Process Biochemistry 67 (2018) 46–54

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Effect of ultrasonic pretreatment on whey protein hydrolysis by alcalase: Thermodynamic parameters, physicochemical properties and bioactivities

Qiongying Wu[a], Xuefen Zhang[a], Junqiang Jia[b,*], Cong Kuang[a], Hongshun Yang[c]

[a] School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, 212018, China
[b] School of Grain Science and Technology, Jiangsu University of Science and Technology, Zhenjiang, 212004, China
[c] Food Science and Technology Programme, c/o Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore, 117543, Republic of Singapore

ARTICLE INFO

Keywords:
Whey protein
Ultrasound
Thermodynamics
Physicochemical properties
ACE inhibitory
Immunomodulatory

ABSTRACT

The effects of ultrasonic pretreatment on the enzymolysis thermodynamics and physicochemical properties of whey protein, and the mechanisms behind those effects, were investigated. Changes in the angiotensin-I converting enzyme (ACE) inhibitory and immunomodulatory activities of whey protein hydrolysates after ultrasonic pretreatment were also determined. Results showed that the ultrasonicated whey protein had a higher in degree of hydrolysis than the non-sonicated protein. After the pretreatment, the activation energy (E_a), enthalpy of activation (ΔH) and entropy of activation (ΔS) of whey protein enzymolysis were decreased by 15.9%, 16.8%, and 16.4%, respectively. There was no significant change in free energy (ΔG) ($P > 0.05$). Physicochemical analysis revealed that ultrasound had induced unfolding of the whey protein, resulting in a 43.7% increase in its surface free sulfhydryl content and a 62.6% increase in surface hydrophobicity. Ultrasound significantly decreased the protein's α-helical content and significantly increased its β-sheets and β-turns ($P < 0.05$). The ACE inhibitory and immunomodulatory activities of the whey protein hydrolysates were significantly increased by ultrasonic pretreatment ($P < 0.05$). These results suggest that ultrasound can be applied to enhance whey protein enzymolysis for the generation of novel bioactive peptides that can be used as drug or functional food ingredient.

1. Introduction

Ultrasound is a sound wave with a frequency above the range of human hearing; that is, above 20 kHz [1]. It can be classified into two fields on the basis of the frequency range: low-energy ultrasound with a frequency in the range of 100 kHz to 1 MHz, and high-energy power ultrasound with a frequency in the range of 20–100 kHz [2]. Compared with low-energy ultrasound, which is used for analyzing and evaluating the physicochemical properties of foods [2,3], high-energy power ultrasound is widely applied to alter the physicochemical properties of foods in various areas [4]. High-energy power ultrasound can cause variable alterations to food structures depending on the ultrasonic cavitation, the rapid formation and collapse of gas bubbles, which can produce high shear and mechanical energy [3]. Recently, high-energy power ultrasound has been used successfully to improve the enzymatic hydrolysis and properties of proteins. Resendiz-Vazquez et al. [5] found that the application of ultrasound at 20 kHz and 400 W increased the emulsifying activity and emulsion stability of a jackfruit seed protein isolate as well as changed the molecular weight of the protein fraction. Wang et al. [4] reported that ultrasonic pretreatment at 20 kHz significantly increased the degree of hydrolysis (DH) of β-conglycinin (7S) and glycinin (11S) and the antioxidative activity of their hydrolysates. Similarly, Wang et al. [6] reported that ultrasonic pretreatment increased both the hydrolysis rate of oat protein hydrolysates and their angiotensin-I converting enzyme (ACE) activity. The changes in the yield and bioactivities of protein hydrolysates after ultrasound treatment were attributed to the molecular unfolding of the protein and exposure of its functional groups [7,8]. As a result, ultrasonic pretreatment offers a potential way of producing bioactive peptides from proteins. However, the mechanisms behind the effects of ultrasound on protein hydrolysis need to be studied further.

Whey protein, an abundant by-product of the dairy industry,

Abbreviations: DH, degree of hydrolysis; ACE, angiotensin-I converting enzyme; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); ANS, 1-anilino-8-naphthalene sulfonate; HHL, Hippuryl-His-Leu; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; FBS, Fetal bovine serum; k_{in}, effective (total) rate constant; A, pre-exponential or collision factor; R, universal gas constant; T, Kelvin temperature; k_B, Boltzmann constant; h, Planck constant; E_a, activation energy; Δ, SSentropy of activation; ΔG, free energies of activation; ΔH, enthalpy of activation; SH, sulfhydryl

* Corresponding author.
E-mail addresses: wuqy1@163.com (Q. Wu), zxf6980092@163.com (X. Zhang), jiajq@just.edu.cn (J. Jia), rzkuangcong@163.com (C. Kuang), chmynghs@nus.edu.sg (H. Yang).

https://doi.org/10.1016/j.procbio.2018.02.007
Received 1 December 2017; Received in revised form 12 January 2018; Accepted 10 February 2018
Available online 14 February 2018
1359-5113/

consists mainly of β-lactoglobulin (55%–60%, w/w), α-lactalbumin (15%–20%, w/w), and bovine serum albumin (5%–10%, w/w) [9]. It has been widely applied in many protein-based food formulations, primarily attributed to its high nutritional value and desirable functional properties [10]. In recent years, many research studies have confirmed the importance of whey protein as a source of bioactive peptides and the association of their hydrolysates with important biological activities. For example, antioxidative and ACE inhibitory and immunomodulatory activities were observed in the hydrolysates obtained from whey protein using alcalase [11–13]. Although whey protein is rich in bioactive peptides, it is not easily broken down by proteases [14]. Therefore, some studies have focused on the ultrasonic pretreatment method to enhance the enzymatic hydrolysis process [14]. However, the literature about the application of ultrasonic pretreatment to improve whey protein hydrolysis is very limited. Thus, the main objective of this study was to determine the effects of ultrasonic pretreatment on the physicochemical properties of whey protein, its enzymolysis thermodynamics, and the hydrolysate bioactivity. This research should contribute to our further study on the mechanism of ultrasound-accelerated enzymatic hydrolysis of whey protein.

2. Materials and methods

2.1. Materials and chemicals

Whey protein powder (86.5% protein content) manufactured from cow milk, which consisted mainly of β-lactoglobulin and α-lactalbumin (α-lactalbumin- to- β-lactoglobulin ratio of 1: 5, w/w), was purchased from Huangchao Chemical Products Co. (Zhengzhou, China). Alcalase was purchased from Novozymes (China) Biotechnology Co. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 1-anilino-8-naphthalene sulfonate (ANS), ACE (from rabbit lung), Hippuryl-His-Leu (HHL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Trading Co. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Gibico (Grand Island, NY, USA). Other reagents were of analytical grade.

2.2. Test of the effect of ultrasonic pretreatment on the degree of hydrolysis (DH) of whey protein

2.2.1. Ultrasonic pretreatment of whey protein

Ultrasonic pretreatment was carried out in an ultrasonic processor (JY92-II, Haishukesheng Ultrasonic Equipment Co., Ningbo, China) with a 1.5 cm flat tip probe (20 kHz). Whey protein (10 g) was suspended in 200 ml of deionized water and mixed with a magnetic stirrer. Then the suspension was put into a 250 ml beaker, and the beaker was kept in an ice bath. The sonication was done at levels of power of 0 W (control), 100 W, 200 W, 300 W, 400 W and 500W for 15 min (pulse durations of 2 s on and 2 s off), respectively. The temperature of the protein solution was observed during ultrasound to ensure temperature below 25 °C. After ultrasonic pretreatment, the treated protein solution was then used to analyze the effect of ultrasound power on the degree of hydrolysis (DH) of whey protein. According to the changes of DH, an optimal power was chosen. Then 200 ml of whey protein solution at a concentration of 5% w/v was treated at above selected ultrasonic power for different time (0, 5, 10, 15, 20, 25 min). Then, the treated protein solution was used to analyze the effect of ultrasound time on DH of whey protein.

2.2.2. Whey protein hydrolysis and determination of DH

In this study, the untreated and treated whey proteins were hydrolysed by alcalase (enzyme to substrate ratio, 5000 U/g of protein) at substrate concentrations of 10 g/L. The other enzymolysis conditions were: hydrolysis temperature 55 °C, pH 8.0, hydrolysis time 30 min. The hydrolysis conditions were chosen according to our previous experiments.

The DH was determined using the pH-stat method [8].

$$DH\,(\%) = \frac{BN_b}{\alpha M_p h_{tot}} \times 100 \tag{1}$$

where B is the base consumption, N_b is the base normality, α is the average degree of dissociation of the α-NH_2 groups in the protein substrate, M_p is the mass of hydrolyzed protein, and h_{tot} is the total number of peptide bonds in the protein substrate (9.05 mmol/g protein).

2.3. Measurement of the effect of ultrasonic pretreatment on the thermodynamics of whey protein hydrolysis by alcalase

2.3.1. Ultrasonic pretreatment test

An aliquot (200 ml) of whey protein solution (10 g/L) was treated with ultrasound at 20 kHz at 300 W for 15 min (pulse durations of 2 s on and 2 s off). In this study, the conditions of ultrasonic pretreatment were chosen according to above single factor experiment. After ultrasonic pretreatment, the treated whey protein was used to analyze thermodynamics of hydrolysis.

2.3.2. Determination of thermodynamics parameters

A 200 ml volume of untreated or ultrasound-pretreated whey protein solution (10 g/L) was adjusted to pH 8.0 and hydrolysed using alcalase (enzyme- to- substrate ratio, 5000 U/g of protein) at the temperatures of 20–50 °C, respectively.

The alcalase hydrolysis of whey protein can be simply described by the first-order kinetic equation, according to Ma et al. [15]:

$$\ln C = -k_{in} t + lnC_0 \tag{2}$$

where t is the hydrolysis time, C_0 is the initial whey protein concentration, C is the whey protein concentration at a determined time t, and k_{in} is the effective (total) rate constant. As it is difficult to measure the decrement of whey protein directly, the reaction rate can be reflected by the decreased amount of peptide bonds in the protein. The total number of peptide bonds (h_{tot}) was estimated to be 9.05 mmol/g, as determined from the amino acid composition [16]. The decreased amount of peptide bonds (h_{dec}) was calculated as a function of the DH value [8]:

$$h_{dec} = h_{tot} \times DH \tag{3}$$

The observed value of k_{in} can be determined by Eq. (4):

$$\ln C_{pb} = -k_{in} t + lnC_{tpb} \tag{4}$$

where C_{pb} is the concentration of peptide bonds in the whey protein at time $t = t$; C_{tpb} is the total concentration of peptide bonds in the whey protein, which is based on the value of h_{tot} (9.05 mmol/g protein).

The temperature dependence of the rate constant k_{in} can be described by Arrhenius equation:

$$k_{in} = Ae^{\frac{-E_a}{RT}} \tag{5}$$

where A is pre-exponential or collision factor, Ea is the activation energy, R is the universal gas constant (8.314 J/mol K), T is the Kelvin temperature. Eq. (5) is solved to give the logarithmic equation,

$$\ln k_{in} = \ln A - \frac{E_a}{RT} \tag{6}$$

Eq. (6) is used to calculate E_a and A by plotting $\ln k_{in}$ versus $1/T$. The plot of $\ln k_{in}$ versus $1/T$ shall give a straight line. The slope and intercept are $-E_a/R$ and $\ln A$ respectively, from which E_a and A can be calculated.

Thermodynamic parameters for alcalase hydrolysis of whey protein were estimated using the Eyring transition state theory, as shown in Eq. (7):

$$k_{in} = \frac{k_B T}{h}\exp\left(-\frac{\Delta G}{RT}\right) = \frac{k_B T}{h}\exp\left(-\frac{\Delta H}{RT} + \frac{\Delta S}{R}\right) \quad (7)$$

where k_B is the Boltzmann constant (1.38×10^{-23} J/K), h is the Planck constant (6.6256×10^{-34} J/s), ΔS is the entropy of activation, ΔG is the free energies of activation, ΔH is the enthalpy of activation. Eq. (7) is solved to give the logarithmic equation,

$$\ln\left(\frac{k_{in}}{T}\right) = -\frac{\Delta H}{R}\cdot\frac{1}{T} + \frac{\Delta S}{R} + \ln\left(\frac{k_B}{h}\right) \quad (8)$$

Eq. (8) is used to calculate ΔH and ΔS by plotting $\ln k_{in}/T$ versus $1/T$. Then ΔG can be calculated according to the values of ΔH and ΔS using Eq. (9):

$$\Delta S = (\Delta H - \Delta G)/T \quad (9)$$

2.4. Test of effect of ultrasonic pretreatment on physicochemical properties of whey protein

2.4.1. Ultrasonic pretreatment of whey protein

Two-hundred milliliter whey protein solution (10 g/L) was treated with ultrasound at 20 kHz at 300 W for 15 min (pulse durations of 2 s on and 2 s off).

2.4.2. Determination of free sulfhydryl (SH) content

The SH content was measured according to the method of Beveridge et al. [17] with some modifications. For measuring the total free SH content, 0.5 ml of whey protein solution (0.2%, w/v) was added to 2.5 ml of Tris-Gly buffer (0.1 M Tris, 0.1 M glycine, 0.5% (w/v) SDS, 4 mM EDTA, 8 M urea, pH 8.0) and 0.02 ml of Ellman's reagent (DTNB in Tris-Gly buffer, 4 mg/ml). The mixture was then incubated at 25 °C for 30 min and measured at 412 nm by a UV-2450 spectrophotometer (Shimadzu, Japan). Surface free SH content was measured using the same system in the absence of urea. SH content was calculated as follow:

$$\text{SH } (\mu\text{M/g}) = (73.53 \times A_{412} \times D)/C \quad (10)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration in mg/ml, and D is the dilution factor, 6.04.

2.4.3. Determination of surface hydrophobicity

Surface hydrophobicity was determined following the method of Wang et al. [18] with some modifications. The proteins were dissolved in 0.1 M phosphate buffer (pH 7.0) at series of concentrations (0.00625%, 0.0125%, 0.025%, 0.05% and 0.1%, w/v), and 20 μl of ANS (8.0 mM in 0.1 M phosphate buffer, pH 7.0) was added to of diluted protein solutions. The fluorescence intensity (FI) was determined using a spectrofluorometer (F-4600, Hitachi Co., Japan) at excitation wavelength of 390 nm and emission wavelength of 470 nm using slit width of 2.5 nm. Surface hydrophobicity was defined as the initial slope of the FI versus the protein concentration plot.

2.4.4. Raman spectroscopic analysis

Raman spectroscopic experiment was determined according to the method of Shao et al. [19] with a some modifications. The Raman spectrum of each sample was measured using a 785 nm excitation laser with 1 mW of power. Each sample was tested in triplicate. The spectrum was obtained in the range of 400–2700 cm^{-1}. Conditions of Raman spectroscopic experiment were as follow: laser wavelength of 785 nm, three scans, exposure time of 10 s, sampling points 1044, sampling temperature 25 °C. The obtained protein spectrum was smoothed and baselined using software, and its intensity was normalized against the phenylalanine peak at 1004 cm^{-1} [20]. To calculate the secondary structure contents of whey protein. The normalized protein spectrum (amide I region, 1610–1700 cm^{-1}) was truncated and deconvoluted using Gaussian and Lorenztian programme of software. The secondary structures were measured as percentages of α-helix, β-turn, β-sheet and random coil following the method of Ngarize et al. [20].

2.5. Test of effect of ultrasonic pretreatment on the bioactivities of whey protein hydrolysate

2.5.1. Preparation of whey protein hydrolysate

Two-hundred milliliter whey protein solution (10 g/L) was treated with ultrasound at 20 kHz at 300 W for 15 min (pulse durations of 2 s on and 2 s off). Then the treated whey protein (untreated whey protein was used as a control) was hydrolysed by alcalase (enzyme to substrate ratio, 5000 U/g of protein) at 55 °C and pH 8.0 for 30 min. The reaction was stopped by heating the mixture in a boiling water bath for 10 min. After centrifugation, the supernatant was lyophilized and stored at −20 °C until used.

2.5.2. Determination of ACE inhibitory activity

ACE inhibitory activity was determined according to our previously described method [21]. Sample solution, HHL and ACE were prepared in 0.1 M borate buffer, pH 8.3, containing 0.3 M NaCl. 10 μl of sample solution, 45 μl of 6.5 mM HHL and 10 μl of 100 mU/ml ACE were placed in test tube and mixed. The mixture was incubated at 37 °C for 30 min. After reaction, HCl (85 μl, 1 M) was added to stop the reaction. For the blank group, HCl (85 μl, 1 M) were added before the incubation in this study. Then ethyl acetate (1 ml) was used to extract hippuric acid from the reaction mixture. The obtained hippuric acid was dissolved in distilled water, and its absorbance value was measured at 228 nm. ACE inhibition activity was calculated as follows:

$$\text{ACE inhibitory activity}(\%) = \frac{C - S}{C - B} \times 100 \quad (11)$$

where *C, S* and *B* are the absorbance value without sample, the absorbance value with sample, and the absorbance value of blank (HCl was added before incubation), respectively.

2.5.3. Determination of immunomodulatory activity

The immunomodulatory activity was determined according to the method of Wu et al. [22] by using the proliferation test of mouse splenocyte. The spleens from male mice were crushed and washed through a sterilized coppermesh (200 mesh) by 5 ml of RPMI-1640 to obtain a suspension of single spleen cells. The mixture with spleen cells was centrifuged at 4000*g* for 10 min at 4 °C. The precipitates (spleen cells) were resuspended in 5 ml of Tris-NH_4Cl buffer (0.14 M NH_4Cl and 20 mM Tris), and incubated at 37 °C for 3 min to lyse erythrocytes. After centrifugation (4000*g* for 10 min at 4 °C), the obtained spleen cells were washed twice in RPMI-1640 medium, and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at a concentration of 5×10^6 cells/ml. Isolated splenocytes (100 μl/well) were seeded in 96-well plates in the presence or absence of sample (100 μl) with different concentrations (50, 100 and 200 μg/ml), and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 48 h. Then 20 μl of MTT solution (5 mg/ml) was added to each well, and incubated at 37 °C for another 4 h. After incubation, the MTT medium was discarded and DMSO (100 μl/well) was added. The absorbance was measured at 570 nm by an EL310 microplate reader (Bio-TKE Instruments, USA). In this paper, Con A (5 μg/ml) was used as the positive control. Each sample was analyzed six times, and the values were averaged.

2.6. Statistical analysis

All values were expressed as the mean ± standard deviations from three replications. Dates were analyzed and statistical significance was determined by SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). For the data of thermodynamic parameters, SH content, secondary structure, ACE inhibitory and immunomodulatory activities of whey protein

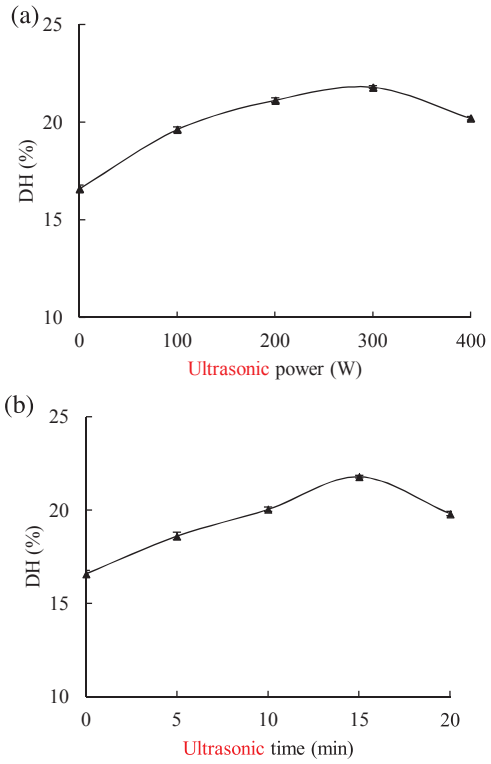


Fig. 1. Effects of ultrasonic power (a) and ultrasonic time (b) on the degree of hydrolysis (DH) of whey protein.

hydrolysates, an analysis of variance (ANOVA) using the general linear model procedure of Statistical Analysis System was performed. The means of variable among different treatments were compared using the Duncan's multiple comparisons. Significant difference between the mean values was determined with a 95% confidence interval ($P < 0.05$).

3. Results and discussion

3.1. Effect of ultrasonic pretreatment on the degree of hydrolysis (DH) of whey protein

Fig. 1 shows the effects of ultrasonic power and ultrasonic time on the DH of whey protein. The DH value initially increased with increasing ultrasonic power and time up to a point beyond which it decreased with the further increase in both ultrasonic parameters, which was similar to the results obtained by Wang et al. [4]. Compared with that of the untreated whey protein, the DH of the ultrasonicated protein was significantly higher. Our results suggested that ultrasonic pretreatment was able to promote the enzymolysis of whey protein. This phenomenon might be due to the disruption of the protein structure by ultrasound, whereupon its enzyme binding sites were exposed [4,8]. Uluko et al. [23] and Resendiz-Vazquez et al. [5] reported that ultrasonic pretreatment increased the DH of milk protein concentrate and jackfruit seed protein isolates, which is in agreement with the results obtained in this present study. On the contrary, another study found that the DH of rice protein was not increased significantly after ultrasonic pretreatment ($P > 0.05$) [7]. The contrasting results may be attributed to apparent differences in the protein type and nature among the different proteins studied.

3.2. Effect of ultrasound on thermodynamic parameters for alcalase hydrolysis of whey protein

The rate constant, an important kinetics parameter, is dependent on the reaction temperature, medium, and catalyst. Its values for the alcalase hydrolysis of the untreated and pretreated whey proteins at 20–50 °C could be obtained by plotting ln C_{pd} versus time (Fig. 2). The alcalase hydrolysis reactions for both protein samples obeyed first-order kinetics, with regression coefficients (R^2) greater than 0.90. The rate constants (k_{in}) based on Eq. (4), at various temperatures, are shown in the inserted table of Fig. 2. The k_{in} values for hydrolysis of the untreated and pretreated whey proteins increased with increasing temperature, which might be due to an enhancement of the collision frequency between the whey protein and alcalase molecules at higher temperatures [15]. However, compared with the untreated protein, the ultrasonicated sample had a significantly higher k_{in} value. This phenomenon might be caused by secondary structural changes of the whey protein induced by the ultrasonication, whereupon more cleavage sites were exposed, allowing improved contact with alcalase [4].

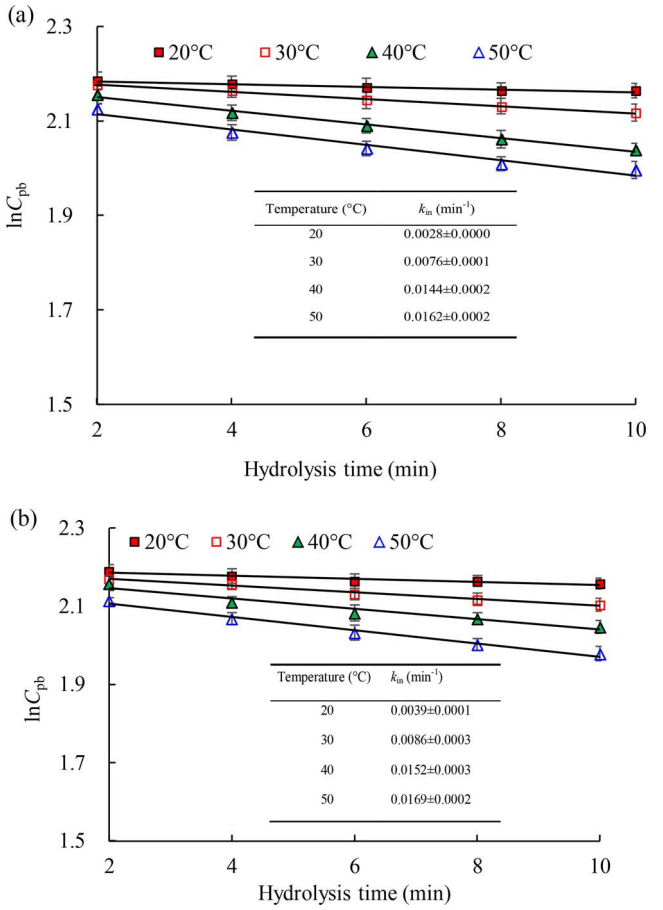
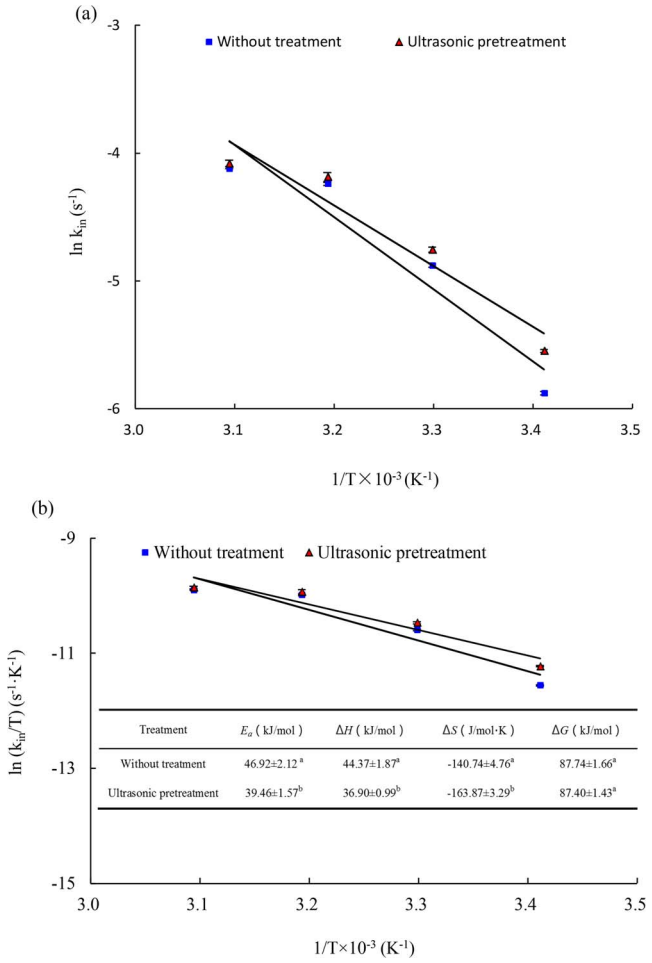


Fig. 2. First-order plots for untreated (a) and ultrasound-pretreated (b) whey protein hydrolysis by alcalase at various temperatures. The inserted table showed the reaction rate constants (k_{in}) for the hydrolysis of untreated (a) and pretreated (b) whey proteins. The regression coefficients (R^2) of the curves obtained at 20, 30, 40 and 50 °C for untreated whey protein was 0.938, 0.995, 0.991 and 0.963, respectively. The R^2 of the curves obtained at 20, 30, 40 and 50 °C for pretreated whey protein was 0.902, 0.987, 0.984 and 0.984, respectively.

The activation energy (E_a) for the hydrolysis reaction, which is the minimum energy required to start a chemical reaction, was calculated by determining the slope ($-E_a/R$) of the Arrhenius plot (ln k_{in} versus 1/T, where T is the temperature) in the range of 20–50 °C (Fig. 3a). E_a was found to be 46.92 and 39.46 kJ/mol for the untreated and pretreated whey proteins, respectively. Compared with the untreated protein, the ultrasonicated protein had a significantly lower E_a value ($P < 0.05$), which revealed that ultrasound had strongly decreased the energy barrier required for whey protein hydrolysis [24]. To calculate the entropy of activation (ΔS) and the enthalpy of activation (ΔH) for whey



Treatment	E_a (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
Without treatment	46.92±2.12 ^{a}	44.37±1.87^{a}	-140.74±4.76^{a}	87.74±1.66^{a}
Ultrasonic pretreatment	39.46±1.57^{b}	36.90±0.99^{b}	-163.87±3.29^{b}	87.40±1.43^{a}

Fig. 3. Arrhenius plot of ln k_{in} versus 1/T (a), and Eyring plot of ln (k_{in}/T) versus 1/T (b). The inserted table showed thermodynamic parameters for untreated and ultrasonic-treated whey protein hydrolysis by alcalase in temperature range 20–50 °C; data expressed are the mean ± standard deviation; mean values with different letters in the same column are significantly different ($P < 0.05$).

protein hydrolysis, the Eyring transition state theory was used. The thermodynamic parameters calculated are summarized in the inserted table of Fig. 3b. After ultrasonic pretreatment, the ΔH and ΔS values for whey protein hydrolysis were significantly decreased ($P < 0.05$), while the free energy (ΔG) for hydrolysis did not alter significantly ($P > 0.05$). The reduction of ΔH and ΔS values might be due to an increase in the affinity between the whey protein and enzyme caused by the stretching and unfolding of the protein molecules by the ultrasonication. Similar results were reported by Cheng et al. [25], who found that the thermodynamic parameters (i.e., E_a, ΔH, and ΔS) of potato protein enzymolysis were reduced by ultrasonic pretreatment while ΔG increased only slightly. Jin et al. [26] also reported that ultrasonic pretreatment decreased the E_a, ΔH, and ΔS values of corn gluten meal enzymolysis, but had little effect in on the ΔG value. Our results demonstrated that ultrasound can remarkably improve the efficiency of whey protein enzymolysis, and thus can be used as a powerful tool for enzyme-catalyzed protein hydrolysis.

3.3. Effect of ultrasonic pretreatment on the physicochemical properties of whey protein

3.3.1. Changes in the sulfhydryl content of whey protein

As shown in Fig. 4, the content of surface free sulfhydryl (SH) and total free SH in untreated whey protein was 5.81 and 27.32 μmol/g, respectively. After ultrasonic pretreatment, the content of surface free

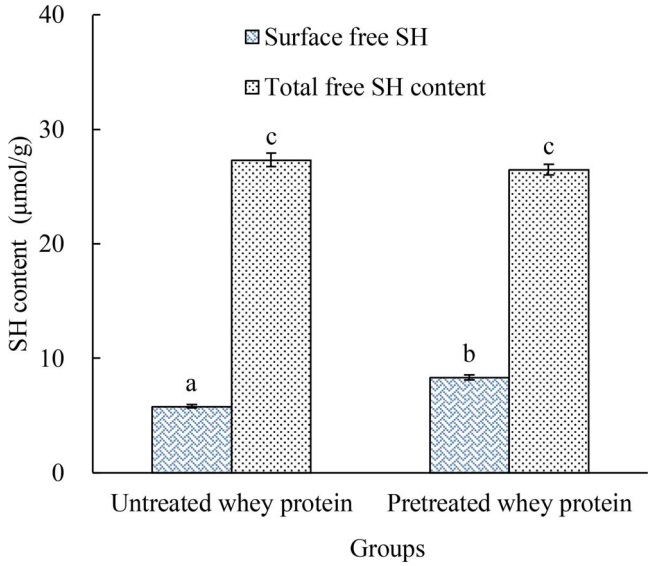


Fig. 4. Contents of surface free SH and total free SH in untreated and ultrasound-pretreated whey proteins. Ultrasonic pretreatment conditions: pretreated sample, 200 ml; pretreatment time, 15 min (pulse durations of 2 s on and 2 s off); ultrasonic power, 300 W. Results represent the means of three determinations ± standard deviation. Values with different lowercase letters are significantly different for Surface free SH and total free SH ($P < 0.05$).

SH was increased significantly ($P < 0.05$), which might be due to the ultrasound-mediated unfolding of the protein molecules, thereby exposing the SH groups to the outer surface of the protein molecules [4]. Similar results of exposed SH content changes after ultrasonic treatment have been observed in chicken actomyosin [27] and a sunflower meal protein isolate [28]. Because there were no significant differences in the total free SH content, the disulfide bond of whey protein was not affected by the ultrasonic pretreatment.

3.3.2. Changes in the surface hydrophobicity of whey protein

The surface hydrophobicities of the untreated and ultrasound-pretreated (300W, 15 min) whey proteins were analysed by using the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (Fig. 5). The surface hydrophobicity was measured from the initial slope of the plot of fluorescence intensity versus whey protein concentration, and was 502.1 and 816.6 for the untreated and pretreated whey proteins, respectively. This indicated that ultrasound could induce unfolding of the protein molecule, destroy hydrophobic interactions of the molecule, and cause greater exposure of the interior hydrophobic groups [8].

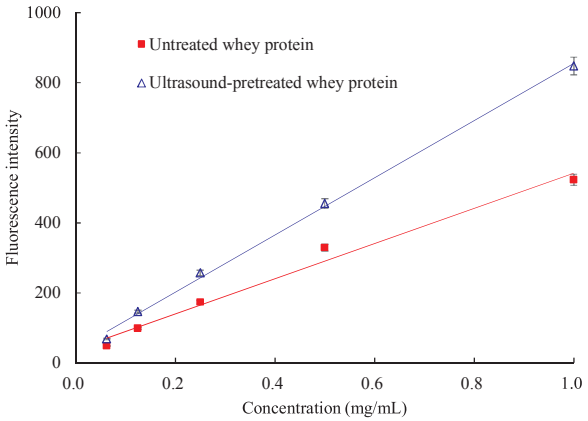


Fig. 5. Fluorescence intensities of untreated and ultrasound-pretreated whey proteins (using ANS as a fluorescence probe) at different concentrations. Ultrasonic pretreatment conditions: pretreated sample, 200 ml; pretreatment time, 15 min (pulse durations of 2 s on and 2 s off); ultrasonic power, 300 W.

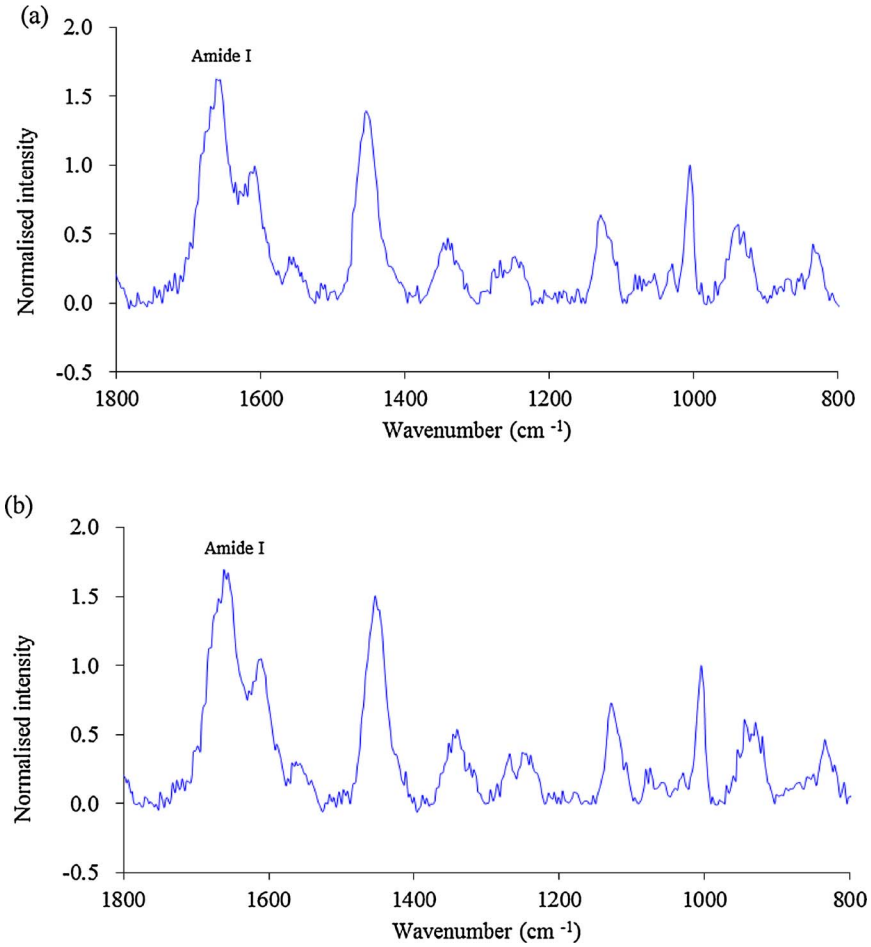


Fig. 6. Raman spectra (800–1800 cm^{-1}) of untreated (a) and ultrasound-pretreated (b) whey protein after normalization (the spectra were normalized to the phenylalanine peak at 1004 cm^{-1}).

Other studies have revealed that ultrasonic pretreatment could also increase the surface hydrophobicity of some proteins, such as sunflower protein [28], tilapia (*Oreochromis niloticus*) protein [29], and duck liver protein [30].

3.3.3. Changes in the secondary structure of whey protein

Raman spectroscopy was used to study the secondary structure of the whey protein. The typical Raman spectra of the untreated and pretreated whey proteins in the 800–1800 cm^{-1} range are shown in Fig. 6. The absorption bands within the 1600–1700 cm^{-1} range are the characteristic absorption peaks of amide I, which is assigned to C═O stretching/hydrogen bonding coupled with −COO [31]. The different bands of amide I were assigned to specific protein secondary structures according to previous studies [20,32,33]. In whey protein, the bands at 1650–1660, 1670–1675, 1680–1685, and 1660–1670 cm^{-1} (Fig. 7) can be assigned to α-helices, β-sheets, β-turns and random coil structures, respectively. As shown in Fig. 7, the nine major bands related to secondary structure were evident in the spectra of untreated and pretreated whey proteins, respectively. On the basis of the amide I band profile analysis, the percentages of secondary structures in the untreated and pretreated whey proteins were obtained (Table 1). Untreated whey protein was composed of 46.23% α-helices, 11.25% β-sheets, 16.26% β-turns and 26.25% random coils, whereas ultrasound-pretreated whey protein was made up of 44.75% α-helices, 12.28% β-sheets, 16.60% β-turns and 26.37% random coils. Ultrasonic pretreatment had significantly decreased the α-helical content and increased the β-sheets and β-turns in the protein ($P < 0.05$). Similar results were reported by Li et al. [34], who also showed that ultrasonic treatment had significantly decreased the α-helical content and increased the formation of β-sheets and β-turns in breast meat protein of pale, soft, exudative-like chickens. The reduction in the α-helical proportion contributes to protein molecule unfolding, whereas the increase of β-sheets and β-turns contributes to protein aggregates [34,35]. The results may offer an explanation for the improvement in functional properties based on molecule unfolding of the protein. In addition, the result showed that the α-helix is the major secondary structure in whey protein.

3.4. Effects of ultrasonic pretreatment on the bioactivity of whey protein hydrolysate

The effects of ultrasonic pretreatment on the ACE inhibitory activity of the whey protein hydrolysate are shown in Fig. 8. Compared with that of the untreated hydrolysate, ultrasonication significantly increased the ACE inhibitory activity of the pretreated hydrolysate ($P < 0.05$). This phenomenon might be caused by the surface hydrophobicity changes of the protein (see Fig. 5). The molecular unfolding of the whey protein caused by ultrasound increased its surface hydrophobicity, which facilitated the release of ACE inhibitory peptides during the enzymatic hydrolysis [36]. Similar findings were reported by Zhou et al. [37], who reported that ultrasonic pretreatment of defatted wheat germ proteins could accelerate the release of ACE inhibitory peptides during alcalase hydrolysis. Furthermore, Uluko et al. [23] reported that ultrasonic pretreatment had a significant effect on the ACE inhibitory activity of hydrolysates of milk protein concentrate, and found that a short ultrasonic pretreatment time coupled with a high

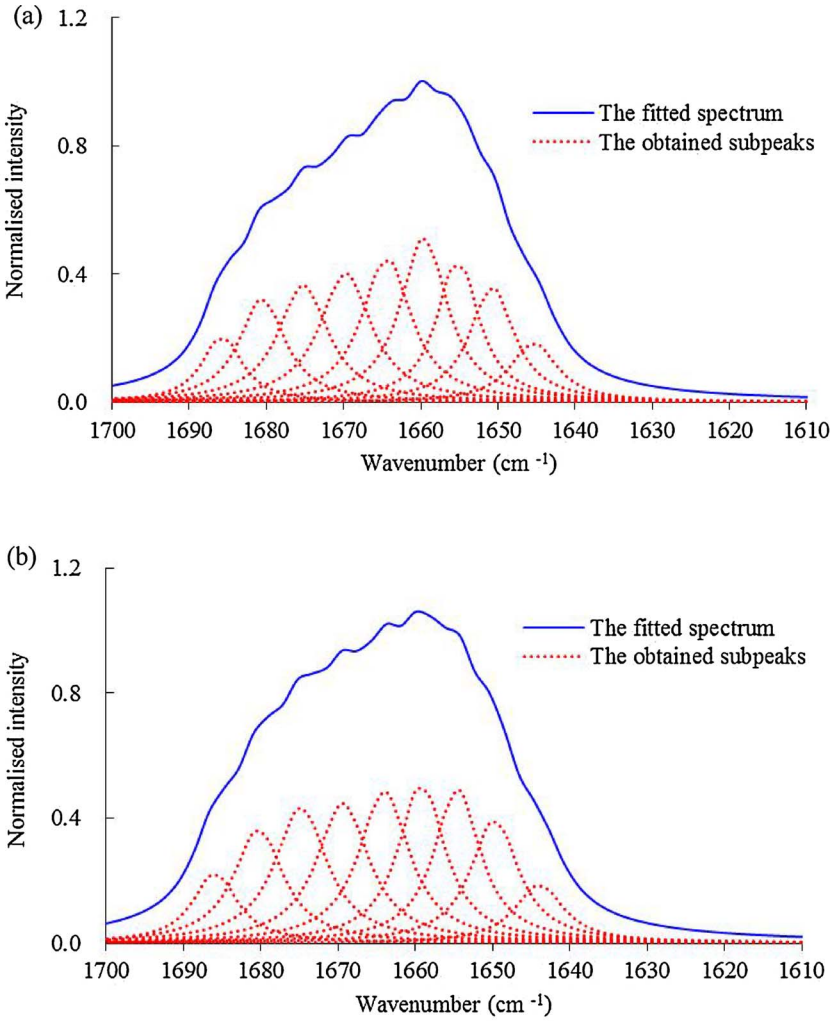


Fig. 7. Fitting Raman spectra of untreated (a) and ultrasound-pretreated (b) whey protein at amide I region (1610–1700 cm^{-1}) after deconvolution.

Table 1
Secondary structure contents of untreated and ultrasound-pretreated whey protein.

Proteins	Secondary structure contents (%)			
	α-helixes	Random coils	β- sheets	β-turns
Untreated whey protein	46.23 ± 0.06[a]	26.25 ± 0.12[a]	11.25 ± 0.21[a]	16.26 ± 0.10[a]
Ultrasonic-treated whey protein	44.75 ± 0.13[b]	26.37 ± 0.19[a]	12.28 ± 0.16[b]	16.60 ± 0.20[b]

Data expressed are the mean ± standard deviation (SD). Mean values with different letters in the same column are significantly different ($P < 0.05$).

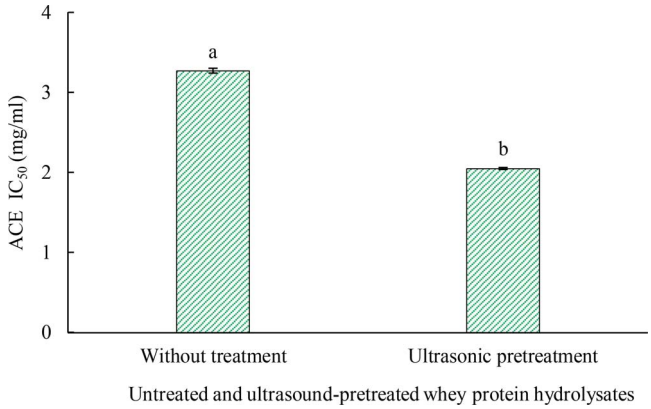


Fig. 8. ACE-inhibitory activities of untreated and ultrasound-pretreated whey protein hydrolysates. Results represent the mean ± standard deviation (SD). Means with different superscripts are significantly different ($P < 0.05$).

enzyme-to-substrate ratio yielded high ACE-inhibitory activity.

The spleen is the largest immune organ in the body, and in vitro evaluation of the splenocytes can reflect the systemic immune status. The effects of the untreated and ultrasound-pretreated whey protein hydrolysates on mouse splenocyte proliferation are shown in Table 2. Both types of hydrolysates promoted splenocyte proliferation, which was increased in a dose-dependent manner at concentrations ranging from 25 to 100 μg/ml of hydrolysate. However, the splenocyte proliferation effect of ultrasound-pretreated hydrolysate was significantly higher ($P < 0.05$). In addition, 100 μg/ml of the pretreated hydrolysate produced a splenocyte proliferation activity close to that of the positive control group (Con A). Although the mechanism remains unclear, it is a fact that the immunomodulatory capacity of bioactive peptides is greatly influenced by their physicochemical characteristics, such as length, charge, and hydrophobicity [38,39]. Chalamaiah et al. [40] reported that lower molecular weight peptides with higher hydrophobic residues can exert higher immunomodulatory activities. It was therefore speculated that the ultrasonic treatment used to increase the surface hydrophobicity of whey protein and promote its hydrolysis

Table 2
Effects of untreated and ultrasound-pretreated whey protein hydrolysates on mouse splenocyte proliferation.

Sample	Absorbance at 570 nm Dose (μg/ml)				
	0	5	25	50	100
Control	0.244 ± 0.018	–	–	–	–
Positive control (Con A)	–	1.102 ± 0.006	–	–	–
Untreated hydrolysate	–	–	0.745 ± 0.009[a]	0.905 ± 0.012[a]	0.950 ± 0.019[a]
Ultrasound-pretreated hydrolysate	–	–	0.790 ± 0.011[b]	0.955 ± 0.007[b]	1.050 ± 0.016[b]

Data expressed are the mean ± standard deviation (SD). Mean values with different letters in the same column are significantly different ($P < 0.05$).

would ultimately lead to an increase in the release of immunomodulatory peptides with hydrophobic residues and low molecular weight. To the best of our knowledge, no previous work has been reported on the effect of ultrasonic pretreatment on the immunomodulatory activity of whey protein hydrolysates. The fact suggested that the ultrasound-pretreated whey protein hydrolysate showed strong splenocyte proliferation activity suggests that the humoral immune function would be enhanced as well.

4. Conclusions

This work evaluated the changes in the enzymolysis thermodynamics and physicochemical properties of whey protein after ultrasonic pretreatment and the effects on the ACE inhibitory and immunomodulatory activities of its hydrolysate. The thermodynamics analysis revealed that ultrasonic pretreatment decreased the E_a, ΔH and ΔS values for whey protein enzymolysis, thereby improving the affinity of the enzyme to the whey protein and hydrolysis. Ultrasound (20 kHz, 300 W, and 15 min) induced the unfolding of whey protein by high cavitation shear forces, which caused increases in the surface free SH content and surface hydrophobicity. Ultrasound also changed the protein's secondary structure, where the content of α-helices was significantly decreased, while the β-sheets and β-turn were significantly increased. Furthermore, the in vitro ACE inhibitory and immunomodulatory activities of the whey protein hydrolysates were significantly increased by the ultrasonic pretreatment. The results of this study should provide a theoretical basis for the industrial production of peptide drugs from whey protein by ultrasonication.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

This work was supported by National Natural Science Foundation of China (No. 31401641).

References

[1] S.U. Kadam, B.K. Tiwari, C. Alvarez, C.P. O'Donnell, Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides, Trends Food Sci. Technol. 46 (1) (2015) 60–67.
[2] J. O'Sullivan, B. Murray, C. Flynn, I. Norton, The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins, Food Hydrocolloids 53 (2016) 141–154.
[3] R.P. Frydenberg, M. Hammershoj, U. Andersen, M.T. Greve, L. Wiking, Protein denaturation of whey protein isolates (WPIs) induced by high intensity ultrasound during heat gelation, Food Chem. 192 (2016) 415–423.
[4] Y. Wang, Z. Wang, C.L. Handa, J. Xu, Effects of ultrasound pre-treatment on the structure of beta-conglycinin and glycinin and the antioxidant activity of their hydrolysates, Food Chem. 218 (2017) 165–172.
[5] J.A. Resendiz-Vazquez, J.A. Ulloa, J.E. Urias-Silvas, P.U. Bautista-Rosales, J.C. Ramirez-Ramirez, P. Rosas-Ulloa, L. Gonzalez-torres, Effect of high-intensity ultrasound on the technofunctional properties and structure of jackfruit (*Artocarpus heterophyllus*) seed protein isolate, Ultrason. Sonochem. 37 (2017) 436–444.
[6] B. Wang, G.G. Atungulu, R. Khir, J. Geng, H. Ma, Y. Li, B. Wu, Ultrasonic treatment effect on enzymolysis kinetics and activities of ace-inhibitory peptides from oat-isolated protein, Food Biophys. 10 (3) (2015) 244–252.
[7] X. Yang, Y. Li, S. Li, A.O. Oladejo, S. Ruan, Y. Wang, S. Huang, H. Ma, Effects of ultrasound pretreatment with different frequencies and working modes on the enzymolysis and the structure characterization of rice protein, Ultrason. Sonochem. 38 (2017) 19–28.
[8] J. Jia, H. Ma, W. Zhao, Z. Wang, W. Tian, L. Luo, R. He, The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein, Food Chem. 119 (1) (2010) 336–342.
[9] V. Ambrosi, G. Polenta, C. Gonzalez, G. Ferrari, P. Maresca, High hydrostatic pressure assisted enzymatic hydrolysis of whey proteins, Innov. Food Sci. Emerg. Technol. 38 (2016) 294–301.
[10] X. Shen, T. Fang, F. Gao, M. Guo, Effects of ultrasound treatment on physicochemical and emulsifying properties of whey proteins pre- and post-thermal aggregation, Food Hydrocolloids 63 (2017) 668–676.
[11] R.J.S. de Castro, H.H. Sato, Advantages of an acid protease from *Aspergillus oryzae* over commercial preparations for production of whey protein hydrolysates with antioxidant activities, Biocatal. Agric. Biotechnol. 3 (3) (2014) 58–65.
[12] E.L. da Costa, J.A. da Rocha Gontijo, F.M. Netto, Effect of heat and enzymatic treatment on the antihypertensive activity of whey protein hydrolysates, Int. Dairy J. 17 (6) (2007) 632–640.
[13] J.-J. Ma, X.-Y. Mao, Q. Wang, S. Yang, D. Zhang, S.-W. Chen, Y.-H. Li, Effect of spray drying and freeze drying on the immunomodulatory activity, bitter taste and hygroscopicity of hydrolysate derived from whey protein concentrate, Lwt-Food Sci. Technol. 56 (2) (2014) 296–302.
[14] L. Abadía-García, E. Castaño-Tostado, L. Ozimek, S. Romero-Gómez, C. Ozuna, S.L. Amaya-Llano, Impact of ultrasound pretreatment on whey protein hydrolysis by vegetable proteases, Innov. Food Sci. Emerg. Technol. 37 (2016) 84–90.
[15] H. Ma, L. Huang, J. Jia, R. He, L. Luo, W. Zhu, Effect of energy-gathered ultrasound on Alcalase, Ultrason. Sonochem. 18 (1) (2011) 419–424.
[16] L. Huang, H. Ma, L. Peng, Z. Wang, Q. Yang, Enzymolysis kinetics of garlic powder with single frequency countercurrent ultrasound pretreatment, Food Bioprod. Process. 95 (2015) 292–297.
[17] T. Beveridge, S.J. Toma, S. Nakai, Determination of SH- and SS-groups in some food proteins using Ellman's reagent, J. Food Sci. 39 (1974) 49–51.
[18] L. Wang, Y. Xu, S. Zhou, H. Qian, H. Zhang, X. Qi, M. Fan, Interaction between *Vaccinium bracteatum* Thunb. leaf pigment and rice proteins, Food Chem. 194 (2016) 272–278.
[19] J.-H. Shao, Y.-F. Zou, X.-L. Xu, J.-Q. Wu, G.-H. Zhou, Evaluation of structural changes in raw and heated meat batters prepared with different lipids using Raman spectroscopy, Food Res. Int. 44 (9) (2011) 2955–2961.
[20] S. Ngarize, H. Herman, A. Adams, N. Howell, Comparison of changes in the secondary structure of unheated, heated, and high-pressure-treated beta-lactoglobulin and ovalbumin proteins using fourier transform Raman spectroscopy and self-deconvolution, J. Agric. Food Chem. 52 (21) (2004) 6470–6477.
[21] Q. Wu, J. Du, J. Jia, C. Kuang, Production of ACE inhibitory peptides from sweet sorghum grain protein using alcalase: hydrolysis kinetic, purification and molecular docking study, Food Chem. 199 (2016) 140–149.
[22] F. Wu, H. Yan, X. Ma, J. Jia, G. Zhang, X. Guo, Z. Gui, Comparison of the structural characterization and biological activity of acidic polysaccharides from *Cordyceps militaris* cultured with different media, World J. Microbiol. Biotechnol. 28 (5) (2012) 2029–2038.
[23] H. Uluko, H. Li, W. Cui, S. Zhang, L. Liu, J. Chen, Y. Sun, Y. Su, J. Lv, Response surface optimization of angiotensin converting enzyme inhibition of milk protein concentrate hydrolysates in vitro after ultrasound pretreatment, Innov. Food Sci. Emerg. Technol. 20 (2013) 133–139.
[24] R. Kadkhodaee, M.J.W. Povey, Ultrasonic inactivation of Bacillus alpha-amylase. I. effect of gas content and emitting face of probe, Ultrason. Sonochem. 15 (2) (2008) 133–142.
[25] Y. Cheng, Y. Liu, J. Wu, P.O. Donkor, T. Li, H. Ma, Improving the enzymolysis efficiency of potato protein by simultaneous dual-frequency energy-gathered ultrasound pretreatment: thermodynamics and kinetics, Ultrason. Sonochem. 37 (2017) 351–359.
[26] J. Jin, H. Ma, W. Qu, K. Wang, C. Zhou, R. He, L. Luo, J. Owusu, Effects of multi-frequency power ultrasound on the enzymolysis of corn gluten meal: kinetics and thermodynamics study, Ultrason. Sonochem. 27 (2015) 46–53.
[27] R. Saleem, R. Ahmad, Effect of low frequency ultrasonication on biochemical and structural properties of chicken actomyosin, Food Chem. 205 (2016) 43–51.
[28] M.A. Malik, H.K. Sharma, C.S. Saini, High intensity ultrasound treatment of protein

isolate extracted from dephenolized sunflower meal: effect on physicochemical and functional properties, Ultrason. Sonochem. 39 (2017) 511–519.
[29] J. Tian, Y. Wang, Z. Zhu, Q. Zeng, M. Xin, Recovery of Tilapia (*Oreochromis niloticus*) protein isolate by high-intensity ultrasound-aided alkaline isoelectric solubilization/precipitation process, Food Bioprocess Technol. 8 (4) (2015) 758–769.
[30] Y. Zou, L. Wang, P. Li, P. Cai, M. Zhang, Z. Sun, C. Sun, Z. Geng, W. Xu, X. Xu, D. Wang, Effects of ultrasound assisted extraction on the physiochemical, structural and functional characteristics of duck liver protein isolate, Process Biochem. 52 (2017) 174–182.
[31] Y. Kobayashi, S.G. Mayer, J.W. Park, FT-IR and Raman spectroscopies determine structural changes of tilapia fish protein isolate and surimi under different comminution conditions, Food Chem. 226 (2017) 156–164.
[32] Z.-L. Kang, F.-S. Chen, H.-J. Ma, Effect of pre-emulsified soy oil with soy protein isolate in frankfurters: a physical-chemical and Raman spectroscopy study, Lwt-Food Sci. Technol. 74 (2016) 465–471.
[33] O.O. Oshokoya, R.D. Jiji, Parallel factor analysis of multi-excitation ultraviolet resonance Raman spectra for protein secondary structure determination, Anal. Chim. Acta 892 (2015) 59–68.
[34] K. Li, Z.-L. Kang, Y.-Y. Zhao, X.-L. Xu, G.-H. Zhou, Use of high-intensity ultrasound to improve functional properties of batter suspensions prepared from PSE-like chicken breast meat, Food Bioprocess Technol. 7 (12) (2014) 3466–3477.
[35] Z. Zhang, Y. Yang, P. Zhou, X. Zhang, J. Wang, Effects of high pressure modification on conformation and gelation properties of myofibrillar protein, Food Chem. 217 (2017) 678–686.
[36] J. Jia, Q. Wu, H. Yan, Z. Gui, Purification and molecular docking study of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide from alcalase hydrolysate of ultrasonic-pretreated silkworm pupa (*Bombyx mori*) protein, Process Biochem. 50 (5) (2015) 876–883.
[37] C. Zhou, H. Ma, X. Yu, B. Liu, A.E.-G.A. Yagoub, Z. Pan, Pretreatment of defatted wheat germ proteins (by-products of flour mill industry) using ultrasonic horn and bath reactors: effect on structure and preparation of ACE-inhibitory peptides, Ultrason. Sonochem. 20 (6) (2013) 1390–1400.
[38] B.P. Singh, S. Vij, S. Hati, Functional significance of bioactive peptides derived from soybean, Peptides 54 (2014) 171–179.
[39] H. Hou, Y. Fan, B. Li, C. Xue, G. Yu, Z. Zhang, X. Zhao, Purification and identification of immunomodulating peptides from enzymatic hydrolysates of Alaska pollock frame, Food Chem. 134 (2) (2012) 821–828.
[40] M. Chalamaiah, W. Yu, J. Wu, Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: a review, Food Chem. 245 (2018) 205–222.