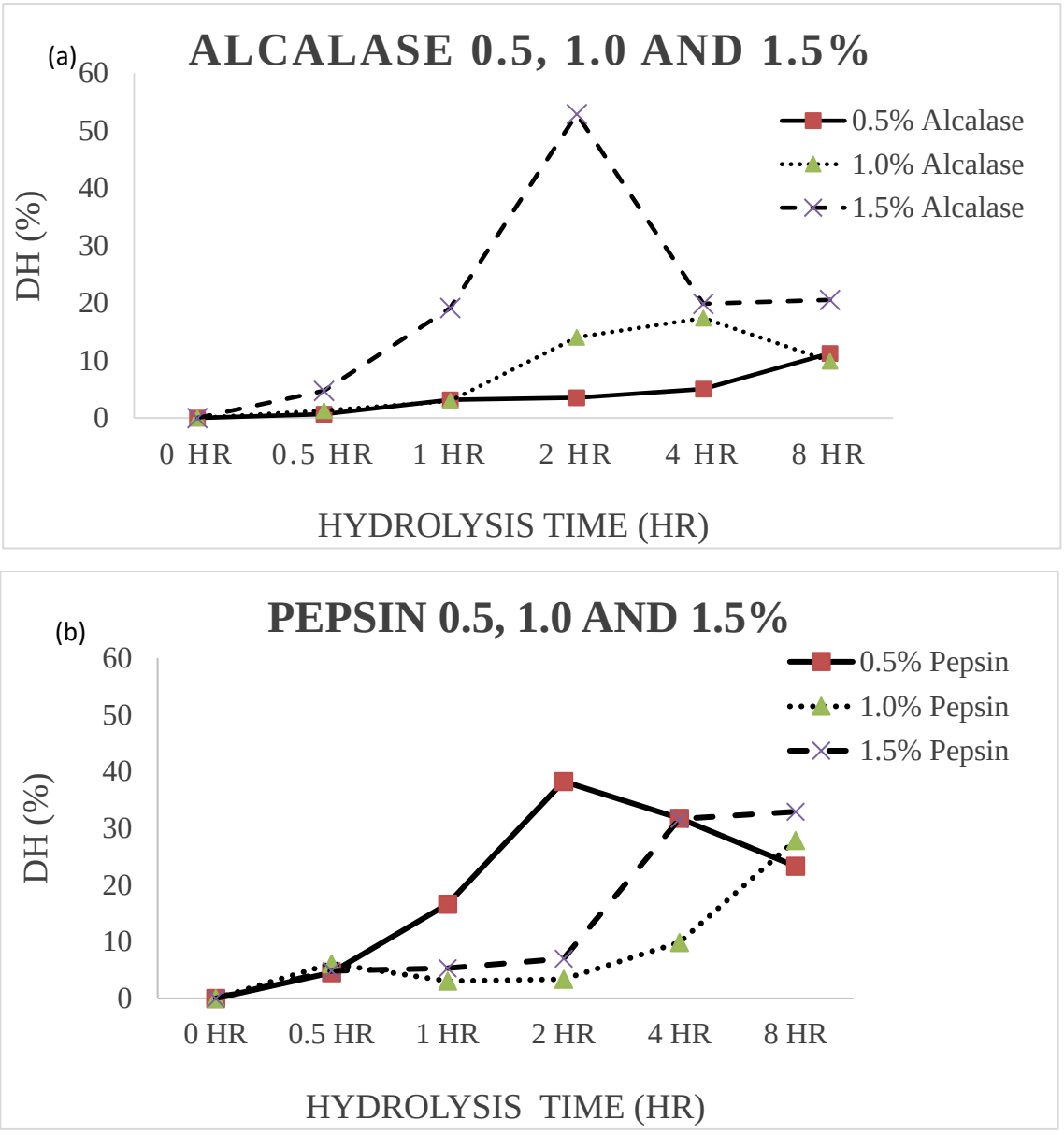

Figure 2. Enzymatic hydrolysis of soy protein isolate with:
(a) Alcalase at 50 ^{o}C, pH 8.0 and time intervals of 0 - 8 h.
(b) Pepsin at 37 ^{o}C, pH 2.0 and time intervals of 0 - 8 h.

All hydrolytic curves showed an increasing rate of hydrolysis, with 1.5% E/S attaining the highest rate in the first 2h (52.87%), but the proteolysis rate decreased gradually over time. Adler-Nissen [30] attributed the reduction in hydrolysis rate to the competition between unhydrolysed protein and the peptides being constantly formed during hydrolysis. Xiang *et al* [15] experienced a vivid change after 180 min (3h) of hydrolysis of SPI with alcalase, which also gave the highest DH value of 18.36% at the concentration of 2.5% E/S. The highest concentration in this experiment obtained at 1.5% E/S (52.87%) did not confer any substantial anti-allergic activity but the lowest concentration (0.5%) did. Alcalase has been used not only for the production of protein hydrolysates with better functional and nutritional characteristics but also for the generation of bioactive peptides with ACE inhibitory activity [31].

The highest degree of hydrolysis of soy protein hydrolysates so far recorded until date is 39.5% [13], while most other scientists detected a rate between 1-25% [32]. However, in Fig.2 (b), the degree of hydrolysis obtained from pepsin was much higher (38.24%) at a lower protease

concentration of 0.5%, compared to DH (3.54%) attained by alcalase and at same time (2h). This suggests that enzyme type affects the degree of protein hydrolysis in addition to the functionality/activity of such hydrolysate as further confirmed with the cell line (RBL-2H3) anti-allergy test. Moreover, other SPHs treated with pepsin concentrations of 1.0% and 1.5% revealed the possibility of increasing yield of hydrolysis after the first 2h.

Protein recovery of SPHs

Higher DH indicates more hydrolysed soy protein could be obtained. The yields of protein hydrolysates with different concentrations of alcalase and pepsin are presented in Table 2. Although many factors can affect the yield of hydrolysis, the concentration and type of enzyme used contributed significantly to the yield and anti-allergic property of the final product. High protein recovery by alcalase-treated and pepsin-treated SPHs (87.70% and 89.30% respectively) at 0.5% E/S makes prospective commercial applications cost effective.

Protein recovery was measured as the amount of protein (%N × 6.25) present in the hydrolysates relative to the initial amount of protein present in the reaction mixture. Data are reported as the means ± SD, n=3

Table 2. Protein recovery from SPHs subjected to different hydrolysis conditions

Enzyme concentration (%)	**Time (Hour)**	**Protein recovery**	
		(%)-Alcalase	**(%)-Pepsin**
0.5	0	73.71±0.002	71.75±0.011
	0.5	75.60±0.001	82.64±0.019
	1	85.50±0.003	88.62±0.007
	2	86.37±0.001	60.68±0.006
	4	87.70±0.013	86.66±0.002
	8	86.02±0.001	89.30±0.021
1.0	0	76.95±0.029	73.51±0.002
	0.5	85.90±0.007	83.69±0.000
	1	85.79±0.011	85.97±0.004
	2	85.18±0.001	87.19±0.003
	4	85.81±0.004	84.92±0.004
	8	86.76±0.015	84.93±0.004
1.5	0	76.95±0.029	73.89±0.003
	0.5	85.90±0.007	84.22±0.003
	1	85.79±0.011	84.22±0.001
	2	85.18±0.001	85.79±0.003
	4	85.81±0.004	86.67±0.007
	8	86.76±0.015	85.79±0.004

Ability of SPHs to inhibit β-hexosaminidase release by IgE-antigen complex-stimulated RBL-2H3 cells and their proliferation

To the best of our knowledge, anti-allergic activity of soy protein hydrolysates using RBL-2H3 cells has not been reported yet in previous studies despite its excellent functional properties and uses in food and meat industries. In order to investigate the ability of SPHs to inhibit degranulation, RBL-2H3 cells were primed with anti-DNP IgE, treated with SPHs, stimulated with DNP-BSA, and afterwards measured the release of *β*-hexosaminidase. As shown in Table 3-4, stimulation with the IgE-antigen complex induced the release of *β*-hexosaminidase, which was significantly inhibited (39.22%, $P<0.05$) by alcalase-educed SPHs at the conditions of 0.5% E/S, 4h of hydrolysis using 0.1 mg/mL concentration as compared to other conditions. Other concentrations of E/S as well as the time differences did not yield any significant inhibitory results. Interestingly, SPHs prepared with pepsin showed more promise, as there was a much stronger inhibitory effect (72.51%) at the same conditions (0.5% E/S, 4h of hydrolysis using 0.1 mg/mL concentration) compared to SPHs treated with alcalase (39.22%). Other E/S concentrations of pepsin-educed SPHs as well as the time differences also yielded more significant inhibitory results than SPHs prepared with alcalase.

Rat basophilic leukaemia RBL-2H3 cells are mucosal mast cells that have been used more often in the study of anti-allergic activity of diverse functional food products or medicines. When RBL-2H3 cells are primed with anti-DNP IgE, stimulation with an antigen such as DNP-BSA induces them to rapidly degranulate and produce cytokines, including histamines [33]. *β*-hexosaminidase is stored in the secretory granules of RBL-2H3 cells and is released simultaneously with histamine when the cells are immunologically activated, it is therefore generally accepted that *β*-hexosaminidase is a degranulation marker of mast cells and can reflect changes in other inflammatory factors [34]. RBL-2H3 cells were exposed to various enzyme concentrations of alcalase-educed SPHs ranging from 0.5% to 1.5% E/S for 4h. As shown in Table 3-4, RBL-2H3 cell viability was not affected by the 0.1 mg/mL concentration of SPHs obtained from different time and E/S intervals, except for a slight cytotoxic effect demonstrated by increasing E/S concentrations in pepsin-educed hydrolysates. However, if cell survival is more than 80%, it is considered non-cytotoxic [35, 36]. The results obtained also support the hypothesis that hydrolysed proteins and peptides demonstrate better bioactive activity than their parent proteins [37]. Some reports have already been made on certain biological activities such as antioxidant, anti-thrombotic, angiotensin-converting enzyme (ACE) inhibition, antihypertensive, and immunomodulatory properties for soy peptides [24, 38-39]. Xiang *et al* [15] also evaluated immunoregulatory properties of SPHs prepared with alcalase and insoluble soy protein, only to find out that apart from being more efficacious than other enzymes, they had the highest immunomodulatory activity on proliferation of murine splenic lymphocytes and phagocytic effect of peritoneal macrophages. However, in this research, pepsin has been employed in the production of SPHs without any interference from other enzymes and has been discovered to exhibit anti-allergic activity.

Table 3. Inhibition rate of *β*-hexosaminidase release by SPHs obtained from different hydrolysis conditions of alcalase with regard to E/S and time

	0.001 mg/ml		0.01 mg/ml		0.1 mg/ml SPH		1.0 mg/ml SPH		10.0 mg/ml SPH	
E/S & Time Conditions	**% Inh**	**% prolif**	**% Inh**	**% prolif**	**% Inh**	**% prolif**	**% Inh**	**% prolif**	**% Inh**	**% prolif**
0.5% Alc										
0 min					8.67±0.357	100.01±0.068	-	97.93±0.017	-	96.06±0.022
30 min					-	100.64±0.069	-	90.69±0.006	-	91.61±0.020
1 h					-	101.92±0.072	-	92.02±0.006	-	104.42±0.005
2 h					-	104.24±0.073	-	92.70±0.014	-	90.07±0.022
4 h					39.22±0.207	92.07±0.053	33.22±0.014*	102.96±0.006	-	102.99±0.031
8 h					-	103.65±0.069	-	97.08±0.006	-	95.49±0.017
K. fumarate	53.77±0.007*	87.48±0.007	58.92±0.002*	87.67±0.008	108.22±0.004*	89.51±0.003				
1.0% Alc										
0 min					-	106.53±0.069	-	99.45±0.006	-	103.93±0.009
30 min					-	99.89±0.070	-	94.58±0.014	-	93.49±0.034
1 h					25.38±0.228	97.97±0.058	22.29±0.014*	93.80±0.014	-	96.48±0.039
2 h					-	96.41±0.057	-	89.76±0.008	-	91.56±0.016
4 h					-	101.72±0.070	-	91.84±0.004	-	99.42±0.003
8 h					-	99.09±0.074	-	96.41±0.012	-	79.26±0.006
1.5% Alc										
0 min					-	101.45±0.071	-	97.84±0.017	-	108.07±0.026
30 min					-	100.37±0.074	-	101.87±0.004	-	104.42±0.020
1 h					-	98.98±0.090	-	98.63±0.016	-	108.04±0.037
2 h					-	99.88±0.071	-	96.84±0.010	-	96.90±0.024
4 h					-	97.91±0.067	-	97.33±0.014	-	99.52±0.013
8 h					-	92.50±0.078	-	93.27±0.020	-	89.98±0.018

Release inhibition rate is expressed as a percentage of sample treated cells vs. PBS-treated control cells (considered as 100% complete release). Data are reported as the means ± SD, n=4, * Samples with a significant difference ($p < 0.05$ & 0.01 for the SPH and K. fumarate samples respectively) compared to the control. Remark: % Inh = Inhibition rate of *β*-hexosaminidase release (%); % prolif = Cell Proliferation (%); K. fumarate = Ketotifen fumarate (Standard

Table 4. Inhibition rate of β-hexosaminidase release by SPHs obtained from different hydrolysis conditions of pepsin with regard to E/S and time

	0.001 mg/ml		0.01 mg/ml		0.1 mg/ml SPH		1.0 mg/ml SPH		10.0 mg/ml SPH	
E/S & Time Conditions	**% Inh**	**% Prolif**	**% Inh**	**% Prolif**	**% Inh**	**% prolif**	**% Inh**	**% prolif**	**% Inh**	**% prolif**
0.5% Pepsin										
0 min					36.55±0.014*	94.14±0.075	-	101.00±0.023	-	86.24±0.009
30 min					59.06 ±0.012*	99.56±0.075	66.37±0.007*	94.43±0.024	-	86.09±0.012
1 h					-	96.34±0.077	69.30±0.016*	92.64±0.017	-	81.17±0.019
2 h					-	102.75±0.094	-	90.12±0.010	-	90.28±0.028
4 h					72.51±0.012*	95.95±0.065	-	98.83±0.021	25.53±0.012*	95.94±0.017
8 h					65.21±0.018*	104.70±0.071	45.03±0.019*	97.37±0.026	3.60±0.019	91.13±0.022
K. fumarate	53.77±0.007*	87.48±0.007	58.92±0.002*	87.67±0.008	108.22±0.004*	89.51±0.003				
1.0% Pepsin										
0 min					-	79.27±0.058	-	99.96±0.005	-	79.32±0.022
30 min					-	99.47±0.086	-	85.50±0.005	-	79.39±0.011
1 h					-	94.60±0.068	-	84.35±0.005	-	88.45±0.021
2 h					54.39±0.019*	95.64±0.073	-	88.45±0.001	-	86.93±0.014
4 h					-	99.28±0.076	-	83.31±0.009	-	88.02±0.017
8 h					-	95.12±0.092	20.8±0.026*	81.53±0.005	-	84.72±0.012
1.5% Pepsin										
0 min					-	103.29±0.071	-	83.12±0.074	-	99.52±0.014
30 min					-	94.08±0.069	-	98.98±0.017	-	98.73±0.014
1 h					-	97.59±0.069	72.47±0.007*	94.05±0.025	2.12±0.021	100.14±0.028
2 h					-	108.25±0.067	60.23±0.010*	87.72±0.038	-	93.05±0.012
4 h					-	102.33±0.067	-	88.22±0.007	-	88.16±0.016
8 h					-	83.70±0.052	28.36±0.024*	76.71±0.023	-	86.77±0.004

Release inhibition rate is expressed as a percentage of sample treated cells vs. PBS-treated control cells (considered as 100% complete release). Data are reported as the means ± SD, n=4, * Samples with a significant difference ($p < 0.05$ & 0.01 for the SPH and K. fumarate samples respectively) compared to the control. Remark: % Inh = Inhibition rate of β-hexosaminidase release (%); % prolif = Cell Proliferation (%); K. fumarate = Ketotifen fumarate (Standard)

Amino acid composition of SPHs prepared with pepsin

Gleanings from aforementioned experiments led to the analysis of the amino acid compositions of SPH prepared with pepsin, which was then compared to SPI (shown in Table 5). The quantitative differences in amino acid compositions between them can be attributed to the hydrolytic process involved in the preparation of SPH that might have contributed to the disparity. Certain chemical reactions, including protein denaturation and mallard reaction, may lead to loss of amino acids. Additionally, an increase in applied energy and decrease in water (such as freeze-drying) caused a significant reduction of arginine (38.32%) in this experiment. Whereas percentage losses of lysine, arginine, histidine, cysteine, methionine and tryptophan in an extrusion process had been previously reported to be 30-40%, 7-21%, 15%, 8-21%, 14%, and 14% respectively [40], and established lysine to be the most sensitive amino acid to damage during processing and storage [41]. However, there is an increase in Ala, Asp, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, and Gln compositions, with the essential amino acids (EAAs) being entirely intact in the hydrolysed soy protein prepared with pepsin. It has been reported that plant proteins do not contain high amounts of EAAs compared to animal proteins [42], thereby making the result obtained in this experiment conceivable. Finally, this study relates harmoniously with the report of Fleddermann *et al* [43] who studied some cereal proteins, including SPI and reported the similarity of their bioavailability in human nutrition based on the requirement of FAO/WHO/UNO for preschool children (1-2 y) [44], and further maintained that soy protein exhibited true digestibility, especially when hydrolysed into smaller peptides.

Table 5. Comparison of AAs from pepsin-educed SPH with SPI

Amino acid composition	**SPI (%)**	**SPH (%)**	**Essential Amino Acids**	**Non-Essential Amino Acids**
Alanine	3.02	5.54	Histidine	Alanine
Arginine	48.08	9.76	Isoleucine	Arginine*
Aspartic acid	4.51	6.56	Leucine	Aspartic acid
Cystine	0.59	0.31	Lysine	Cystine*
Glutamic acid	3.64	7.22	Methionine	Cysteine*
Glycine	2.38	4.56	Phenylalanine	Glutamic acid
Histidine	1.42	2.15	Threonine	Glutamine*
Hydroxylysine	0.00	0.00	Tryptophan	Glycine*
Hydroxyproline	0.05	0.15	Valine	Proline*
Isoleucine	4.15	6.32		Serine*
Leucine	4.24	8.88		Tyrosine*
Lysine	3.90	8.65		Asparagine*
Methionine	2.43	2.99		Hydroxylysine
Phenylalanine	3.24	4.95		Hydroxyproline
Proline	3.72	7.41		
Serine	3.25	5.70		
Threonine	2.95	3.76		
Tryptophan	0.74	0.80		
Tyrosine	2.31	2.74		
Valine	2.30	3.55		
Asparagine	0.00	0.00		
Cysteine	0.18	0.00		
Glutamine	2.89	8.00		
TOTAL	100	100		

SPI = soy protein isolate; SPH = soy protein hydrolysate;

*conditionally essential (not usually required in the human diet, but do become essential under certain circumstances such as illness and stress)

CONCLUSION

Observations on the effects of SPHs on RBL-2H3 cell degranulation prompted our hypothesis that soy protein isolate hydrolysed with alcalase, having the conditions of 0.5% E/S and hydrolysis time of 4h at 0.1 mg/mL concentration, may reduce allergic symptoms. In the same vein, if hydrolysed with pepsin and subjected to the conditions of 0.5% E/S and hydrolysis time of 4h and at 0.1 mg/mL concentration, similar results can be attained. Furthermore, the most potent SPH sample obtained in this study yielded essential amino acids (EAAs) suitable for quality human nutrition. Additionally, these results indicated that soy protein hydrolysates could be used as potential sources of low cost hypo or anti-allergic protein (with 86.66% and 87.70% protein recovery, and β-hexosaminidase release inhibition rate of 72.51% and 39.22% for pepsin and alcalase respectively) to produce functional immunomodulatory food products.

List of Abbreviations: SPI, Soy Protein Isolate; SPH, Soy Protein Hydrolysate; RBL, Rat Basophilic Leukemia; PNAG, *P*-nitrophenyl N-acetyl-β-D-glucosaminide; FBS, Fetal Bovine Serum; DNP, Dinitrophenol, PBS, Phosphate-buffered saline.

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