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The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein

Junqiang Jia [a], Haile Ma [a,b,c,*], Weirui Zhao [a], Zhenbin Wang [a], Wanmin Tian [a], Lin Luo [a], Ronghai He [a]

[a] School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China
[b] Jiangsu Provincial Research Center of Bio-process and Separation Engineering of Agri-products, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China
[c] School of Food and Biological Engineering, Henan University of Science and Technology, 48 Xiyuan Road, Luoyang 471003, China

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ABSTRACT

The effects of ultrasonic treatment during proteolysis on kinetic characterisation of the hydrolysis of defatted wheat germ protein (DWGP), and on ACE-inhibitory activity of the hydrolysate, were investigated. The effects of ultrasonic pretreatment on the release of peptides with ACE-inhibitory were also studied. The results showed that the value of k_A for DWGP hydrolysis under ultrasonic irradiation increased by about 22.2%, and K_M decreased about 13.0%, compared with that obtained without ultrasound. Analysis of ACE-inhibitory activity indicated that ultrasound during enzyme treatments had less effect on the ACE-inhibitory activity, while ultrasonic pretreatment caused a 21.0–40.7% increase in ACE-inhibitory activity of DWGP hydrolysate. Analyses of hydrophobicity, microstructure, and amino acid composition revealed that ultrasonic pretreatment could accelerate the release of hydrophobic amino acids from DWGP during enzymatic hydrolysis. In conclusion, ultrasonic treatment during proteolysis could facilitate the enzymatic hydrolysis of DWGP, whereas ultrasonic pretreatment could promote the release of ACE-inhibitory peptides from DWGP during enzymatic hydrolysis.

1. Introduction

The angiotensin I-converting enzyme (E.C. 3.4.15.1; ACE) is a zinc metallopeptidase that participates in the synthesis of a hypertensive peptide, angiotensin II, and in the degradation of a hypotensive peptide, bradykinin (Cushman, Cheung, Sabo, & Ondetti, 1982). This enzyme plays a key physiological role in the control of blood pressure, in the rennin–angiotensin system (Ganten, Unger, & Lang, 1984). Consequently, ACE inhibitors may exert an antihypertensive effect. For this reason, many studies have been directed toward the attempted synthesis of ACE inhibitors, such as captopril, alacepril, and lisinopril, which are currently used in the treatment of hypertensive patients, but these substances may provoke undesirable side effects, such as coughing, loss of taste, and renal impairment (Kapel, Rahhou, Lecouturier, Guillochon, & Dhulster, 2006). Therefore, finding safer, more innovative and economical ACE inhibitors is necessary for the prevention and remedy of hypertension. Bioactive peptides have been identified as the functional compounds in food products. For example, a placebo-controlled study in hypertensive human subjects demonstrated a significant reduction in blood pressure after daily ingestion of 95 ml of Calpis sour milk containing the potent ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro (Hata et al., 1996). These peptides are inactive within the sequence of parent proteins, but they can be released by enzyme hydrolysis *in vivo* or *in vitro*. Recently, many ACE-inhibitory peptides have been isolated from processed foods and enzymatic hydrolysates, such as milk (Yamamoto & Takano, 1999), wheat germ (Matsui et al., 2000), and corn gluten meal (Yang, Tao, Liu, & Liu, 2007).

Wheat germ (WG), a byproduct of the flour milling industry, is reported to be one of the potential sources of plant proteins. However, the WG protein source has poor utility for human applications. Defatted wheat germ (DWG) is a highly nutritive protein material, with a relatively high protein content (27.8–30 g/100 g) (Arshad, Anjum, & Zahoor, 2007), and is one of the most attractive sources of vegetable proteins. Several authors have shown that defatted wheat germ protein (DWGP) is a potential protein resource for preparation of ACE-inhibitory peptides (Matsui, Li, & Osajima, 1999; Matsui et al., 2000).

Ultrasound has attracted more and more attention in food science and technology, due to its promising effects, in the food industry. Ultrasound can mainly be classified into two fields: high frequency low-energy ultrasound in the MHz range, and low frequency high-energy power ultrasound in the kHz range. The former is usually used as an analytical technique for providing information about the physicochemical properties of foods, such as composition, structure and physical state (Mason, Paniwnyk, &

* Corresponding author. Address: School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China. Tel.: +86 511 88790958; fax: +86 511 88780201.
E-mail address: mhl@ujs.edu.cn (H. Ma).

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Lorimer, 1996). However, the latter, which is used in the food industry, is relatively new and has been used in the laboratory or processing plant to effect novel changes in the physicochemical properties of foods in various areas, such as nanoemulsion preparation (Kentish et al., 2008), ultrasound-assisted extraction (Ma et al., 2008), and improvement of foaming properties (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008). More recently the interest of food technologists has turned to the use of power ultrasound in altering enzyme activities. Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of a number of food enzymes (Kadkhodaee & Povey, 2008). However, in some cases, solutions containing enzymes have been found to have increased activity following short exposures to ultrasound (Lee, Nguyen, Koo, & Ha, 2008). Furthermore, some authors have recently reported that ultrasound can accelerate the enzymatic hydrolysis of solid leather waste (Song, Tao, & Chen, 2008).

Little is known about the effects of ultrasonic treatment on the proteolysis pattern of DWGP, or on the release of peptides with ACE-inhibitory properties. Therefore, the objective of this work was to evaluate the impacts of ultrasonic treatment during enzymatic hydrolysis on kinetics of the hydrolysis of DWGP, and on ACE-inhibitory activity of hydrolysate. The effects of ultrasonic pretreatment on the release of peptides with ACE-inhibitory, on amino acid composition of hydrolysate, on surface hydrophobicity of DWGP, and on the microstructure of DWGP and hydrolysis residue were also analysed.

2. Materials and methods

2.1. Materials and chemicals

DWG (protein content, 27.6 g/100 g) was purchased from Anyang Mantianxue Protein Co. (Henan, China). ACE (from rabbit lung) was purchased from Sigma–Aldrich Trading Co. (Shanghai, China), and its activity was determined by using Hippuryl-His-Leu (Sigma–Aldrich Trading Co., Shanghai, China) as a substrate. Alcalase 2.4L FG was purchased from Novozymes (China) Biotechnology Co., and its activity was 132,507 U/g (by Folin-phenol method) (Zhou, 1995). All other chemicals and solvents were of analytical grade.

2.2. Protein isolates preparation

Protein isolates were prepared from DWG according to the method of Zhu, Zhou, and Qian (2006) with some modifications. DWG (10 g) was dispersed by stirring for 10 min in 100 ml of deionized water. The suspension was treated with α-amylase at 55 °C (E/S 1/100, wt/wt) for 2 h, and then its pH was adjusted to 9.5 by using 1 M NaOH. After stirring for 30 min, the suspension was centrifuged at 2500*g* and 20 °C for 15 min. The supernatant was adjusted to pH 4.0 with 1.0 M HCl to precipitate the proteins, and centrifuged again at 2500*g* and 20 °C for 15 min. The precipitates were washed several times with distilled water (pH 4.0), dispersed in a small amount of distilled water, and adjusted to pH 7.0 by using the 0.1 M NaOH. The dispersed product was freeze-dried. The protein content was determined by the micro-Kjeldahl method ($N \times 5.83$).

2.3. Sonication during enzymatic hydrolysis

An aliquot (500 ml) of DWGP solution (the concentrations were 1.5, 3, 6, 12, 24 g/l, respectively) was prepared and adjusted to pH 8.0 with 1 M NaOH, and then incubated in a water bath at 50 °C. After 10 min, 50 mg (6625.4 U) of alcalase were added. The mixtures of protein and enzyme were immediately treated using an ultrasonic cell crusher (GA92-IIDB, Shangjia Biotechnology Co., Wuxi, China) with a 0.6 cm flat tip probe at 20 kHz at ultrasonic power 40 W for 210 min (pulse durations of on-time 2 s and off-time 4 s). The ultrasonic power was obtained from a digital wattmeter. During the enzymatic reaction process, the pH was maintained at 8.0 by the addition of 1 M NaOH and the reaction temperature was controlled at 50 ± 5 °C using a water bath. At the end of the incubation period, the enzymatic hydrolysis was stopped by boiling for 10 min.

2.4. Ultrasound pretreatment of DWGP and preparation of enzymatic hydrolysates

An aliquot (200 ml) of protein solution (the concentrations were 1 g/100 ml) was prepared, and then the solution of protein was immediately treated using an ultrasonic cell crusher (GA98-IIID, Shangjia Biotechnology Co., Wuxi, China) with a 2.0 cm flat tip probe at 20 kHz at different levels of power output (0 W, 900 W, 1200 W, 1500 W, and 1800 W), whose ultrasonic power was obtained from a digital wattmeter, for 20 min (Pulse durations of on-time 2 s and off-time 2 s). After sonication, the treated protein solution was adjusted to pH 8.0 with 1 M NaOH, and then incubated in a water bath at 50 °C. After 10 min, the alcalase was added at an enzyme to substrate ratio of 3500 U/g of protein. The mixtures of protein and enzyme were incubated at 50 °C to start the enzymatic hydrolysis reaction. The pH was maintained at 8.0 by the addition of 1 M NaOH in the enzymatic reaction process. At the end of the incubation period, the enzymatic hydrolysis was stopped by boiling for 10 min. Then the mixture of protein and enzyme was centrifuged at 2500*g* for 15 min at 20 °C. The supernatant (hydrolysate) was collected and stored at 4 °C.

2.5. Measurement of initial reaction rate and kinetic parameters

Initial reaction rate were measured by the method of Galvão, Pinto, Jesus, Giordano, and Giordano (2009) with some modifications. The initial reaction rate were determined using graphical extrapolation by plotting concentrations of hydrolysed protein (P) as a function of different hydrolysis times at the initial stage of hydrolysis (three levels), and then the initial reaction rate was obtained from the slope of the plot. P was calculated as a function of the degree of hydrolysis (DH), and DH was calculated according to the pH-stat method of Adler-Nissen (1986):

$$DH\ (\%) = \frac{h}{h_{\mathrm{tot}}} \times 100 = \frac{BN_b}{\alpha M_p h_{\mathrm{tot}}} \times 100 \quad (1)$$

$$P = \mathrm{c} \times \mathrm{DH} \times 0.01 \quad (2)$$

where B is the base (NaOH) consumption in ml, N_b is the normality of the base, α is the average degree of dissociation of the α-NH_2 groups in the protein substrate (0.874 for 50 °C and pH 8.0), M_p is the mass of hydrolysed protein (g), h_{tot} is the total number of peptide bonds in the protein substrate (7.8 m equiv./g protein), and c is the protein concentration.

Kinetic parameters of DWGP hydrolysis were determined according to the process described by Song et al. (2008). According to this method, a formula, which was used to determine the kinetic parameters of the enzymatic hydrolysis of DWGP under ultrasound irradiation, was applied in this research:

$$V = \frac{k_A E_T S_0}{K_M + S_0} \quad (3)$$

where V is reaction rate, k_A is the average value of apparent breakdown rate constant (binding frequency between substrate and enzyme), E_T is the enzyme concentration in the reaction system

(0.1 g/l), S_0 is the initial substrate concentration, and K_M is an apparent constant analogous to the Michaelis–Menten constant.

Kinetic parameters of the enzymatic reaction can be estimated from data of initial hydrolysis rates by the direct linear method of the Lineweaver–Burk plot. In order to determine the values of k_A and K_M, Eq. (3) was rearranged as the following form:

$$\frac{1}{V} = \frac{K_M}{k_A E_T} \cdot \frac{1}{S_0} + \frac{1}{k_A E_T} \quad (4)$$

Thus a plot of the reciprocal of initial rate versus the reciprocal of protein concentration for experiments at a fixed enzyme concentration (E_T = 0.1 g/l) should give a straight line. The intercept with the ordinate gives $1/k_A E_T$ and the slope is $K_M/k_A E_T$, from which k_A and K_M can be calculated.

2.6. Surface hydrophobicity

Surface hydrophobicity of DWGP dispersions (20 mg/ml) was measured according to Kato and Nakai (1980) using the fluorescence probe, 1-anilino-8-naphthalene-sulfonate (ANS) (Sigma–Aldrich Trading Co., Shanghai, China), with slight modifications, as described in a previous publication (Li, Chen, & Mo, 2007). Ultrasound-pretreated/unpretreated DWGP dispersions (20 mg/ml) were diluted with phosphate buffer (pH 7.0) to a final concentration of 0.5 mg/ml. Then, 20 ml of ANS (8.0 mM in sample buffer) was added to 4 ml of the diluted sample and mixed. Relative fluorescence intensity (RFI) was measured immediately at room temperature using a fluorescence spectrophotometer (Cary Eclipse, Varian Co., USA) at 390 nm (excitation wavelength, slit = 5.0 nm), 400–650 nm (emission wavelength, slit = 5.0 nm) and 10 nm/s of scanning speed. Surface hydrophobicity in the sample was expressed as the relative fluorescence intensity with the 0.5 mg/ml protein concentration.

2.7. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured by the method of Cushman and Cheung (1971), with slight modifications, as described in a previous publication (Muguerza et al., 2006). An aliquot (45 μl) of hippuryl-His-Leu (HHL) sodium borate buffer (6.5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) were mixed with 10 μl of sample solution (sample in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) and pre-incubated for 5 min at 37 °C. The reaction was initiated by adding 10 μl of ACE (ACE in 0.1 M borate buffers containing the 0.3 M NaCl, pH 8.3) and the reaction was carried out at 37 °C for 30 min. The reaction was stopped by adding 85 μl of 1 M HCl to the samples except for the blank (85 μl of 1 M HCl were added before the pre-incubation). The hippuric acid formed was extracted by adding ethyl acetate (1000 μl) to the mixture with vigorous shaking for 2 min. After centrifugation at 4000*g* for 10 min, 800 μl of the ethyl acetate layer was collected and then dried at 100 °C for 20 min. The hippuric acid was re-dissolved in distilled water (800 μl) and determined in a spectrophotometer at 228 nm. The ACE inhibition activity was calculated using the following equation:

$$\text{ACE inhibition activity } (\%) = \frac{C - S}{C - B} \times 100 \quad (5)$$

where C is the optical density without sample (buffer for samples), and S is the optical density in the presence of both ACE and sample. B is the optical density of blank (hydrochloric acid was added before ACE). ACE inhibition was also expressed in terms of IC_{50}, defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

2.8. Scanning electron microscopy (SEM)

Sample preparation of SEM was as follows: DWGP dispersions (1 g/100 ml) with/without ultrasonic pretreatment were centrifuged at 2500*g* and 20 °C for 20 min. Then the residue (DWGP) was collected. DWGP dispersions (1 g/100 ml), with or without ultrasonic pretreatment, were subjected to enzymatic hydrolysis with alcalase. Then the mixture of protein and enzyme was centrifuged at 2500*g* for 10 min at 20 °C. The residue (hydrolysis residue) was collected. The above collected residues were dried by a freeze-dryer (ALPHA 1-2, Martin Christ Inc., Osterode, Germany).

A thin layer of the sample granules was mounted on the copper sample-holder, using a double sided carbon tape and coated with gold of 10 nm thicknesses to make the samples conductive. SEM studies were carried out using a scanning electron microscope (JSM-7001F, JEOL, Tokyo, Japan) at acceleration voltage of 15 kV.

2.9. Amino acid analysis

The determination of total amino acids composition was obtained after acid hydrolysis of the DWGP hydrolysates. The samples were hydrolysed with 6 M HCl for 24 h at 110 °C in a sealed tube. The amino acids were subjected to RP-HPLC analysis (Agilent 1100, USA) after precolumn derivatization with o-phthalaldehyde (OPA) (Jarrett, Cooksy, Ellis, & Anderson, 1986) or with 9-fluorenylmethyl chloroformate (FMOC) (Näsholm, Sandberg, & Ericsson, 1987). Methionine and cysteine were determined separately, by their oxidation products, according to the performic acid procedure of Moore (1963) before hydrolysis in 6 M HCl. Tryptophan was determined after alkaline hydrolysis by isocratic ion-exchange chromatography with OPA derivatization followed by fluorescence detection (Ravindran & Bryden, 2005). By this technique, asparagine and glutamine cannot be individually quantified. Both are indistinctly measured with aspartic and glutamic acid, respectively.

2.10. Statistical analysis

The results were expressed as means ± S.E.M. ANOVA, followed by the Tukey test, used for statistical comparisons among groups, with a value of $P < 0.05$ indicating significance. All calculations and comparisons were done using the SAS statistical software package (V.8.3, SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Effects of sonication of DWGP during alcalase treatment on kinetic parameters and on ACE-inhibitory activity of hydrolysates

3.1.1. Effects of sonication of DWGP during alcalase treatment on initial reaction rate and on kinetic parameters

Effects of ultrasonic treatment, during proteolysis, on the initial reaction rate for various substrate concentrations and on kinetic parameters for the enzymatic hydrolysis of DWGP are shown in Table 1. Values of initial hydrolysis rate for different DWGP concentrations are obtained from the slopes of the time courses at the initial stage. In this work, the concentration range of DWGP was from 1.5 to 24 g/l, the hydrolysis temperature was controlled at 50 °C, and pH was controlled at 8.0. Moreover, the enzymatic reaction was performed without/with ultrasound treatment (40 W of ultrasonic power). Since correlations of linear regression are between 0.9769 and 0.9981 for different DWGP concentrations at the initial stage, the linearity is good. The results also show that the hydrolysis follows first order kinetics (Song et al., 2008). For different DWGP concentrations (1.5, 3, 6, 12, 24 g/l), the value of the initial hydrolysis rate with ultrasonic treatment at 40 W was

Table 1
Effects of ultrasound treatment on the initial reaction rate for various substrate concentrations and on kinetic parameters for the enzymatic hydrolysis of DWGP.

	Substrate concentration (g/l)					K_M/k_AE_T	$1/k_AE_T$	k_A (min^{-1})	K_M (g/l)
	1.5	3.0	6.0	12.0	24.0				
Without ultrasound									
Rate/V (g/l·min^{-1})	0.0165	0.0241	0.0303	0.0352	0.0447		–		
Correlation	0.9919	0.9767	0.9940	0.9932	0.9981				
Kinetic parameters			–			58.1	22.2	0.45	2.62
With ultrasound									
Rate/V (g/l·min^{-1})	0.0220	0.0305	0.0404	0.0460	0.0511		–		
Correlation	0.9893	0.9848	0.9903	0.9843	0.9977				
Kinetic parameters			–			41.4	18.2	0.55	2.28
Percentage of increase	33.3	26.6	33.3	30.7	14.3	–	–	22.2	−13.0

Note: E_T = 0.1 g/l.

higher than that of the control (without ultrasonic treatment), and initial hydrolysis rate with ultrasonic treatment increased about 14.3–33.3%. That is to say, ultrasonic treatment during proteolysis can accelerate the enzymatic hydrolysis of DWGP. The phenomenon was in agreement with the findings of Song et al.(2008), who observed that ultrasonic treatment during proteolysis could enhance the enzymatic hydrolysis of leather waste and give a better digestion yield of fleshing and shaving. Furthermore, it was also reported that ultrasonic treatment during proteolysis led to a smooth increase of reaction rate of soy oil lipase-catalysed hydrolysis at relatively higher enzyme concentration than that with a shaking condition (Liu et al., 2008). The reason may be attributable to ultrasonic treatment during proteolysis, which induces easier dispersion and less agglomeration of enzyme.

Kinetic parameters of the enzymatic reaction can be estimated by the direct linear method of the Lineweaver–Burk plot. It is found that $1/V$ is linear to $1/S_0$ by plotting $1/V$ versus $1/S_0$ (data not shown). The results of linear regression analysis are shown in Table 1. k_A represents the binding frequency between substrate and enzyme, and K_M represents an apparent constant analogous to the Michaelis–Menten constant. As shown in Table 1, the value of k_A for the enzymatic hydrolysis of DWGP with ultrasonic treatment increases by 22.2%, and K_M decreases by 13.0%, compared with that obtained without ultrasonic treatment. The increase of k_A (with ultrasonic treatment) is due to enhancing of the mixing of substrate and proteases under ultrasonic irradiation. The result was in agreement with the findings of Mason et al. (1996), who reported that ultrasonic treatment during proteolysis appeared to be particularly useful in increasing the transport of substrate to the enzyme. The decrease of K_M (with ultrasonic treatment) may be due to a substantially higher affinity of the enzyme (alcalase) for DWGP than for that without ultrasonic treatment. The results show that ultrasonic treatment during proteolysis can accelerate enzymatic hydrolysis.

3.1.2. Effects of sonication of DWGP during alcalase treatment on ACE-inhibitory activity of hydrolysates

The ACE-inhibitory activity of the hydrolysate was determined *in vitro* and expressed in terms of IC_{50}, defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. The IC_{50} was determined using graphical extrapolation by plotting ACE inhibition as a function of different hydrolysate concentrations. The change in ACE-inhibitory activity with initial substrate concentrations in the enzymatic hydrolysate of DWGP, using alcalase (alcalase concentrations, 13.3 U/ml of substrate solution) without/with ultrasonic treatment at 40 W is shown in Fig. 1.

As the initial substrate concentrations extended from 1.5 g/l to 24 g/l, the IC_{50} value of the enzymatic hydrolysate without ultrasonic treatment increased from 0.55 mg/ml to 2.95 mg/ml, and that of the enzymatic hydrolysate with ultrasonic treatment increased from 0.42 mg/ml to 2.22 mg/ml, while inhibition of ACE decreased, as indicated by an increase of IC_{50}. Thus, ACE-inhibitory activities of the hydrolysates markedly decrease with increasing initial substrate concentrations. That is to say, the ACE-inhibitory activities of the hydrolysates increase with the enzyme to substrate (E/S) ratio while the same alcalase solution was used. This was in agreement with the results reported by Chiang, Tsou, Tsai, and Tsai (2006) on production of soy protein hydrolysate using alcalase, who found that the ACE-inhibitory activity had a tendency to increase with increased E/S ratio. Comparing with the control, ACE-inhibitory activities of the hydrolysates obtained from DWGP with ultrasonic treatment among 1.5 g/l, 3.0 g/l, 6.0 g/l and 12.0 g/l of initial substrate concentrations had no significant differences ($P > 0.05$), but there was a significant difference ($P < 0.05$) at 24.0 g/l of initial substrate concentration. A possible reason was that DWGP was not adequately hydrolysed when the initial substrate concentration was 24.0 g/l or over, while ultrasonic treatment during proteolysis could facilitate the reaction (Song et al., 2008). The results showed that ultrasonic treatment during proteolysis had less effect on the ACE-inhibitory activity.

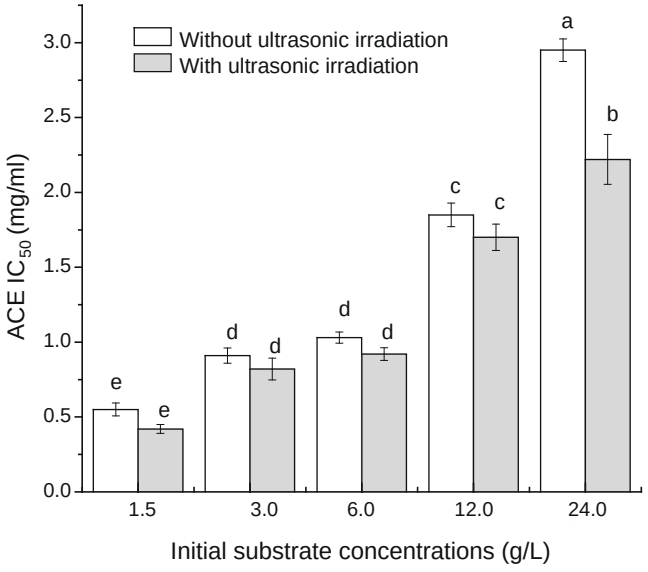

Fig. 1. Effect of ultrasonic treatment during proteolysis on ACE-inhibitory activity for the hydrolysates of DWGP hydrolyzed by alcalase. Ultrasonic treatment conditions: treated sample, 500 ml; ultrasonic power, 40 W; treatment time, 210 min (Pulse durations of on-time 2 s and off-time 4 s). Hydrolysis conditions: pH, 8.0; temperature, 50 °C; alcalase dosage, 13.3 U/ml of DWGP solution; hydrolysis time, 210 min. □, control (without ultrasonic treatment); ▨, with ultrasonic treatment. Results represent the means of three determinations ± standard deviation. Means with different superscripts are significantly different ($P < 0.05$).

3.2. Effects of ultrasonic pretreatment on ACE-inhibitory activity of hydrolysates

ACE-inhibitory activity for the hydrolysates of unpretreated and ultrasonic-pretreated (900–1800 W) DWGP hydrolysed by alcalase is shown in Fig. 2. The IC_{50} value of the hydrolysate of DWGP with alcalase was 0.42–0.26 mg/ml after ultrasonic pretreatment at 900–1800 W of ultrasonic power. The ACE-inhibitory activity increased with the increase of ultrasonic power from 900 W to 1500 W, while the ACE-inhibitory activity declined slightly when ultrasonic power was extended to 1800 W. In a previous work, we found that ultrasonic pretreatment had less effect on DH of DWGP (date not shown). However, ultrasonic pretreatment caused a 21.0–40.7% increase in ACE-inhibitory activity of hydrolysate compared to that without ultrasonic pretreatment. The result illustrates that ultrasonic pretreatment is an effective way to increase the ACE-inhibitory activity of hydrolysate of DWGP.

3.3. Mechanism of ultrasonic pretreatment on increasing ACE-inhibitory activity of hydrolysates

3.3.1. Effects of ultrasonic pretreatment on the surface hydrophobicity of DWGP

The emission fluorescence spectra of unpretreated and ultrasonic-pretreated DWGP were obtained using ANS as a fluorescence probe (Fig. 3). The fluorescence spectrum is mainly attributed to the Trp, Tyr and Phe residues, particularly the Trp residue, and the fluorescence quantum yield of these residues decreases as their exposure to solvent increases (Wang et al., 2008).

The fluorescence peak (450–550 nm) intensity of DWGP increased with increase of ultrasonic power from 0 W (Control) to 1500 W at pretreatment time 20 min, reaching the maximum (about 27% of the control) when ultrasonic power was 1500 W, indicating that ultrasonic pretreatment could induce molecular unfolding of protein, destroy hydrophobic interactions of protein molecules, cause more hydrophobic groups and regions inside the molecules to expose outside (Gulseren, Guzey, Bruce, & Weiss, 2007; Jambrak et al., 2008), and thus increase ANS fluorescence intensity of DWGP. The fluorescence intensity declined slightly when ultrasonic power was extended to 1800 W, suggesting that

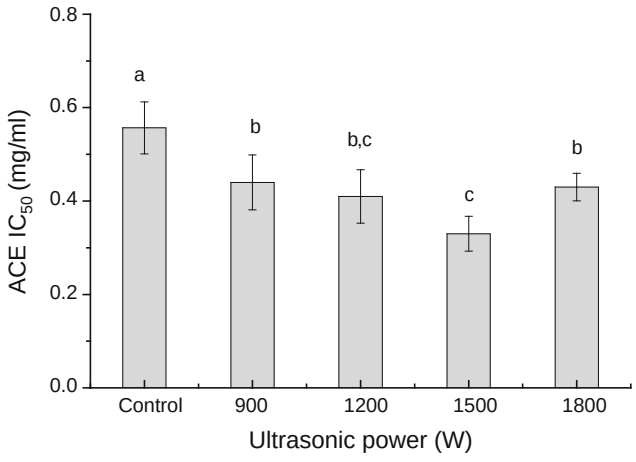

Fig. 2. ACE-inhibitory activity for the hydrolysates of unpretreated and ultrasonic-pretreated (900–1800 W) DWGP hydrolyzed by alcalase. Hydrolysis conditions: pH, 8.0; temperature, 50 °C; enzyme–substrate ratio, 3500 U/g of protein; substrate concentration, 1% (w/v); hydrolysis time, 90 min. Ultrasonic pretreatment conditions: pretreated sample, 200 ml; pretreatment time, 20 min (Pulse durations of on-time 2 s and off-time 2 s). Results represent the means of three determinations ± standard deviation. Means with different superscripts are significantly different ($P < 0.05$).

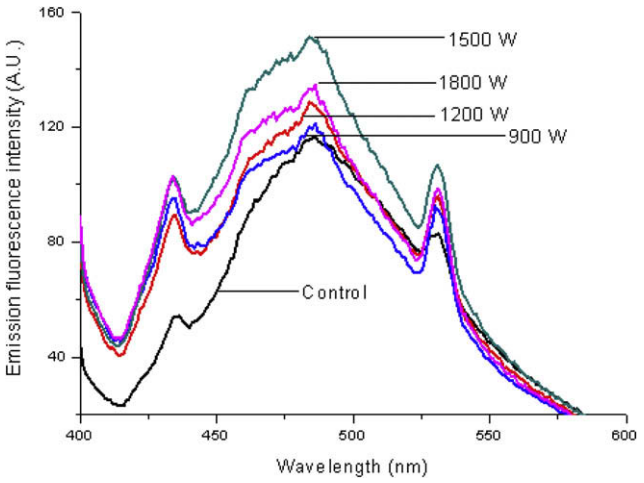

Fig. 3. Emission fluorescence spectra of untreated and ultrasonic-pretreated (900–1800 W) DWGP (using ANS as a fluorescence probe). Ultrasonic pretreatment conditions: pretreated sample, 200 ml; pretreatment time, 20 min (pulse durations of on-time 2 s and off-time 2 s).

the exposed hydrophobic groups might re-associate or aggregate to form a more stable structure (Li et al., 2007). The results may offer an explanation for the observed increase in ACE-inhibitory activity of hydrolysate of ultrasonic-pretreated DWGP since molecular unfolding of protein may lead to more release of hydrophobic amino acids during proteolysis (Lourenço da Costa, Antonio da Rocha Gontijo, & Netto, 2007).

3.3.2. Effects of ultrasonic pretreatment on the physical structure of DWGP and on its hydrolysis residues

The microstructure of the unpretreated DWGP, the DWGP pretreated by ultrasound and their alcalase hydrolysis residues were examined by scanning electron microscopy. DWGP without ultrasonic pretreatment is heterogeneous in size, ranging from as small as sub-μm to as big as about 20 μm in diameter (Fig. 4A). After ultrasonic pretreatment, DWGP size became smaller, and its maximum diameter was approximately 10 μm (Fig. 4B). According to the previous reports, the formation of aggregates is due to the formation of non-covalent interactions between protein molecules, e.g. electrostatic and hydrophobic interactions (Visessanguan, Ogawa, Nakai, & An, 2000). Therefore, high power ultrasound may cut off the electrostatic and hydrophobic interactions between protein molecules which result in DWGP particle breakage. The SEM image presented in Fig. 4C and D depicts that hydrolysed DWGP, after ultrasonic pretreatment, becomes looser than that without ultrasonic pretreatment. The result suggests that ultrasonic pretreatment may loosen the tissue of DWGP aggregate, and help the enzyme alcalase to attack the interior of DWGP aggregate easily, which results in the release of hydrophobic amino acids from DWGP aggregate.

3.3.3. Amino acid composition of the hydrolysates of DWGP without/with ultrasonic pretreatment

In order to further elucidate the mechanism of ultrasonic pretreatment on the release of ACE-inhibitory peptides from DWGP, amino acid composition of hydrolysate of DWGP without/with ultrasonic pretreatment was examined (Table 2). As shown in Table 2, the hydrolysate of DWGP that had undergone ultrasonic pretreatment showed a higher relative concentration of hydrophobic amino acids (6.5%), with particularly noticeable increase (42.86%) in Pro content, as compared with the hydrolysate of unpretreated DWGP. Alcalase-proteolysis may lead to production of a rich hydrophobic amino acid content in the C-terminus

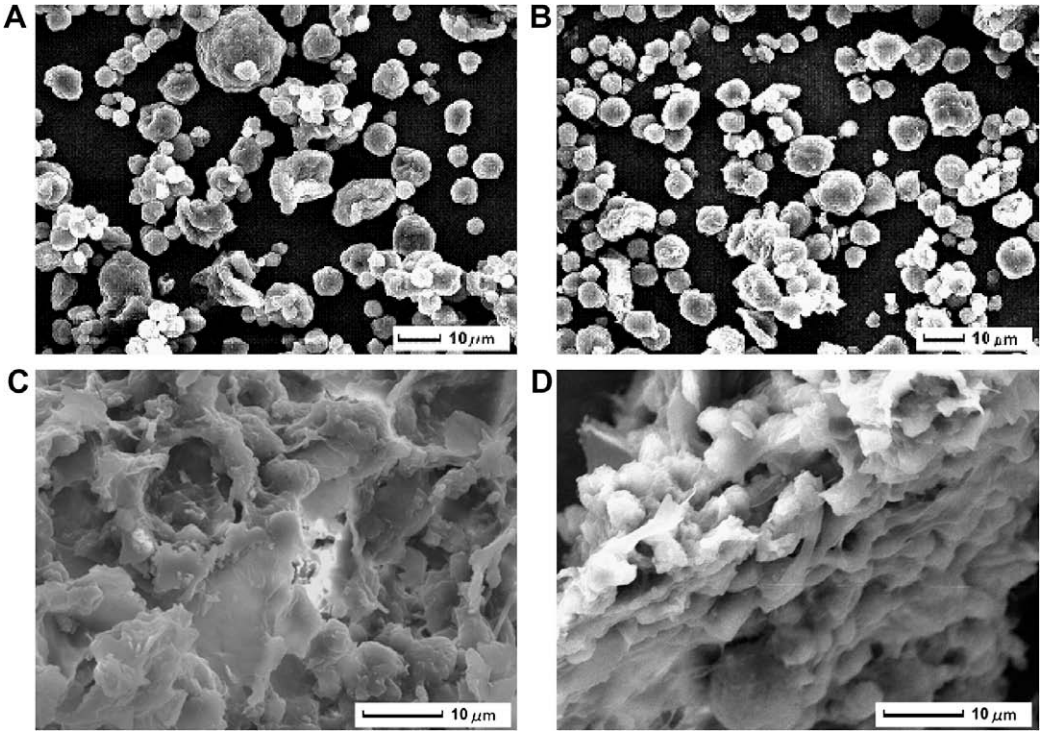

Fig. 4. Scanning electron micrographs of unpretreated DWGP (A) and pretreated DWGP (B) by ultrasound, and their hydrolysis residues with alcalase (C, hydrolysis residues of unpretreated DWGP; D, hydrolysis residues of ultrasonic-pretreated DWGP). Ultrasonic pretreatment conditions: pretreated sample, 200 ml; ultrasonic power, 1500 W; pretreatment time, 20 min (Pulse durations of on-time 2 s and off-time 2 s). Hydrolysis conditions: pH, 8.0; temperature, 50 °C; enzyme–substrate ratio, 3500 U/g of protein; substrate concentration, 1% (w/v); hydrolysis time, 90 min. Magnification: A and B, ×1000; C and D, ×2000.

because alcalase preferentially cleaves C-terminal hydrophobic residues (Ala, Val, Leu, Ile, Phe, Pro, Trp, and Met) (Lourenço da Costa et al., 2007). Thus, it is evident that enzymatic hydrolysis of DWGP, pretreated by ultrasound tends to produce more peptides with a C-terminal hydrophobic amino acid residue than does hydrolysis of unpretreated DWGP, since ultrasonic pretreatment causes more hydrophobic groups and regions inside the molecules to be exposed outside. Furthermore, the hydrolysate of ultrasonic-pretreated DWGP has a 42.9% increase in Pro content compared to that of unpretreated DWGP (Table 2). The result indicates that the hydrolysate of ultrasonic-pretreated DWGP may contain more peptides with a C-terminal Pro residue.

Table 2
Amino acid composition of the hydrolysates of DWGP without/with ultrasonic pretreatment hydrolyzed by alcalase (pH 8.0, 50 °C, 3500 U/g of protein, 1% (w/v) of substrate concentration, 90 min).

Amino acid	% of hydrolysate	
	No pretreatment	With ultrasonic pretreatment
Asp + Asn	7.93	7.53
Glu + Gln	12.4	11.6
Ser	5.07	4.4
His	3.09	2.86
Gly	5.91	5.95
Thr	4.46	4.23
Arg	9.43	9.28
Ala	6.44	6.35
Tyr	3.53	3.29
Cys	0.62	0.55
Val	7.32	7.64
Met	1.96	1.97
Phe	5.71	5.70
Ile	5.29	5.62
Leu	9.00	9.11
Lys	7.06	7.11
Pro	4.76	6.80
Trp	0.74	0.71
Hydrophobic amino acids[a]	41.2	43.9

[a] Ala, Val, Leu, Ile, Phe, Pro, Trp, and Met; ultrasonic pretreatment condition: treated sample, 200 ml; ultrasonic power, 1500 W; pretreatment time, 20 min (pulse durations of on-time 2 s and off-time 2 s).

According to the structure–activity features of the ACE-inhibitory peptide, the preferred ACE inhibitors are those peptides that contain hydrophobic amino acids on the C-terminal (Li, Le, Shi, & Shrestha, 2004). Furthermore, several studies have revealed the importance of C-terminal Pro residues on the activity of ACE-inhibitory peptide. Mizuno, Nishimura, Matsuura, Gotou, and Yamamoto (2004) reported that the casein hydrolysate of *Aspergillus oryzae*, which expressed potent antihypertensive effects in spontaneously hypertensive rats, mainly contains short peptides of X-Pro and X-Pro-Pro sequences. Pro plays a key role in stabilities of ACE-inhibitory peptides too. ACE-inhibitory peptides having Pro residues, especially at the C-terminal, are generally resistant to degradation by digestive enzymes (Li et al., 2004). Thus, ultrasonic pretreatment is beneficial to the release of ACE-inhibitory peptides from DWGP with alcalase.

4. Conclusion

The use of ultrasonic treatment during proteolysis has affected the kinetics of the hydrolysis of DWGP, whereas it has less effect to the ACE-inhibitory activity of the hydrolysate. Compared with that obtained without ultrasonic treatment, the value of k_A for the enzymatic hydrolysis of DWGP, under ultrasonic irradiation, increases by 22.2%, and K_M decreases by 13.0%. The use of ultrasonic pretreatment has affected the release of peptides with ACE-inhibitory activity from DWGP during enzymatic hydrolysis, which caused a 21.0–40.7% increase in ACE-inhibitory activity of the hydrolysate. The ANS fluorescence spectra and scanning electron micrographs

revealed that ultrasonic pretreatment could result in an increase of surface hydrophobicity of DWGP and loosening of the protein tissue, which facilitate the release of hydrophobic amino acids during enzymatic hydrolysis. Amino acid composition further showed that the hydrolysate of ultrasound-pretreated DWGP had more hydrophobic amino acids and Pro, which play important roles in the activities of ACE-inhibitory peptides, than that without ultrasonic pretreatment. The results showed that ultrasonic treatment during proteolysis could facilitate the enzymatic hydrolysis, whereas ultrasonic pretreatment can promote the release of ACE-inhibitory peptides from DWGP during enzymatic hydrolysis.

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