



Preparation and in vitro bioactive evaluation of cashew-nut proteins hydrolysate as a potential source of anti-allergy peptides

Di Chen¹ · Yang Shu¹ · Jian Chen¹ · Xianying Cao¹

Revised: 7 August 2020 / Accepted: 1 October 2020
© Association of Food Scientists & Technologists (India) 2020

Abstract This work proposes a novel potential source of antiallergens based on bioactive peptides. Cashew-nut protein hydrolysate with antiallergic activity was prepared from cashew nuts through protease treatment. The change in the antiallergic activity of cashew-nut protein hydrolysate during in vitro simulated digestion was investigated. Cashew-nut protein hydrolysates were prepared through treatment using five different enzymes, namely, Alcalase, Protamex, Neutrase, papain, and bromelin. According to the results of molecular weight distribution, more small molecular weight peptides could be obtained by selecting Alcalase protease than other proteases, and the degree of hydrolysis, trichloroacetic acid-soluble peptide yield and hyaluronidase inhibitory rate of the hydrolysate were $17.0 \pm 61.52\%$, $26.28 \pm 0.13\%$ and $62.06\% \pm 5.07\%$, which were significantly higher than those of other proteases. Therefore, Alcalase is the most suitable protease for the preparation of cashew-nut hydrolysates. Cashew-nut protein hydrolysates prepared with Alcalase under optimum conditions were fractionated through ultrafiltration. Fractions with low molecular weight exhibited the highest hyaluronidase inhibitory rate (90.57%) among all fractions. The inhibition of hyaluronidase activity during digestion showed that cashew-nut protein hydrolysate III (CPH III)

has persistent antiallergic activity. Therefore, CPH III could serve as a potential source of functional peptides with health-promoting effects.

Keywords Cashew-nut protein hydrolysate · Hydrolysis conditions · In vitro digestibility · Hyaluronidase inhibitory rate

Introduction

The cashew nut contains approximately 15–25% protein. Previous study showed cashew nut protein was nutritional protein which had well-balanced in essential amino acids (Liu et al. 2018). Three conserved allergenic seed storage proteins had been characterized in cashew nut, including the 7S globulin Ana o 1, 11S globulin Ana o 2, and the 2S albumin Ana o 3. Mattison et al. (2014) indicated that high molecular weight cashew nut proteins except 2S albumin Ana o 3 was degraded in digestion by assessed with an in vitro digestion model.

Allergic reactions occur when the immune system overreacts to normal substances. They manifest as certain conditions, such as atopic dermatitis, asthma, and allergic rhinitis. The incidence of allergic reactions has increased annually, which makes it imperative to develop efficient therapies (Shin et al. 2015). Hyaluronidase is an enzyme that mainly participates in allergic reactions and cleaves the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue (D. He et al. 2001). The compounds which have hyaluronidase inhibition activity can be expected to have anti-allergic effects because the anti-allergic agents show anti-hyaluronidase activity (Fujitani 2001). Antiallergic agents include clinical drugs and food-derived bioactive substances, such as polyphenols,

Di Chen contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13197-020-04838-z>) contains supplementary material, which is available to authorized users.

✉ Xianying Cao
cxying_02@126.com

¹ College of Food Science and Engineering, Hainan University, Haikou 570228, China

flavonoids, and polysaccharides (Kawai et al. 2007). The enzymolytic products of peanut proteins stimulate T cells and are used in immunotherapy for food allergies (Bøgh et al. 2009). Meanwhile, immunotherapy with the enzymatic hydrolysates of cashew-nut proteins can decrease available body temperature and relieved allergic symptoms relative to placebo therapy (Kulis et al. 2012).

Protein hydrolysis products obtained through food processing contain bioactive peptides as potential antiallergic agents. Thus, the conditions of protein hydrolysis have been controlled to acquire other bioactive peptides (Yust et al. 2003). Protein hydrolysates are prepared through chemical methods or enzymatic reactions (Singh et al. 2019). In view of biosafety, the former method cannot provide products that are applicable for the food industry, whereas the latter method yields products with enhanced specific bioactivity and reduces the allergenicity of intact proteins (Luo et al. 2014). Intact proteins own low allergenicity and lack bioactivity, whereas peptides produced through protein hydrolysis are bioactive given that they are easily absorbed in the gastrointestinal tract and blood (Erdmann et al. 2008). Protein hydrolysates that are rich in short peptides have a high nutritional value (Olagunju et al. 2018). Peptides with low molecular weight (M_w) have immunomodulatory (Agyei et al. 2016), anti-inflammatory (Sun et al. 2016), and antibacterial (Li et al. 2016) properties. Thus, enzymatic hydrolysis is a favorable choice for producing low- M_w peptides with various biological activities (Cotabarren et al. 2019).

In this study, the effects of enzyme type, pH, time, substrate concentration, enzyme/substrate ratio (E/S), and temperature on the degree of hydrolysis (DH) and trichloroacetic acid-soluble peptide yield (TCA-YSP) were evaluated. Cashew-nut protein hydrolysate (CPH) was further separated through ultrafiltration to obtain the several fractions with hyaluronidase inhibitory activity. In addition, the antiallergic activity of the fraction with high hyaluronidase inhibition capacity during digestion was studied.

Materials and methods

Materials

The cashew nut was purchased from the factory which picked cashew nut from Jianfeng Ridge in Ledong County, Hainan, China. The cashew-nut powder was obtained through crusher and 60 mesh sieve. Alcalase, Protamex, Neutrase, papain, pepsin and bromelin were bought from Beijing Solarbio Science & Technology (Beijing, China). The trichloroacetic acid (TCA), hyaluronidase and sodium hyaluronate was purchased from Aladdin Industrial

Corporation (Shanghai, China). The other reagents were both analytically pure.

Preparation of Cashew-nut protein hydrolysate (CPH)

Enzymatic hydrolysis

Cashew-nut protein was mixed with distilled water (1:25, w/v), and enzymatically hydrolyzed (dosage of enzyme 6% and hydrolysis time 6 h) to obtain hydrolysates. Different hydrolysis conditions were employed as follows: Alcalase (pH 10.0, 60 °C), Protamex (pH 7.0, 50 °C), Neutrase (pH 7.0, 50 °C), papain (pH 7.0, 50 °C), bromelin (pH 8.0, 55 °C) and Pepsin (pH 2.0, 37 °C). Enzymatic reaction was terminated by heating at 95 °C for 10 min and then centrifuged at 5000 r/min for 20 min (Singh et al. 2019). The supernatant was collected for further analysis.

Optimization of enzymatic hydrolysis conditions

The single factor experiments including pH (9, 9.5, 10, 10.5, 11, 11.5, 12 and 12.5), time (1, 2, 3, 4, 5 and 6 h), E/S (5, 6, 7, 8, 9 and 10%), temperature (35, 40, 45, 50, 55, 60 and 65 °C) and substrate concentration (30, 40, 50, 60 and 70 g/L) were investigated to optimize hydrolysis conditions of cashew nut protein. TCA-YSP was as the response value to determine the optimum conditions of by response surface methodology (RSM). The factors and levels of response surface methodology shown in Table 1.

M_w distribution of CPH

The M_w of samples obtained by different proteases were evaluated by high performance gel permeation chromatography (HPGPC) as previously described (Tang et al. 2016). It was determined using Ultrahydrogel 500 (7.8 mm × 300 mm) and Ultrahydrogel 120 (7.8 mm × 300 mm) and a 2414 refractive index (RI) detector installed into the a Waters e2695. the mobile phase was 0.05% sodium azide at a flow rate of 0.6 mL/min.

Table 1 Factors and levels used in response surface analysis for alcalase

Factors	Coding level		
	− 1	0	1
(A)Temperature/°C	35	42.5	50
(B)pH	10.5	11.5	12.5
(C)Substrate concentration /g/L	30	40	50

Ultrafiltration system

CPH was separated into three M_w fractions by ultrafiltration at 4 °C through a membrane with the molecular weight of 10 kDa and 3 kDa. Fractionates were named as follows: CPH I (> 10 kDa), CPH II (3–10 kDa) and CPH III (< 3 kDa). All the CPH obtained from the hydrolysates were lyophilized.

Scanning electron microscopy (SEM)

The protein and hydrolysates were coated with gold powder and then placed on a specimen holder. The samples were captured at 600× magnification by a SEM system (JOEM 3000 N, Japan).

Simulated digestion in vitro

The saliva digestion model was built by the Yuan's method (2019), with minor modification. CPH III solution of 20 mL (3 mg/mL) was blended with 6 mL of saliva gathered from healthy volunteers. The mixture was put in a beaker at 37 °C for 0 min (S1) 15 min (S2) and 30 min (S3). The gastric digestion was simulated with the method of Ketnawa and Ogawa (2019). The mixture was incubated at 37 °C for 30 min (G1), 60 min (G2) and 120 min (G3). The intestinal solution was added to initiate the intestinal stage after the pH of G3 sample was adjusted to 6.8 with 0.5 M NaOH. The sample was maintained for 30 min (I1), 60 min (I2) and 120 min (I3) and the reaction was kept in boiling water for 10 min to terminate. The in vitro digestibility of sample calculated as follows:

$$\text{Digestibility (\%)} = \frac{(A - B)}{A} \times 100 \quad (1)$$

A: the total protein content of the sample before digestion (mg/mL). B: the residual protein content after digestion (mg/mL).

Degree of hydrolysis (DH)

The DH was analyzed by formol titration according to the review of Silvestre (1997). The mixture was adjusted to pH 8.2 using 0.05 M NaOH. The total free amino groups of cashew-nut protein was determined by the above method when 0.05 g of the cashew nut protein was digested in the condition of vacuum with 6 mL HCl (6 M) at 110 °C for 24 h. The number of free amino acids and the DH were calculated as follows:

$$\text{free amino groups (\%)} = \left(A \times B \times \frac{14.007}{C} \times 1000 \right) \times 100 \quad (2)$$

$$\text{DH(\%)} = \left[\frac{\text{free amino groups of supernatant (\%)}}{\text{total free amino groups (\%)}} \right] \times 100 \quad (3)$$

A: volume of NaOH (mL) used. B: the concentration of the titration solution (0.05 M NaOH). C: volume of sample (mL).

Trichloroacetic acid-soluble peptide yield (TCA-YSP)

TCA-YSP was performed through the reported methods (Wang et al. 2018). Each sample (2 mL) was added to 2 mL aliquot of an enzymatic hydrolysate of 10% (w/w) TCA. The mixture was kept for 30 min at 4 °C and centrifuged at 4000 r/min for 15 min. The peptide content in the the supernatant was determined by using the biuret method. The total protein content of the sample was determined by using Kjeldahl method. TCA-YSP was calculated as follows:

$$\text{TCA-YSP(\%)} = \frac{A}{B} \times 100 \quad (4)$$

A: the peptide content of the supernatant (mg/mL). B: the total protein content of the sample before digestion (mg/mL).

Hyaluronidase inhibitory rate

Hyaluronidase inhibitory rate was measured through determining the amount of β-N-acetylglucosamine formed and determined according to the reported methods (Agyei et al. 2016; Madan and Nanda 2018). Different concentrations of samples had been incubated with hyaluronidase (300 unit/mL) at 37 °C for 20 min in advance, which was incubated with CaCl₂ (2.5 M) at 37 °C for 20 min. After incubation, 500 μL of sodium hyaluronate (0.5 mg/mL) dissolved in 0.2 M acetate buffer (pH 5.6) was added and the mixture was incubated in a water bath at 37 °C for 40 min. Then the mixture reaction was stopped by adding 100 μL of 5 M sodium hydroxide and placed for 10 min in an ice water bath. Next, 500 μL of acetylacetone (3.5 mL of acetylacetone dissolved in 50 mL of 1 M sodium carbonate solution) was added, then the mixture was incubated for 15 min in boiling water and 10 min in an ice water bath. After placed kept in 25 °C for 10 min, 1.0 mL of p-dimethylaminobenzaldehyde solution (0.8 g of acetylacetone dissolved in 15 mL of concentrated hydrochloric acid

and 15 mL of anhydrous alcohol) was added to the reaction mixture for 30 min at 25 °C. The absorbency of the reaction mixture was determined at 530 nm by using a the spectrophotometer. The samples were replaced by the acetate buffer for the control, while the hyaluronidase solution was replaced by acetate buffer for the blank. Hyaluronidase inhibitory activity was calculated as follows:

$$\begin{aligned} &\text{Inhibition rate of hyaluronidase (\%)} \\ &= \frac{(A - B) - (C - D)}{(A - B)} \times 100 \end{aligned} \quad (5)$$

A: OD (optical density) of control. B: OD of control blank. C: OD of sample. D: OD of sample blank.

Statistical analysis

All the experiments were done in triplicate and the data were reported as mean $\pm$ standard deviation (SD). The differences in means were analyzed using the one-way ANOVA test through SPSS (SPSS Inc, Chicago, IL, USA). The RSM was analyzed using Design Expert 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

Results and discussion

Selection of the most appropriate enzymes for obtaining CPH

The cashew-nut protein was 33.33% in defatted cashew-nut powder. Similar results were obtained by Guo (2018), which also proved that The protein content in defatted cashew-nut flour were 31.9%. This result indicates that defatted cashew-nut powder is a potential source of peptides. The solubility of cashew nut proteins was found to be extremely sensitive to pH, and the maximum protein solubility was observed at pH 9.0.

To prepared the more small molecule peptide from cashew nut proteins, the high-efficiency proteases were screened from three different aspects of DH, TCA-YSP and M_w . The CPH produced using Alcalase had DH and TCA-YSP values of $17.06\% \pm 1.52\%$ and $26.28\% \pm 0.13\%$, respectively (Fig. 1a). However, the DH and TCA-YSP values of Neutrase was $5.29\% \pm 0.56\%$ and $23.47\% \pm 0.47\%$. The DH and TCA-YSP values of Protamex was $7.16\% \pm 0.71\%$ and $21.76\% \pm 0.47\%$. The bromelain's DH and TCA-YSP values $9.39\% \pm 0.65\%$ and $21.94\% \pm 0.61\%$. The papain's DH and TCA-YSP values $2.96\% \pm 0.27\%$ and $13.18\% \pm 0.34\%$. After statistical analysis, the DH and TCA-YSP values of the CPH produced by using Alcalase were significantly ($p < 0.05$) higher than those of CPH produced by using other

proteases. The results of this work are similar to those of He (2019), who proved that Alcalase with a maximum DH of 15.8% was the most efficient protease for obtaining the highest yield of peptides.

TCA-YSP and DH are related to the cut sites of specific enzymes and the solubility of the substrate. The high TCA-YSP and DH values of the CPH obtained with Alcalase were attributed to the broad specificity of Alcalase, which can decompose proteins into low- M_w peptides (Osman et al. 2016). In addition, the CPH with the highest solubility was obtained when cashew-nut protein was hydrolyzed with Alcalase under optimum conditions (Ogunwolu et al. 2009). Therefore, Alcalase provided the CPH with the highest TCA-YSP and DH.

The different M_w distributions reflect marked differences in the degree of protein decomposition. The M_w distribution of CPH was determined through HPGPC (Fig. 1c). M_w decreased as the retention time was prolonged. After the same enzymolysis time and under the optimal conditions of each enzyme, it can be seen from Fig. 4 that the bromelain hydrolysate contained high macromolecular peptides and relatively low small molecule peptides. The content of small molecule peptides in the papain hydrolysis solution was the lowest. However, the molecular weight distribution of peptides in the Neutrase, Protamex and Alcalase hydrolysis solution was close, and the content of small molecule peptides was high. Therefore, it was determined from the three aspects about DH, TCA-YSP and M_w , which found that Alcalase obtained more small molecule peptides and the its hydrolyzing capacity was the strongest.

The hyaluronidase inhibition rate of the enzymatic hydrolysate was measured to determine the protease that can produce the enzymatic hydrolysate with anti-allergic effect (Sawabe et al. 1992). The high hyaluronidase inhibition rate indicates the enzymatic hydrolysate produced by protease has high anti-allergic effect. Kakegawa reported that a good correlation exists between histamine release from mast cells and hyaluronidase inhibition. The active substance exhibit antiallergic activity if it inhibits hyaluronidase (Kakegawa et al. 1985). The CPH produced by using Alcalase presented the highest hyaluronidase inhibition rate of $62.06\% \pm 5.07\%$ ($p < 0.05$), followed by that produced using pepsin ($50.11\% \pm 1.64\%$), Protamex ($47.40\% \pm 9.8\%$), and Neutrase ($43.13\% \pm 3.39\%$) (Fig. 1b). The CPH produced by using Alcalase had the best hyaluronidase inhibitory activity and thus the strongest antiallergic activity among all other proteases. This result may be attributed to the high DH of the CPH prepared by using Alcalase. To sum up, Alcalase produced more small peptides than the other enzymes, and the CPH obtained by using Alcalase had strong hyaluronidase inhibition activity.

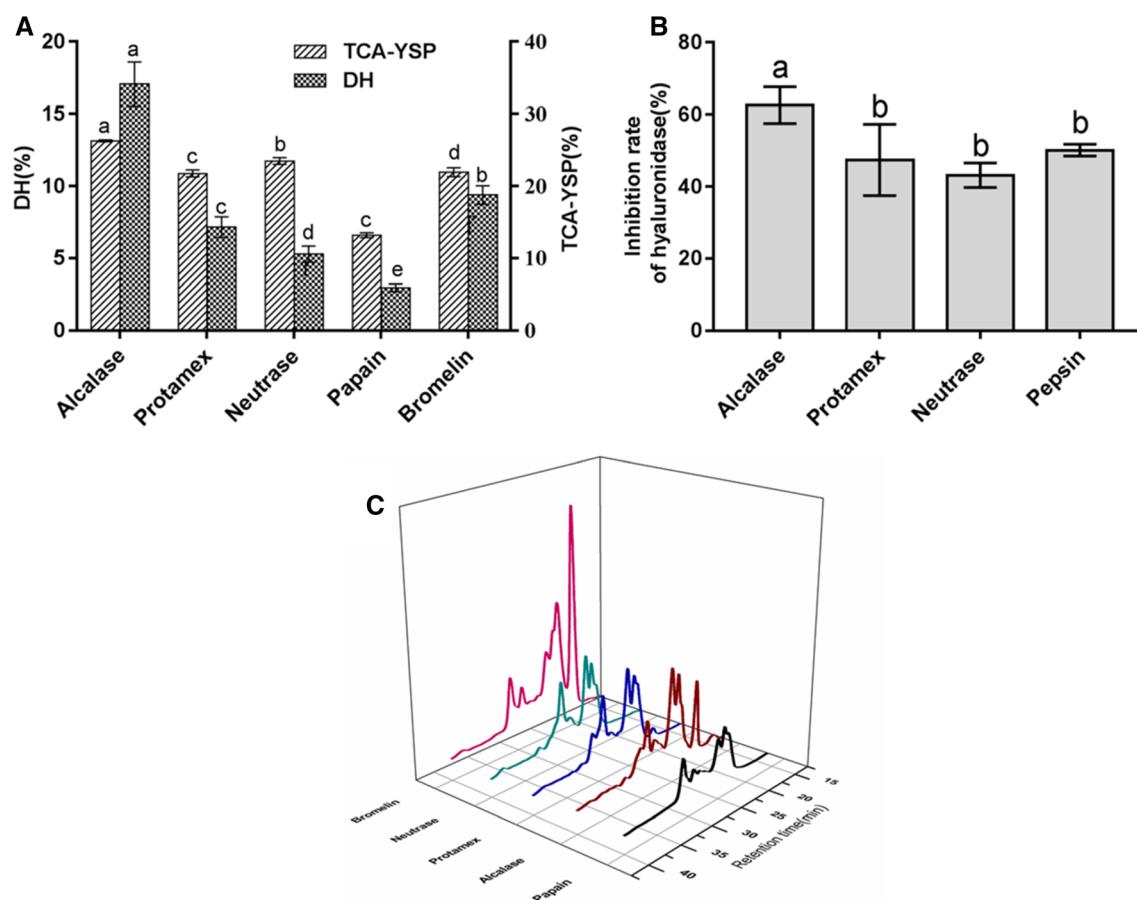


Fig. 1 (a) Degree of hydrolysis (DH) and TCA-soluble peptide yield (TCA-YSP) of hydrolysates prepared by different enzyme; (b) Inhibition rate of hyaluronidase of enzymatic hydrolysates; (c) M_w

distribution of hydrolysates obtained by different proteases. Different letters represent significant differences ($p < 0.05$). Data represent means $\pm$ S.D. ($n = 3$)

Determination of optimum enzymatic hydrolysis conditions

The optimum enzymatic hydrolysis conditions, including pH, time, E/S ratio, temperature, and substrate concentration, were investigated to maximize peptide production from cashew-nut protein.

The DH increased (11.34% to 16.89%) as the pH increased (9 to 11.5) but decreased to 8.11% at pH 12.5 (Fig. 2a). Meanwhile, TCA-YSP peaked at pH 11.5 and 12 and then drastically decreased at pH 12.5. Changing the pH of the substrate solution changes protein denaturation and solubility because pH influences the conformation and charge distribution of molecules and the accessibility of inner peptide bonds to enzymes (Stefanović et al. 2014).

The DH gradually increased from 12.62% to 19.27% as the reaction time was prolonged from 1 to 5 h (Fig. 2b). However, the DH negligibly changed when incubation time was extended beyond 5 h. The increment in the DH during hydrolysis indicates that the amounts of free amino acids in the CPH gradually increased. The stabilization of the DH

after 5 h may be attributed to enzyme inhibition and substrate limitation.

Cashew-nut proteins were hydrolyzed with the enzyme concentrations of 5% to 10% (Fig. 2c). The DH of CPH significantly ($p < 0.05$) increased when the E/S ratio increased from 5 to 8% but became constant when the E/S ratio exceeded 8%. The DH decreased when enzyme concentration ranged from 5 to 7% given the shortage of catalytic sites required for hydrolysis. However, the DH and TCA-YSP negligibly changed ($p > 0.05$) when the E/S ratio exceeded 8% because enzyme aggregation reduced the number of active sites for substrate attachment on enzymes.

The incubation temperature range of 35–65 °C was evaluated (Fig. 2d). The temperatures of 40 °C and 45 °C were the optimum temperatures for obtaining high DH values, and 40 °C was the optimum temperature for obtaining the highest TCA-YSP value. However, the DH and TCA-YSP remarkably declined when the reaction temperature increased to 50 °C. Similar results were reported by Salwance, who revealed that the DH also

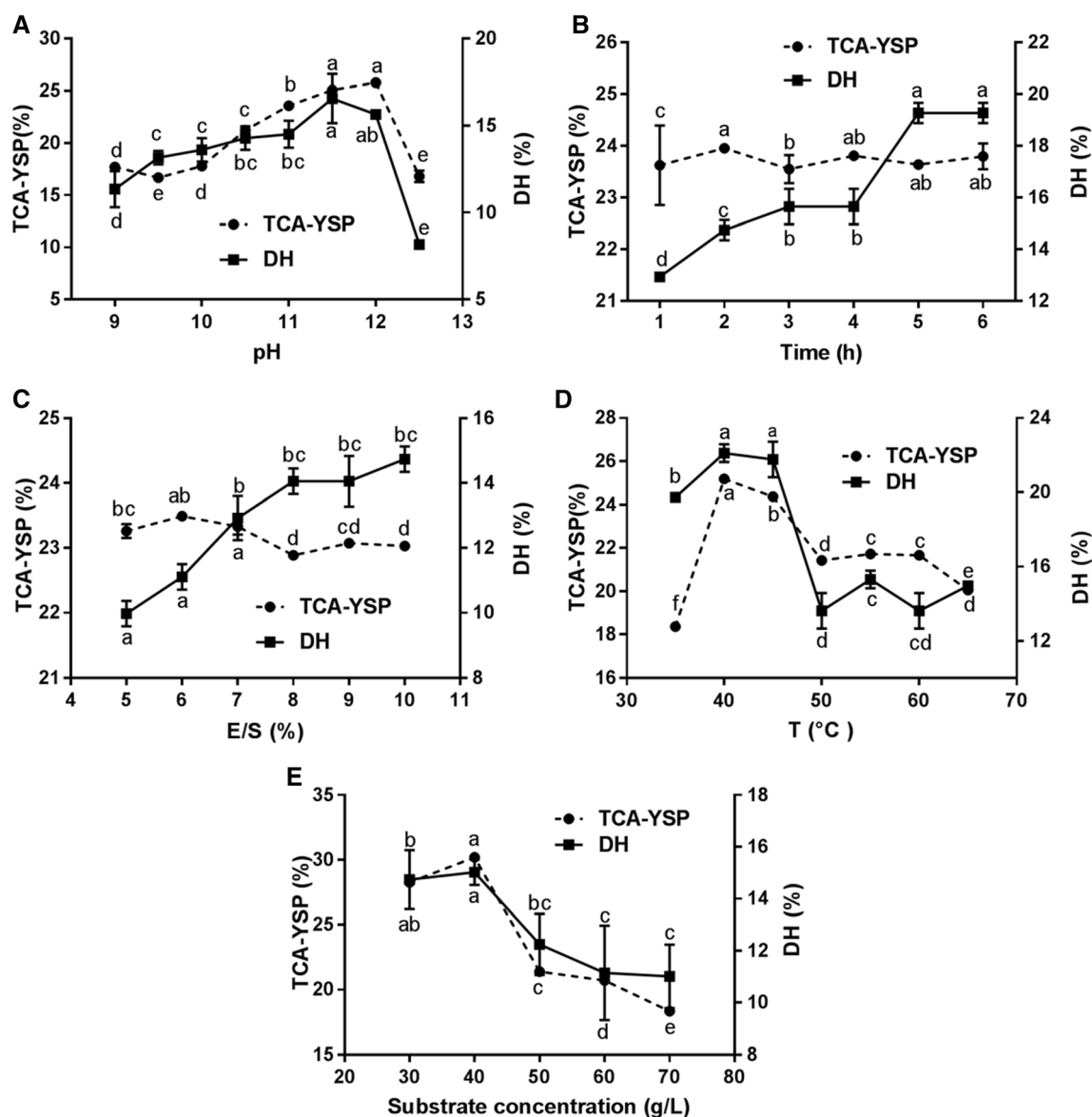


Fig. 2 Effects of different factors on the TCA-YSP and DH: (a) time; (b) substrate concentration; (c) E/S; (d) pH; and (e) temperature of the reaction. Values with different characters are significantly different ($p < 0.05$). Data are expressed as mean $\pm$ S.D. ($n = 3$)

increases when the temperature is increased from 30 to 40 °C (Salwanee et al. 2013). The reduction in the DH and TCA-YSP likely resulted from the thermal denaturation of enzymes (Klingaman et al. 2017).

The influence of substrate concentration on the DH and TCA-YSP was investigated (Fig. 2e). The DH and TCA-YSP increased as the substrate concentration increased from 30 g/L to 40 g/L but then decreased significantly ($p < 0.05$) when the substrate concentration was further increased. The DH and TCA-YSP of CPH at the optimum substrate concentration of 40 g/L were 15.02% and 30.19%, respectively, and were in agreement with a previous work (Deng et al. 2018). The increment in DH and TCA-YSP values with the increase in substrate

concentration increases the possibility that the enzyme can access the substrate. However, the significant decline ($p < 0.05$) in the DH and TCA-YSP values at substrate concentrations of more than 40 g/L may be attributed to enzyme saturation and reduced enzyme concentration (Deng et al. 2018).

The optimum enzymatic hydrolysis conditions was determined as 5 h, 8% E/S ratio, 35 °C, pH 12.5, and 40 g/L substrate through RSM. The response surface analysis was added in supplementary material. The TCA-YSP was 28.19% under that hydrolysis conditions, predicted through RSM. In order to verify the optimum conditions, the TCA-YSP of hydrolysate was 27.59% using that conditions, close

to the predicted value. Therefore, the optimum conditions predicted by RSM was accurate and reliable.

Ultrafiltration fractionation of the CPH

The fraction with high antiallergic activity was selected for further purification to obtain antiallergic peptides. The size of the peptide contained in the CPH III component was mainly concentrated in less than 3 kDa. Hyaluronidase activity was inhibited by CPH and the fractions obtained through ultrafiltration (CPH I, CPH II, and CPH III) (Fig. 3a). The hyaluronidase inhibitory rate of CPH III was 90.57% and was drastically higher than that of CPH and other ultrafiltration fractions at the same concentration (50 mg/mL) ($p < 0.05$). Meanwhile, the minimum hyaluronidase inhibitory rate of CPH I was only 57.86%. The M_w of the hydrolysate is one of the important factors that influences peptide activity and is highly correlated with the bioactivities and absorption efficiencies of peptides in the body (Jin et al. 2016). Hydrolysates that contain numerous short-chain peptides could have high nutritional values and intense biological activities, such as antimicrobial (Muhialdin and Algboory 2018) and antiallergy (Chung et al. 2018) activities. Low M_w can improve and accelerate the intestinal absorption, passage through the intestinal barrier, and action of peptides (Ketnawa et al. 2016). Antiallergy peptides are small peptides that comprise less than 20 amino acids (Uchida et al. 2019).

CPH III at concentrations of 5–50 mg/mL had significant hyaluronidase inhibitory activity ($p < 0.05$). By contrast, low concentrations of CPH III had low hyaluronidase inhibitory rates (Fig. 3b). Thus, the inhibitory activity of hyaluronidase was concentration-dependent and increased as peptide concentration was increased. The SEM of cashew-nut protein shown a structure with granular integrity, uneven surface and significant protrusions (Fig. 4a). However, the shape and surface of cashew-nut protein

hydrolysate changed significantly after enzymolysis, which was smooth, irregular and angular fragments (Fig. 4b). These results indicated that the cashew-nut protein was degradation after enzymolysis. The destruction of protein structure and the reduction in M_w are suggestive of protein degradation. These results further indicate that CPH derived its activity from the numerous small peptides that it contained.

Characteristics of protein and CPH III in vitro digestion

Digestibility and TCA-YSP determine the availability of proteins for intestinal absorption and reflect the efficiency of product utilization through oral consumption (Almeida et al. 2015). The variations in the TCA-YSP and in vitro digestibility of cashew-nut protein and CPH III at nine stages were determined (Table 2). The digestibility of cashew-nut protein and CPH III during the gastric stage was significantly higher than that during the saliva stage ($p < 0.05$) given the presence of digestive enzymes in the gastric digestion stage. The digestibility of protein and CPH III significantly increased ($p < 0.05$) after 120 min of intestinal digestion.

The TCA-YSP is used to monitor changes in peptide content of cashew protein and CPH III during digestion. The TCA-YSP of protein increased with the prolongation of digestion time during the digestion stage (Table 2). In addition, the TCA-YSP of the protein after digestion was significantly higher ($p < 0.05$) than that before digestion. Similarly, Ketnawa reported that the TCA-YSP of fermented soybeans increases during the digestion stage (Ketnawa and Ogawa 2019). However, the TCA-YSP of CPH III gradually decreased during the digestion stage. The high TCA-YSP of CPH III could be ascribed to the large number of soluble peptides that it initially contained. TCA-YSP may decrease when peptides are precipitated by

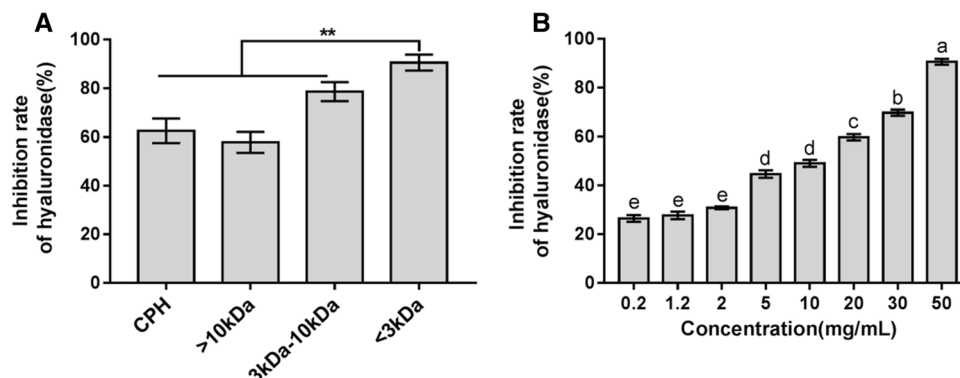


Fig. 3 (a) Hyaluronidase inhibitory activity of CPH and ultrafiltration fractions. Mean assigned with ** denotes a statistically significant difference at $p < 0.01$ respect to hyaluronidase inhibitory

activity of CPH III. (b) Inhibition rate of hyaluronidase of different concentrations of CPH III. Bars with different alphabets have mean values ($n = 3$) that are significantly different ($p < 0.05$)

Fig. 4 Scanning electron micrographs (SEM) of cashew nut protein (a) and CPH III (b) with magnification of 600 ×

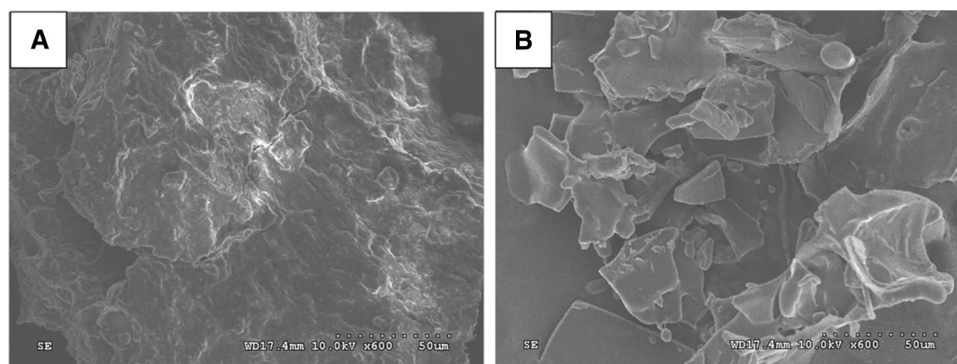


Table 2 Changes in TCA-YSP and protein digestibility of the protein and CPH III during the *in vitro* digestion

Digestive stage	Digestibility (%)		TCA-YSP (%)	
	cashew nut protein	CPH III	cashew nut rotein	CPH III
S1	5.50 ± 0.02 ^c	13.71 ± 0.01 ^c	1.66 ± 0.18 ^f	14.40 ± 0.36 ^b
S2	5.72 ± 0.01 ^c	13.71 ± 0.01 ^c	1.48 ± 0.23 ^f	14.98 ± 0.06 ^a
S3	5.26 ± 0.07 ^c	13.67 ± 0.01 ^c	2.70 ± 0.09 ^e	14.81 ± 0.21 ^a
G1	70.80 ± 0.72 ^b	50.36 ± 0.57 ^b	4.72 ± 0.12 ^d	8.65 ± 0.07 ^c
G2	71.60 ± 1.03 ^b	51.08 ± 0.88 ^b	5.02 ± 0.14 ^c	8.55 ± 0.18 ^c
G3	70.80 ± 0.46 ^b	48.96 ± 0.57 ^b	6.19 ± 0.22 ^b	8.07 ± 0.04 ^d
I1	82.81 ± 0.74 ^a	82.06 ± 1.01 ^a	7.50 ± 0.05 ^a	5.13 ± 0.17 ^e
I2	83.74 ± 0.66 ^a	83.00 ± 1.00 ^a	7.60 ± 0.19 ^a	5.41 ± 0.16 ^e
I3	83.78 ± 0.70 ^a	83.10 ± 1.01 ^a	7.65 ± 0.21 ^a	5.34 ± 0.18 ^e

Mean ± SD (*n* = 3) and different letters in same column are significantly different (*p* < 0.05)

TCA upon combination with digestive enzymes (Chen et al. 2013).

The molecular weight of cashew nut protein decreased by protease hydrolysis and produced the low molecular weight peptides. Low molecular weight peptides could improve their absorption in the interior and accelerate their transformation through internal interactions to produce physiological effects (Wang and Li 2017). Other studies had shown that only the molecular weight greater than or equal to 3.5 kDa had the ability to cross-link with IgE antibodies on mast cells to cause allergic reaction, yet the small molecular oligopeptides had low cross-linking capabilities with IgE, and even competitively absorb the binding sites of sensitizers (Liu et al. 2012).

Hyaluronidase inhibitory rates at different digestion stages were investigated (Fig. 5). The hyaluronidase inhibitory rate initially increased and then decreased at the saliva phase and then significantly increased (*p* < 0.05) at 120 min with further pepsin digestion. However, the hyaluronidase inhibitory activity of samples was more stable during the intestinal phase than that at the saliva and gastric phase. The change in hyaluronidase inhibitory rate during digestion indicates that CPH III participates in antiallergy responses and that the antiallergic activity of peptides improved during digestion. Sanjukta also showed

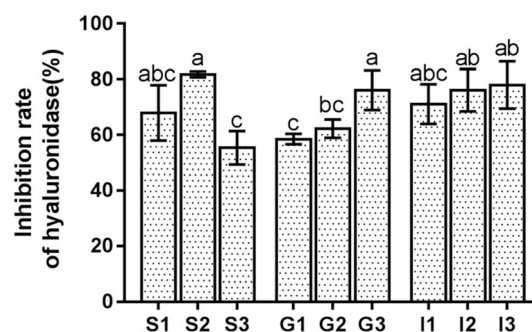


Fig. 5 Hyaluronidase inhibitory rate of samples were measured after CPH *in vitro* saliva, gastric and intestinal digestion. Significant differences are highlighted with different letters (*p* < 0.05). Data are expressed as mean ± S.D.(*n* = 3)

that some bioactive peptides were released through gastrointestinal digestion (Sanjukta et al. 2015). Thus, the antiallergy properties of CPH III persisted after digestion.

To sum up, CPH III had inhibitory and maintained hyaluronidase activity during digestion, which showed that it contained a large amount of digestive stable anti-allergic peptide and could further developed peptide drugs for the treatment of allergic reactions. It had been reported the small molecular weight peptides, artificially synthesized or separated from hydrolyzate, could be used as medicines to

treat allergic reactions (Moldaver and Larché 2011; Larché 2007; Ko et al. 2016). Small molecular weight peptides achieved anti-allergic effects by significantly reducing the cross-linking ability of IgE bound to mast cells and basophils or inhibiting the production of IgE antibodies by inducing Treg cells to release immunomodulatory cytokine IL-10.

Conclusion

The CPH obtained with Alcalase had the highest DH, TCA-YSP, and hyaluronidase inhibitory activity. These characteristics indicate that CPH had low M_w and antiallergic activity. The optimum enzymatic hydrolysis conditions (5 h, 8% E/S ratio, 35 °C, pH 12.5, and 40 g/L substrate) were determined through RSM. Then, CPH was prepared under optimum conditions and further fractionated through ultrafiltration. CPH III (< 3 kDa) showed the highest hyaluronidase inhibitory rate and is a novel antiallergy peptide. CPH III retained its antiallergy property after digestion. Therefore, the CPH III can be used as a potential source of an anti allergic peptides for development of drugs to treat allergic reactions.

Acknowledgments This work was supported by “the Natural Science Foundation of Hainan Province (2019RC004 and 2017CXTD001)”, oriented subject of the State Key Laboratory of Marine Resource Utilization in South China Sea (Hainan University) (DX2017005).

References

- Agyei D, Ongkudon CM, Wei CY, Chan AS, Danquah MK (2016) Bioprocess challenges to the isolation and purification of bioactive peptides. *Food Bioprod Process* 98:244–256
- Almeida CC, Monteiro MG, Costa-Lima BRC, Alvares TS, Conte-Junior CA (2015) In vitro digestibility of commercial whey protein supplements. *LWT Food Sci Technol* 61(1):7–11
- Bøgh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills ENC, Madsen CB (2009) Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clin Exp Allergy* 39(10):1611–1621
- Chen N, Zhao M, Sun W (2013) Effect of protein oxidation on the in vitro digestibility of soy protein isolate. *Food Chem* 141(3):3224–3229
- Chung JH, Kong JN, Choi HE, Kong KH (2018) Antioxidant, anti-inflammatory, and anti-allergic activities of the sweet-tasting protein brazzein. *Food Chem* 267:163–169
- Cotabarren J, Rosso AM, Tellechea M, García-Pardo J, Rivera JL, Obregón WD, Parisi MG (2019) Adding value to the chia (*Salvia hispanica* L.) expeller: production of bioactive peptides with antioxidant properties by enzymatic hydrolysis with Papain. *Food Chem* 274:848–856
- Deng Y, Butré CI, Wierenga PA (2018) Influence of substrate concentration on the extent of protein enzymatic hydrolysis. *Int Dairy J* 86:39–48
- Erdmann K, Cheung BWY, Schröder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J Nutr Biochem* 19(10):643–654
- Fujitani N (2001) Inhibitory effects of microalgae on the activation of hyaluronidase. *J Appl Phycol* 13:489–492
- Guo X, Huang H, Liu D, Zhong C (2018) Improving particle dispersity and CO₂ separation performance of amine-functionalized CAU-1 based mixed matrix membranes with polyethyleneimine-grafting modification. *Chem Eng Sci* 189:277–285
- He D, Zhou A, Wei W, Nie L, Yao S (2001) A new study of the degradation of hyaluronic acid by hyaluronidase using quartz crystal impedance technique. *Talanta* 53(5):1021–1029
- He W, Yang R, Zhao W (2019) Effect of acid deamidation-alcalase hydrolysis induced modification on functional and bitter-masking properties of wheat gluten hydrolysates. *Food Chem* 277:655–663
- Jin J, Ma H, Wang B, Yagoub AE-GA, Wang K, He R, Zhou C (2016) Effects and mechanism of dual-frequency power ultrasound on the molecular weight distribution of corn gluten meal hydrolysates. *Ultrason Sonochem* 30:44–51
- Kakegawa H, Matsumoto H, Satoh T (1985) Activation of hyaluronidase by metallic salts and compound 48/80, and inhibitory effect of anti-allergic agents on hyaluronidase. *Chem Pharm Bull (Tokyo)* 33(2):642–646
- Kawai M, Hirano T, Higa S, Arimitsu J, Maruta M, Kuwahara Y, Ohkawara T, Hagihara K, Yamadori T, Shima Y, Ogata A, Kawase I, Tanaka T (2007) Flavonoids and related compounds as anti-allergic substances. *Allergol Int* 56(2):113–123
- Ketnawa S, Martínez-Alvarez O, Benjakul S, Rawdkuen S (2016) Gelatin hydrolysates from farmed Giant catfish skin using alkaline proteases and its antioxidative function of simulated gastro-intestinal digestion. *Food Chem* 192:34–42
- Ketnawa S, Ogawa Y (2019) Evaluation of protein digestibility of fermented soybeans and changes in biochemical characteristics of digested fractions. *J Funct Foods* 52:640–647
- Klingaman CA, Wagner MJ, Brown JR, Klecker JB, Pauley EH, Noldner CJ, Mays JR (2017) HPLC-based kinetics assay facilitates analysis of systems with multiple reaction products and thermal enzyme denaturation. *Anal Biochem* 516:37–47
- Kulis M, Macqueen I, Li Y, Guo R, Zhong XP, Burks AW (2012) Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. *J Allergy Clin Immunol* 130(3):716–723
- Ko SC, Lee DS, Park W, Yoo J, Yim MJ, Qian ZJ, Lee CM, Oh J, Jung WK, Choi IW (2016) Anti-allergic effects of a nonameric peptide isolated from the intestinegastrointestinal digests of abalone (*Haliotis discus hannai*) in activated HMC-1 human mast cells. *Int J Mol Med* 37(1):243–250
- Larché M (2007) Immunotherapy with allergen peptides. *Allergy Asthma Clin Immunol Off J Can Soc Allergy Clin Immunol* 3(2):53–59
- Liu CM, Peng Q, Zhong JZ, Liu W, Zhong YJ, Wang F (2018) Molecular and functional properties of protein fractions and isolate from cashew nut (*Anacardium occidentale* L.). *Molecules* 23(2):393
- Liu XY, Li L, Liu ZG (2012) The development in mechanisms of food allergy mediated by food allergen. *J Food Saf Qual* 3(4):235–239 (in Chinese)
- Li S, Hao L, Bao W, Zhang P, Su D, Cheng Y, Nie L, Wang G, Hou F, Yang Y (2016) A novel short anionic antibacterial peptide isolated from the skin of *Xenopus laevis* with broad antibacterial activity and inhibitory activity against breast cancer cell. *Arch Microbiol* 198(5):473–482
- Luo Y, Pan K, Zhong Q (2014) Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: partial characterizations. *Food Chem* 155:146–154

- Madan K, Nanda S (2018) In-vitro evaluation of antioxidant, anti-elastase, anti-collagenase, anti-hyaluronidase activities of safranal and determination of its sun protection factor in skin photoaging. *Bioorg Chem* 77:159–167
- Mattison CP, Grimm CC, Wasserman RL (2014) In vitro digestion of soluble cashew proteins and characterization of surviving IgE-reactive peptides. *Mol Nutr Food Res* 58(4):884–893
- Moldaver D, Larché M (2011) Immunotherapy with peptides. *Allergy* 66(6):784–791
- Muhialdin BJ, Algboory HL (2018) Identification of low molecular weight antimicrobial peptides from Iraqi camel milk fermented with *Lactobacillus plantarum*. *PharmaNutr* 6(2):69–73
- Ogunwolu SO, Henshaw FO, Mock HP, Santros A, Awonorin SO (2009) Functional properties of protein concentrates and isolates produced from cashew (*Anacardium occidentale* L.) nut. *Food Chem* 115(3):852–858
- Olagunju A, Omoba O, Enujiugha V, Alashi A, Aluko R (2018) Pigeon pea enzymatic protein hydrolysates and ultrafiltration peptide fractions as potential sources of antioxidant peptides: an in vitro study. *LWT Food Sci Technol* 97:269–278
- Osman A, Goda HA, Abdel-Hamid M, Badran SM, Otte J (2016) Antibacterial peptides generated by Alcalase hydrolysis of goat whey. *LWT Food Sci Technol* 65:480–486
- Salwanee S, Wan Mustapha W, Mamot S, Maskat M, Ibrahim S (2013) Effects of enzyme concentration, temperature, pH and time on the degree of hydrolysis of protein extract from viscera of tuna (*Euthynnus affinis*) by using Alcalase. *Sains Malays* 42(3):279–287
- Sanjukta S, Rai A, Ali AM, Kumaraswamy J, Talukdar NC (2015) Enhancement of antioxidant properties of two soybean varieties of Sikkim Himalayan region by proteolytic *Bacillus subtilis* fermentation. *J Funct Foods* 14:650–658
- Sawabe Y, Nakagomi K, Iwagami S, Suzuki S, Nakazawa H (1992) Inhibitory effects of pectic substances on activated hyaluronidase and histamine release from mast cells. *BBA-Mole Cell Res* 1137(3):274–278
- Shin HS, See HJ, Jung SY, Choi DW, Kwon DA, Bae MJ, Sung KS, Shon DH (2015) Turmeric (*Curcuma longa*) attenuates food allergy symptoms by regulating type 1/type 2 helper T cells (Th1/Th2) balance in a mouse model of food allergy. *J Ethnopharmacol* 175:21–29
- Silvestre MPC (1997) Review of methods for the analysis of protein hydrolysates. *Food Chem* 60(2):263–271
- Singh TP, Siddiqi RA, Sogi DS (2019) Statistical optimization of enzymatic hydrolysis of rice bran protein concentrate for enhanced hydrolysate production by papain. *LWT Food Sci Technol* 99:77–83
- Stefanović A, Jovanović J, Grbavčić S, Šekuljica N, Manojlović VB, Branko B, Knežević-Jugović Z (2014) Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production. *Eur Food Res Technol* 239(6):979–993
- Sun X, Chakrabarti S, Fang J, Yin Y, Wu J (2016) Low-molecular-weight fractions of Alcalase hydrolyzed egg ovomucin extract exert anti-inflammatory activity in human dermal fibroblasts through the inhibition of tumor necrosis factor-mediated nuclear factor κ B pathway. *Nutr Res* 36(7):648–657
- Tang W, Lin L, Xie J, Wang Z, Wang H, Dong Y, Shen M, Xie M (2016) Effect of ultrasonic treatment on the physicochemical properties and antioxidant activities of polysaccharide from *Cyclocarya paliurus*. *Carbohydr Polym* 151:305–312
- Uchida R, Egawa T, Fujita Y, Furuta K, Taguchi H, Tanaka S, Nishida K (2019) Identification of the minimum region of peptide derived from ADP-ribosylation factor1 (ARF1) that inhibits IgE-mediated mast cell activation. *Mole Immunol* 105:32–37
- Wang J, Chi Y, Cheng Y, Zhao Y (2018) Physicochemical properties, in vitro digestibility and antioxidant activity of dry-heated egg white protein. *Food Chem* 246:18–25
- Wang B, Li B (2017) Effect of molecular weight on the transepithelial transport and peptidase degradation of casein-derived peptides by using Caco-2 cell model. *Food Chem* 218:1–8
- Yuan Y, Li C, Zheng Q, Wu J, Zhu K, Shen X, Cao J (2019) Effect of simulated gastrointestinal digestion in vitro on the antioxidant activity, molecular weight and microstructure of polysaccharides from a tropical sea cucumber (*Holothuria leucospilota*). *Food Hydrocoll* 89:735–741
- Yust MM, Pedroche J, Girón-Calle J, Alaiz M, Millán F, Vioque J (2003) Production of ace inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem* 81(3):363–369

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Preparation and *in vitro* bioactive evaluation of cashew-nut proteins hydrolysate as a potential source of anti-allergy peptides

SUPPLEMENTARY MATERIAL

Table of Contents

Table S1. Experimental data for TCA-YSP from the central composite experimental design.

Table S2. Analysis of the response surface methodology regression and variance.

Figure S1. 3D Response surface plot shows the interaction among (A) temperature (T) and pH; (B) Substrate concentration and temperature (T); (C) pH and substrate concentration on the TCA-YSP of CPH.

Figure S2. The mass spectrum of cashew-nut peptide

Model fitting of Response surface analysis

Based on the results of a single factor experiment, three factors of CPH had three levels in a Box-Behnken design (BBD). The effects of three parameters including temperature (35–50 °C), pH (10.5–12.5) and substrate concentration (30–50 g/L) on the TCA-YSP are shown in Table S1. In Table S2, the quadratic regression equation model had a high significance ($p = 0.0022 < 0.01$) while had a low lack-of-fit ($p = 0.2336 > 0.05$). The correlation coefficient of the model was 0.9350 indicating that this model had high precision and was used to analyze and predict the effect the temperature, substrate concentration and pH on TCA-YSP of CPH under various designs. This experiment was designed and analyzed with Design-Expert 8.0.5 and the quadratic polynomial regression equation for the TCA-YSP was as follows:

$$Y = 25.35 - 0.94A + 1.06B - 1.50C - 2.08AB + 0.89AC + 0.31BC + 0.14A^2 - 1.38B^2 - 0.62C^2$$

The significance of the coefficients of the models was evaluated using ANOVA (Table S2). The influence of temperature (A) on the TCA-YSP was significant ($p < 0.05$) while the influences of pH (B) and substrate concentration (C) were very significant ($p < 0.01$). The results show they were the important factors affecting the response. The interaction between temperature and pH (AB) was extremely significant ($p < 0.01$). However, the temperature and substrate concentration (AC; $p < 0.05$) had no significant interaction and there was also no interaction between pH and substrate concentration (BC; $p < 0.05$).

Fig. S1A revealed that the reaction temperature and pH had a strong influence on the curvature of the response surface, in accordance with the values of their quadratic coefficients. The TCA-YSP increased because increasing of temperature and pH accelerate the enzyme activity. The pH had a negative effect on the TCA-YSP with the temperature higher than 47 °C. For the influence of

temperature on TCA-YSP, its quadratic effect was significant and shown in the range of pH from 11.5 to 12.5. The TCA-YSP value decreased with the rise of temperature due to the damaged of tertiary structure of enzymes with the increase of temperatures (Zheng, Xiao, & Roberts, 2016). Fig. S1B shows the three-dimensional surface plots of the interaction effect of temperature and substrate concentration on TCA-YSP. The TCA-YSP is a function of both the substrate concentration and pH. The pH and temperature exist an optimum conditions within the design space (Fig. S1C). The increase of the substrate concentration decrease TCA-YSP given the enzyme concentrations and effective contact reducing between the substrates and enzymes. Additionally, The pH of CPH had a positive influence on the TCA-YSP and its the maximum value at pH 11.5 (Fig. S1C). The decline was attributed to the irreversible change of enzymes three-dimensional structure leading to the enzyme denaturation (Zuorro, Maffei, & Lavecchia, 2016). This phenomenon also could be explained by the fact that pH could impact on the activity of alcalase and the solubility of substrates (Teng, Fang, Song, & Gao, 2011).

The mass spectrum of cashew-nut peptide

The cashew-nut peptide isolated cashew protein hydrolysate was identified with amino acid sequence Ile-Ile-Ala-Ile-Pro-Ala-Gly-Val-Ala-His (IIAIPAGVAH) by mass spectrometry (Figure S2), which donnot have the cleavage sites of pepsin and proteas such as phenylalanine, tryptophan, and tyrosine. The proline residues in peptide may confer unique conformational constraints on the peptide chain, preventing them from being broken down during digestion and retaining activity (Yu, Field, & Wu, 2018).

References

Teng D., Fang Y., Song X., & Gao Y. (2011). Optimization of enzymatic hydrolysis parameters for antioxidant capacity of peptide from goat placenta. *Food and Bioproducts Processing*, 89 (3), 202-208. <https://doi.org/10.1016/j.fbp.2010.05.001>.

- Yu W, Field CJ, Wu J (2018). Purification and identification of anti-inflammatory peptides from spent hen muscle proteins hydrolysate. *Food Chemistry*, 253, 101-107. <https://doi.org/10.1016/j.foodchem.2018.01.093>.
- Zheng Y, Xiao R, Roberts M (2016). Polymer-enhanced enzymatic microalgal cell disruption for lipid and sugar recovery. *Algal Research*, 14, 100-108. <https://doi.org/10.1016/j.algal.2016.01.010>.
- Zuorro A., Maffei G., Lavecchia R (2016). Optimization of enzyme-assisted lipid extraction from *Nannochloropsis* microalgae. *Journal of the Taiwan Institute of Chemical Engineers*, 67, 106-114. <https://doi.org/10.1016/j.jtice.2016.08.016>.

Table S1. Experimental data for TCA-YSP from the central composite experimental design

Model No	A Temperature (°C)	B pH	C substrate concentration (g/L)	TCA-YSP (%)
1	42.5	12.5	30	25.32
2	35	11.5	50	23.09
3	42.5	11.5	40	25.27
4	42.5	11.5	40	24.93
5	42.5	11.5	40	25.32
6	42.5	10.5	50	20.76
7	42.5	11.5	40	24.79
8	35	12.5	40	27.78
9	50	11.5	30	24.88
10	50	11.5	50	22.63
11	50	10.5	40	24.61
12	42.5	12.5	50	23.96
13	42.5	11.5	40	26.46
14	50	12.5	40	22.09
15	42.5	10.5	30	23.36
16	35	11.5	30	28.92
17	35	10.5	40	21.97

Table S2. Analysis of the response surface methodology regression and variance

	Sum of squares	df	Mean Square	F Value	P-value Prob>F	
Model	65.20	9	7.24	11.18	0.0022	significant
A-T	7.14	1	7.14	11.01	0.0128	*
B-pH	8.94	1	8.94	13.80	0.0075	**
C-Substrate concentration	18.10	1	18.10	27.95	0.0011	**
AB	17.36	1	17.36	26.80	0.0013	**
AC	3.20	1	3.20	1.95	0.0615	
BC	0.39	1	0.39	0.60	0.4644	
A ²	0.084	1	0.084	0.13	0.7290	
B ²	8.05	1	8.05	12.43	0.0096	**
C ²	1.60	1	1.60	2.48	0.1596	
Residual	4.53	7	0.65			
Lack of fit	2.81	3	0.94	2.18	0.2336	Not significant
Net error	1.72	4	0.43			
Total dispersion	69.73	16				

**represents extremely significant difference ($p < 0.01$), * represents significant difference ($p < 0.05$).

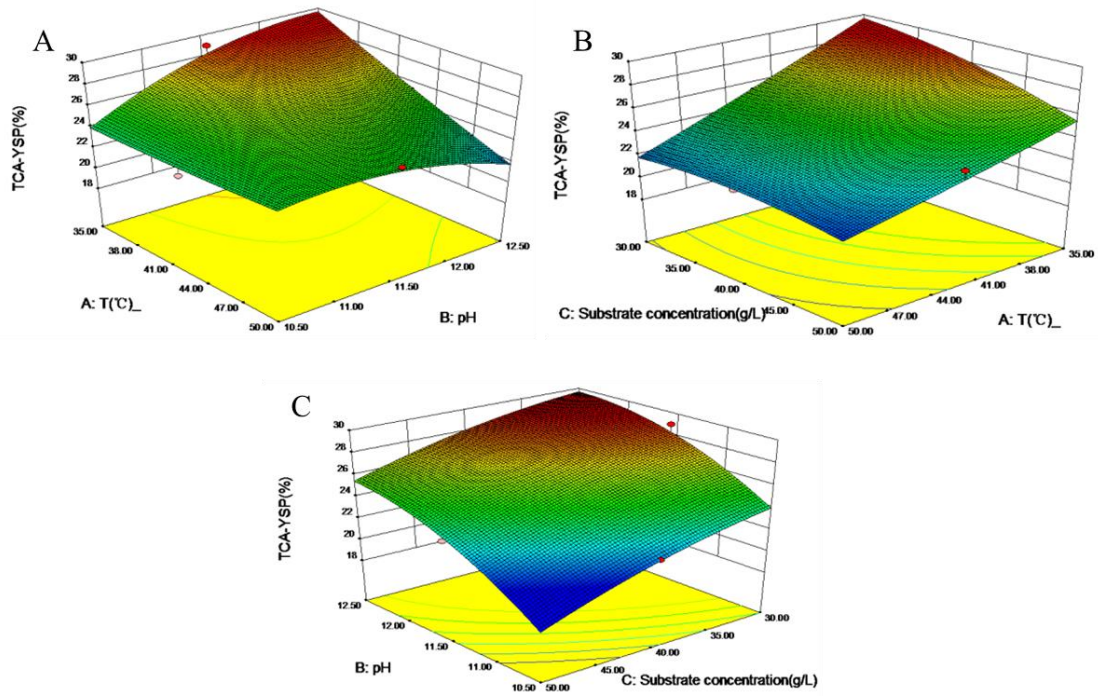


Fig S1. 3D Response surface plot shows the interaction among (A) temperature (T) and pH; (B) Substrate concentration and temperature (T); (C) pH and substrate concentration on the TCA-YSP of CPH

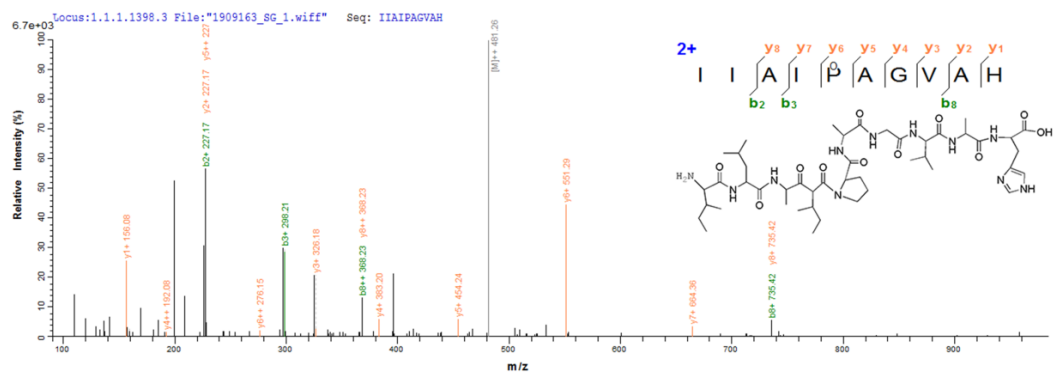


Fig S2. The mass spectrum of cashew-nut peptide