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Original Research Article

Effect of mono- and dual frequency power ultrasound assisted enzymolysis on the degree of hydrolysis and ACE inhibitory activity of Stevia protein hydrolysates

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Abstract

Purpose: *To study the effects of ultrasound pretreatment at different frequencies and working modes, including mono frequency ultrasound (MFU) and dual-frequency ultrasound (DFU), on the degree of hydrolysis (DH) and angiotensin-I-converting enzyme (ACE) inhibitory effect of Stevia protein (SP).*
Methods: *Protein from Stevia leaves was extracted by water (50 g suspended in 1000 mL). The hydrolysis of stevia protein extract (SPE) was carried out using alcalase at 5 % enzyme substrate (E/S) ratio. A study was then carried out to investigate its microstructure and morphology using scanning electron microscopy (SEM).*
Results: *The results showed that ultrasound pretreatment did not increase DH of SP significantly ($p > 0.05$). However, the highest ACE inhibitory activity of stevia protein hydrolysate was obtained at DFU level (20/50 KHz). Overall, ultrasonic frequency mode had a significant influence on ACE inhibitory activity.*
Conclusion: *The frequency selection of ultrasound pretreatment of SP is essential for the preparation of ACE inhibitory peptide.*

Keywords: *Alcalase, Hydrolysis, Ultrasound frequency, Stevia protein, ACE inhibitory peptide*

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INTRODUCTION

In recent years, the demand for protein has increased due to increase in global population [1]. It is estimated that nearly 25% of the world population lack a suitable protein supply [2].

Plant proteins have been studied for likely incorporation into formulated food, because of the limited availability and soaring costs of animal proteins [3]. *Stevia rebaudiana* Bertoni, a herbal medicine belonging to the Asteraceae family originated from Latin America. Recently, it is growing widely in different countries including Japan, Korea, and China as a commercial plant [4]. Studies have shown that dried *stevia* extract is rich in flavonoids, green pigment, amino acids, and other essential nutrients. It has been certified

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that *stevia rebaudiana* leaves contain a considerable amount of protein [5].

Stevia has potential in the tranportation of medicines around the body and may also help to control blood sugar by modulating insulin generation and sensitivity [6]. Hypertension can cause stroke, chronic kidney disease, heart failure and many kinds of problems [7]. It is well known that ACE is responsible for hormones that help control our blood pressure. It converts the hormone angiotensin I to the active vasoconstrictor angiotensin II and inactivates bradykinin. ACE indirectly raises blood pressure by causing blood vessels to constrict [8].

Studies have shown that plant protein is rich in bioactive peptides which attracts a lot of scientists. In addition, hydrolysis by enzymes is largely used in the production of bioactive peptides from different food sources [9,10]. By enzymatic hydrolysis, the physical and chemical properties as well as the micro structure of proteins can be changed.

There are a number of different ultrasound parameters that can increase accessibility of proteolytic enzymes to peptide bonds such as ultrasound frequency [11]. Changes in protein configuration expose more hydrogen bonds during ultra-sonication [12]. However, advanced technologies have been applied to enhance the hydrolysis of protein, functional and bioactive peptides [12,13]. Consequently, the purpose of this research was to examine the effect of single and dual- ultrasound frequency on the DH and ACE inhibitory of stevia protein hydrolysates.

EXPERIMENTAL

Materials

Stevia rebaudiana Bertoni leaf powder was purchased from the (Yancheng Xiaguang Co. Jiangsu, China) in September 2018. It was then kept dried in polyethylene bags at 4 ˚C for further use. Alcalase with an activity of 150,000 U/mL was obtained from Novozymes Biotechnology Co. Ltd. (Shanghai, China). All other reagents used in this study were of analytical grade.

Stevia protein extraction

Stevia protein was extracted according to the method described by Xue *et al* [14] with some modification. The suspension was centrifuged at 4000 g for 20 min and lyophilization for further analysis using a freeze-dryer (ALPHA 1-2, Martin Christ Inc., Germany).

Ultrasound treatment with single frequency ultrasound (SFU)

In this study, ultrasonic frequency can work in single and dual-band. Ultrasound treatment with single frequency (SFU) was at 20, 28, 35, 40 and 50 KHz. The output power was 80 W/L with substrate 50 g/L at 30 °C. The degree of hydrolysis (DH) and angiotensin convert enzyme activity were selected to optimize ultrasound pretreatment parameters. The stevia protein without ultrasound under the same conditions was set as control.

Ultrasound treatment with dual frequency ultrasound (DFU)

The same different model frequency power ultrasound equipment was used to process the sample in this study. The instrument was equipped with four frequency generators (20, 28, 35 and 40 kHz). In this study dual-frequency simultaneous ultrasound (DFU) with 20/28, 20/35, 20/40 and 20/50 kHz was chosen for the sample treatments. The other pretreatment parameters were set and carried out under the same conditions of ultrasound treatment with single frequency ultrasound.

Enzymolysis of Stevia protein (SP)

Before enzymatic hydrolysis, the temperature and pH of SP mixture were increased up to 50°C and 9.0 respectively. Then 5% (E/S) alcalase was added to begin the reaction. During reaction 1 mol/L NaOH continuously added for remaining pH at 9.0. The enzymolysis time was 90 min and at the end of reaction, the mixture was heated (100°C for 10 min) to inactivate alcalase. Finally, the hydrolysates were centrifuged at 12000 ×g for 15 min after cooling to room temperature.

Determination of the degree of hydrolysis (DH)

The degree of hydrolysis (DH) was calculated according to the pH-stat method described by Adler-Nissen [15] using Eqs 1 and 2.

$$DH(\%) = \frac{H}{H_{tot}} \times 100 \quad \text{.....(1)}$$

$$DH(\%) = \frac{N_b \times B \times 100}{\alpha \times M_p \times H_{tot}} \quad \text{..... (2)}$$

where Nb is a normality of sodium hydroxide (mol/L); B is base (sodium hydroxide) consumption (mL); Mp is SP mass (g); H_{tot} is the total content of peptide bonds, which is 9.2 mmol/g protein; α is the average degree of

dissociation of the α-amino groups that is related to the pK of the amino groups at a particular pH and temperature, which is 0.99 at pH 9.0 and 50 °C.

Measurement of ACE inhibitory activity

The measurement of ACE-inhibitory activity of the stevia hydrolysate was based on the assay described by Vermeirssen *et al* [16] Briefly, 50 µL of borate buffer (1.0 mM FAPGG, 80 mM HEPES, 0.1M borate buffer, 300 mM NaCl, pH 8.0) and 40 µL of stevia hydrolysate were mixed in microtiter plate wells, and using 40 µl of distilled water instead of stevia hydrolysate as control.

To initiate the reaction 20 µL of ACE (0.1 U/mL) was added to the solution and kept at 37 °C for 30 min in an incubator (SPX-250B, C. G. Co. Ltd., Jiangsu, China). The reduction in absorbance was read at 340 nm. ACE-inhibitory (I) activity was calculated as shown in Eq 3:

$$I\,(\%) = \left(\frac{Ac - As}{Ac - Ab}\right) \times 100 \quad \text{..... (3)}$$

ACE-inhibitory activity was measured in triplicate at five concentrations. where I is the ACE inhibitory activity (%), Ac is the absorbance of the control group without protein hydrolysates, Ab is the absorbance of the blank group without ACE, and AS is the absorbance of the sample group with ACE and protein hydrolysates.

Determination of soluble protein yield

To measure the soluble protein content in each experiment, the folin phenol procedure was applied as described by Mokhtar *et al* [17]. The absorbance of protein in the reactor was analyzed using a spectrophotometer (T6, Shanghai Purkinje General Instrument Co., China) at 680 nm. The yield (Y) of protein was determined as in Eq 4.

$$Y\,(\%) = \frac{Cs\,V}{M} \times 100 \quad \text{.....(4)}$$

where, y represents the soluble protein yield (%), C s is the soluble protein concentration in the stevia's protein solution (mg/L), V is the volume of the stevia protein solution (L), and m is the protein content of sample (mg).

Amino acid determination

Amino acid profiles of stevia protein samples were determined using RP-HPLC system according to the previous method of Li *et al* [18].

Scanning electron microscopy (SEM) analysis

Stevia protein hydrolysate was observed under SEM (Hitachi - SU1510 – Japan) for morphological characterization. Dried sample particles were fixed on a specific carbon film support, and their shape and surface characteristics were observed using a gaseous secondary electron detector GSED in environmental mode (ESEM).

Statistical analysis

Analysis of variance (ANOVA) was used in this study to compare the influence of various pretreatments of ultrasound of stevia protein at level $p < 0.05$. All statistical analyses were conducted with SPSS 17.0 software (IBM Corporation, USA).

RESULTS

Effects of ultrasound pretreatment with different frequencies and working modes on DH

Figures 1 A-B show the DH of SP. The results indicate that all the ultrasound pretreatment did not improve the DH value of SP significantly ($p > 0.05$). Figure 1 A shows the effects of the MFU pretreatment operating at 20, 28, 35, 40 and 50 kHz on the DH of SP. Figure 1 B shows the effects of DFU of all the frequency combinations (20/28, 20/35, 20/40, 20/50) kHz on the DH of SP.

Angiotensin-I-converting enzyme inhibitory activity of SP hydrolysate was pretreated with MFU and DFU are shown in Figure 2 A and B respectively. The ultrasound pretreatment increased ACE inhibitory effect of SP hydrolysate significantly ($p < 0.05$).

Protein yield

The protein content obtained in stevia is 18.4 % and the protein yield at optimal extraction points of ultrasonic treatments was 56 ± 2.91 %.

Amino acid profile

Amino acids composition is shown in Table 1. The results reveal that the content of essential amino acids have been almost higher than those recommended by FAO and WHO.

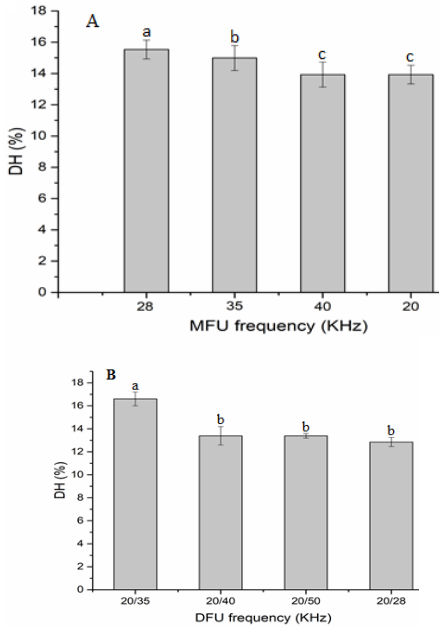

Figure 1: Effect of ultrasound pretreatment at different frequencies and working modes on the DH of SP hydrolysate. (A) MFU and (B) DFU. Results are represented as mean of three determinations ± standard deviation. Means with different superscripts are significantly different ($p < 0.05$)

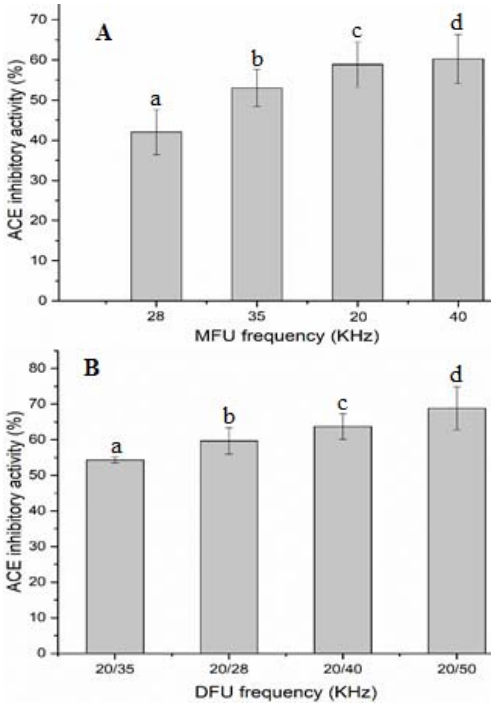

Figure 2: Effect of ultrasound pretreatment at different frequencies and working modes on the ACE inhibitory rate of SP hydrolysate. (A) MFU and (B) DFU Results are represented as mean of three determinations ± standard deviation. Means with different superscripts are significantly different ($p < 0.05$)

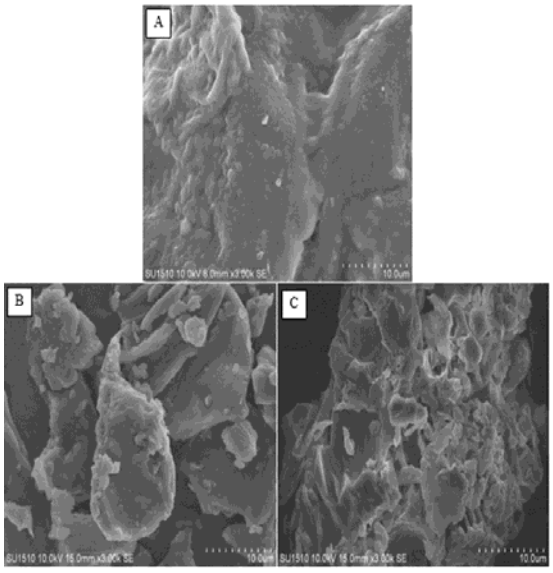

Figure 3: Effects of ultrasound pretreatment with different frequencies and working modes on the microstructure of SP. (A) Native SP; (B) MFU pretreated SP (28 kHz ultrasound working mode); (C) DFU pretreated SP (20/35 kHz ultrasound working mode)

Table 1: Amino acid composition of *Stevia rebaudiana* Bertoni (g/100g protein)[a]

Essential amino acid (EAA)	Value	Recommended level[b]
Histidine	0.43±0.09	0.18
Therionine	0.53±0.13	0.34
Arginine	0.80±0.19	0.35
Valine	0.90±0.11	0.35
Methionine	0.09±0.06	0.25
Phenyl alanine	0.80±0.06	0.63
Isoleucine	0.57±0.12	0.28
Leucine	1.28±0.05	0.66
Lysine	0.83±0.06	0.58
Non-essential amino acid content(g/100g) protein)		
Aspartate	*0.03*±0.01	-
Glutamine	0.03±0.01	-
Serine	0.19±0.07	-
Glycine	0.01±0.02	-
Alanine	0.05±0.01	-
Tyrosine	0.01±0.01	-
Proline	0.04±0.01	-
Cystine	ND	-
Amino acid (%) with different characteristics[c]		
Basic	2.06±0.14	-
Acidic	0.06±0.01	-
Hydrophobic	3.73±0.06	-
Uncharged Polar	0.74±0.06	-

[a] All values given are means of three determinations means ± standard deviation. [b] Lists of FAO/WHO: Daily requirements for humans. [c] Basic: Lysine, arginine, histidine, acidic: aspartic acid, glutamic acid, hydrophobic: alanine, isoleucine, leucine, methionine, phenylalanine, proline, valine, uncharged polar: glycine, serine, threonine, tyrosine, cysteine vbbv

Scanning electron micrographs (SEM)

Stevia rebaudiana bertoni leaves were scanned for structural analyses by scanning electron microscopy and compared with control.

DISCUSSION

The effect of ultrasound pretreatment on the ACE inhibitory activity of SP is significantly increased ($p < 0.05$) compared with the untreated sample by 47.67, 5.55, 33.17, and 51.12 % for 20, 28, 35, 40 kHz respectively. From the results shown in figure 2A, 40 kHz ultrasound frequency was the highest activity. This phenomenon explains changes of the protein that occur by surface hydrophobicity. On the other hand, DFU was also increased significantly ($p < 0.05$) by 72.67% and reached the peak at the frequency combinations of 20/50 kHz compared with the untreated one. The molecular unfolding of the Stevia protein that affected by ultrasound increases its surface hydrophobicity, which enabled the release of ACE inhibitory peptides during the enzymatic hydrolysis. It indicates that the ACE inhibitory activity improved significantly by the DFU compared with the MFU. It can be concluded from the results that the ultrasound pretreatment increases the ACE inhibitory effect of SP hydrolysate. Stevia contained 18 % protein. However, there was a significant ($p < 0.05$) increase of protein (56 ± 2.91%) in the protein extract. As documented, the existence of the protein-lipid complex make disrupts protein hydrolysis [21,22]. Therefore, the differences in the total protein and protein yield after extraction might be caused by the removal of fat and impurities during the extraction, which results in a stable protein which makes it a proteinacious source. The composition of amino acids in SP is presented in Table 1. The amount of two amino acids (phenyl alanine and lysine) were close-to those recommended by FAO and WHO for daily adult- as well as non- essential amino acids (aspartate, serine, glutamine proline, glycine, alanine, cystine and tyrosine).

This indicates that Stevia could be used as a good source of essential amino acids which can be factors to maintain a good health. Similar results were obtained by Mohammad et al [23] who reported that out of twenty amino acids, nine amino acids were identified as glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine and methionine in *stevia* leaves. The total amino acid content (TAA) of the sample was calculated to be 6.23 g/100 g. Leucine was the most abundant amino acid while methionine was the least one. The ultrasound pretreatment samples were examined for structural analyses by SEM and compared with control. As shown in Figure 3, the surfaces of SP showed significant variations in size and shape when viewed by SEM. As a comparison, the surface of control sample Figure 3 A, native SP was rough, taking on island shape and was aggregated due to the intensity of hydrophobicity between the protein molecules. Treated ultrasound samples (Figure B and C) have a porous structure which explained its excellent rehydration property compared to that of control. The reasons for differences in their surfaces could be the changes in interconnection and intermolecular distance caused during the process [24]. It was observed that there was complete parenchyma without any significant destruction on cell walls but with slight ruptures on the surfaces of native stevia protein. Similarly initiated cell rupture and damage, allowed for more of the protein from the powder to be extracted. Further study is needed to assess the yield of total protein between two samples.

CONCLUSION

From this study, we can conclude that stevia protein can be used as a material for generating protein hydrolysate due to its high content of protein. Arginine, valine, methionine, phenyl alanine and lysine are the major amino acid contents of the SP. Furthermore, all the ultrasound pre-treatment including single frequency and dual frequency have significantly influenced the pre-treatment of SP. It is important to notice that stevia protein inhibits the activity of ACE which has been our study's objective. This finding is due to functional properties of stevia protein which can make it easy to use in food and pharmaceutical industry and may potentially serve as a good source of desirable peptide and amino acids.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities

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