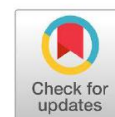




Original Research article

Investigation of ACE Inhibitory Effect and Antioxidant Activity of Peptide Extracted from Spirulina Platensis



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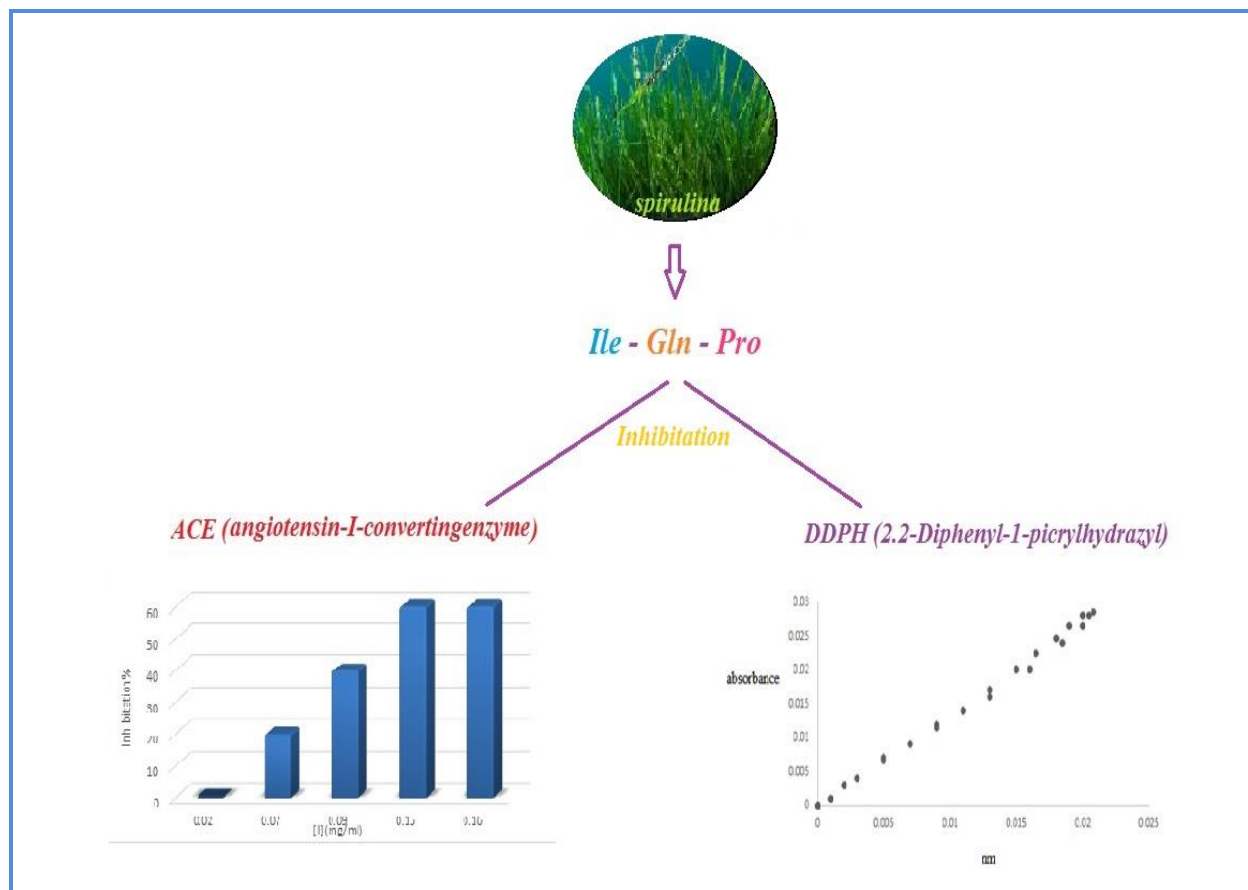
KEYWORDS

Spirulina platensis
Angiotensin I-converting enzyme
Peptide Ile-Gln-Pro

ABSTRACT

Spirulina platensis is a green-blue microalgae and a rich source of many nutrients such as protein, vitamins, and minerals. *Spirulina* contains peptides that have therapeutic and beneficial effects on the human body. In this study, peptide Ile-Gln-Pro from *spirulina platensis* was extracted using alcalase enzyme and purified by ultrafiltration and silica gel columns, and the fractions were identified using RP-HPLC analysis. The molecular weight of the tripeptide was determined by GC/mass spectroscopy. The peptide extracted with a concentration of 6.23 mg/mL and a purity of 75.72%. Then its inhibitory effect on angiotensin I-converting enzyme (ACE) and its antioxidant properties were investigated using DDPH (2,2-diphenyl-1-picrylhydrazyl) assay.

Graphical Abstract



Introduction

Spirulina platensis is one of the most important microalgae, which is used to high-nutritional content in Asian countries and has recently been important in Europe [1]. Antioxidants are the compounds that can give electrons or hydrogen atoms to the free radicals to create a complex that guards the human body from free radicals [2]. The antioxidant activity of the bioactive peptides produced from animal and plant proteins has attracted considerable attention over the last few years. Proverbially, peptides derived from digestion of animal protein products such as eggs [3] and duck meat [4] have showed some antioxidant activity. Also, vegetable proteins have found to be a good source of antioxidants. For example, active peptides from walnuts [5] and maize [6] have been evaluated for their antioxidant valences [2]. Angiotensin *I*-converting enzyme (ACE), an important enzyme part of the renin-angiotensin system (RAS), plays an important role in the adjusting blood pressure and homeostasis of body fluids [7, 8]. ACE inhibitors are widely used to treat high blood pressure and heart failure, containing captopril, zofenopril, enalapril, ramipril, lisinopril, and

benazepril [9]. Paying more attention to peptides is due to their improved environmental activities and less side effects [10]. High blood pressure is one of the most important causes of cardiovascular disease that may result in stroke, coronary artery disease, and sudden death of the heart [11]. In addition, recent research has shown that oxidative stress is a major cause of high blood pressure [12]. Extreme reactive oxygen species also affect the function of cellular sequencing and reduce endothelial nitric oxide biodegradability and increase low-density lipoprotein oxidation in the vascular system [13-15]. Thereby, the need for new ACE inhibitors with oxidative stress control and side effects is lower than natural sources.

Experimental

Preparation of water extracts

Spirulina platensis algae was purchased as dried powder (Super food brand, produced in Iran). In 120 mL of distilled water, 15 g of dried *Spirulina platensis* was suspended and repeatedly frozen and melted in 5 cycles. Then, for destruction of the cell wall, the mixture was subjected to ultrasound at 40 kHz for 5 min. The cell suspension was then incubated for 12 h at 30 °C and centrifuged at 6000 rpm for 30 min. The sediment was discarded and the supernatant was collected. The supernatant was ultrafiltered using a vacuum pump with a 0.45 µm pore size cellulose membrane.

Protein assay

The protein content of the crude was measured using biuret protein assay that used bovine serum as the standard. 2 mL of water extracts was mixed with 2 mL of NaOH 10% (Merck). Then 10 drops of Cu₂SO₄ 10% (Merck) were added slowly from the tube wall. Creating a purple ring signifies the presence of protein.

Preparation enzymatic hydrolysates

The water extracts were digested by 2.4 L of alcalase (~2.4 units/mL) (Sigma) at 2% (w/w) protein concentration and pH 8.5. The crude was incubated at 50 °C for 10 h to enzymatic digestion. This reaction was stopped and the pH was adjusted at 4.0 by adding HCl, then the mixture was cooled down in ice. It was followed by centrifuging at 6000 rpm for 10 min. The enzymatic hydrolysates were ultrafiltered by acetate cellulose filter with pore size of 0.45, 0.2 µm and kept at 4 °C. After ultrafiltration, the sample was passed through the silica gel column (2×10 cm) for further purification and washed with water of chromatography (Lc-Ms Grade). The fractions were detected

at 214 nm by UV-spectrophotometer to identify purified tripeptide fractions (Shimadzu, Japan, model UV-2550 series).

Analysis by chromatography

The extracted tripeptide isolated and identified by Reverse-phase HPLC (SY-8100) on a column C18 (4.6/250 mm; Nacalai Tesque Co. Kyoto, Japan) at a flow rate of 1 mL/min. The mobile phase was included 0.1% trifluoroacetic acid (TFA) in 1% (v/v) acetonitrile (ACN) 99% (v/v) water solution. A standard sample of tripeptide was used as a control and determination of sample withdrawal time.

Mass spectrometry (GC-MS)

The extracted peptide was initially poured into a vacuum rotary, then injected into GC-MS (Shimadzu, Japan, model QP 5050) for mass spectrometry and molecular weight determination. The required temperature was 200 °C and the flow rate was 1.5 mL/min.

ACE inhibitory activity test

To assess inhibitory strength of the peptide, a 2 mL cuvette containing a mixture of 0.1 M Tris-HCl (pH 7.0), 50 mM NaCl, 10 μM ZnCl₂, 0.001 ACE units and 100 μL of different concentrations of inhibited peptide solution were prepared and their absorbance was determined by UV-spectrophotometer (Shimadzu, Japan, model UV-2550).

Antioxidant activity test

Anti-oxidant activity of the peptide was evaluated using DPPH (1-diphenyl-2-picrylhydrazyl). 0.3 mm of DPPH was dissolved in ethanol (100%) and then 3 mL of tripeptide was added to this solution. The solution was stored in the dark and room temperature for 30 min, and then the absorbance was measured by UV spectrometer at 517 nm. Ascorbic acid (vitamin C) was used as a control.

$$\text{RSA\%} = (\text{Absorbance of Control} - \text{Absorbance of sample}) / (\text{Absorbance of Control}) \times 100$$

Results and discussion

The extracted peptide was applied to a reverse-phase HPLC on a C18 column. The mobile phase was included 0.1% trifluoroacetic acid (TFA) in 1% (v/v) acetonitrile (ACN) 99% (v/v). Concentration of the inhibitory extracted peptide was 0.0039 mg/mL. The appearance of the inhibitory peptide peak was between 3 and 4 min at a 214 nm. The synthetic tripeptide by Mimotopes company was used as a standard (Figure 1).

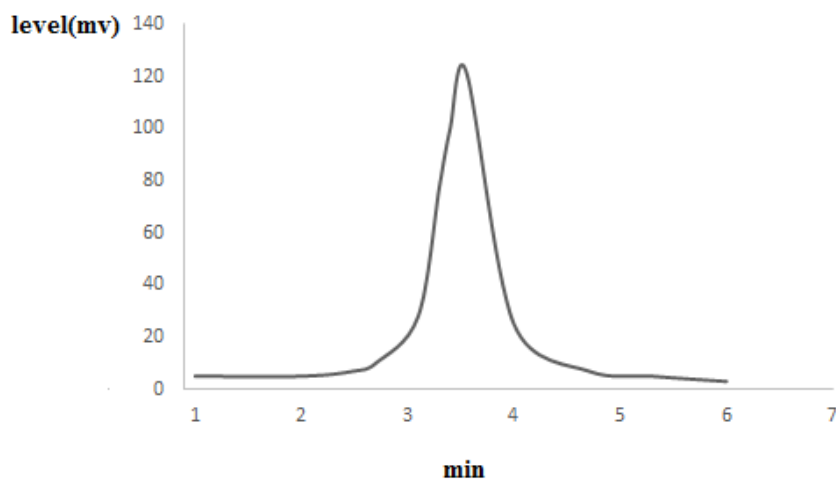


Figure 1. Reverse-phase HPLC of the ACE inhibitory peptide

The digested by alcalase after ultrafiltration and silica gel column, was showed an ACE inhibitory activity about 0.087 mg/mL. The inhibitory percentages with different concentrations of inhibitory peptide and ACE are demonstrated in Figure 2. As the peptide concentration was increased, the inhibition of ACE was enhanced. The IC 50 values at various stages were also measured to illustrate the effectiveness of the purification methods. (Figure 3) shows that the IC 50 value was improved.

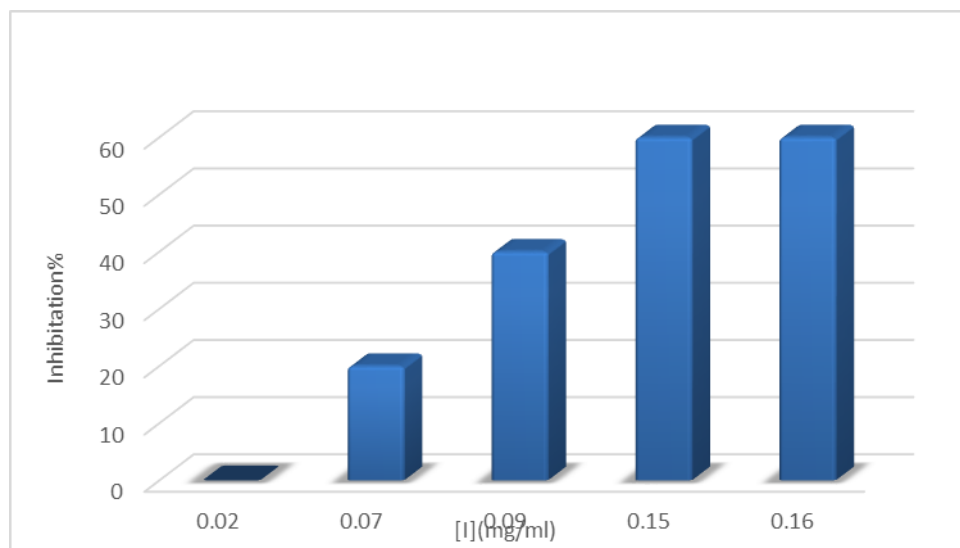


Figure 2. The graph of ACE inhibitory activity for extracted peptide

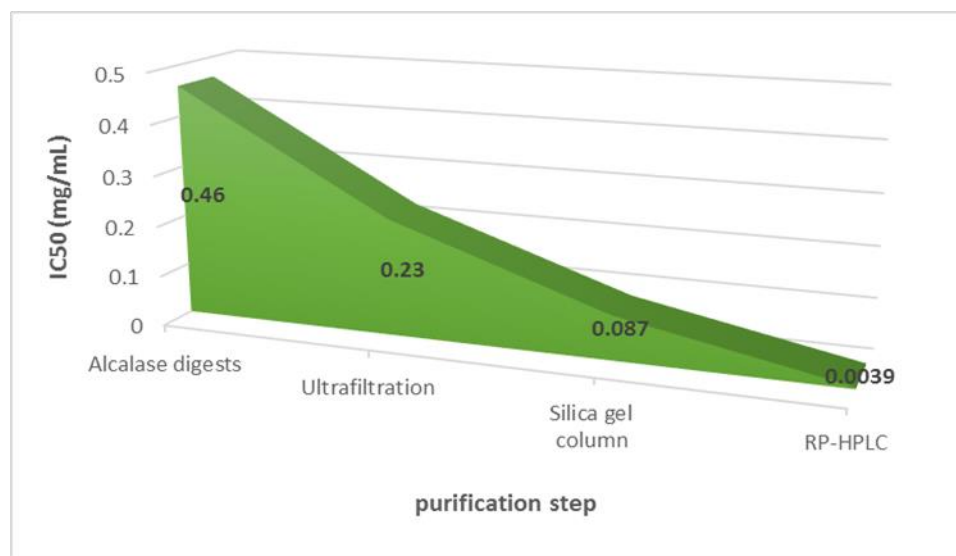


Figure 3. The graph for the amount of IC 50 peptide inhibitors of ACE in different stages of purification

The mechanism of inhibiting ACE inhibitory peptide, Ile-Gln-Pro, was investigated using kinetic studies. The Lineweaver-Burk plot with an intersection on the $1/[s]$ axis showed that the peptide is non-competitive Inhibitor (as shown in Figure 4). Non-competitive inhibition is a kind of enzyme inhibition that decreases the enzyme inhibitor and equally binds the enzyme to one another. And Dixon plot with intersection on the $[I]$ axis also features non-competitive inhibition (Figure 5). The amount of K_i was determined to be about $5.8 \mu\text{M}$.

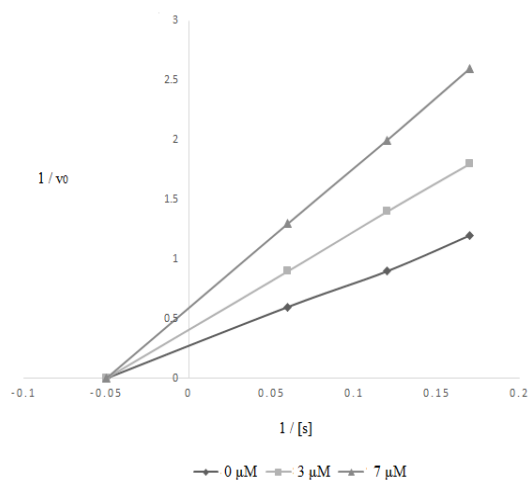


Figure 4. Lineweaver-Burk plot of the reciprocal velocity ($1/v$) against $1/[s]$ in the presence of Ile-Gln-Pro at concentrations of $0 \mu\text{M}$, $3 \mu\text{M}$, $7 \mu\text{M}$

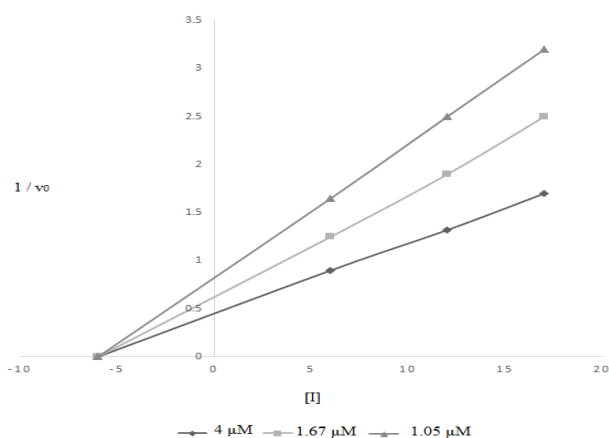


Figure 5. Dixon plot of $1/v$ against the concentration of Ile-Gln-Pro, $[I]$, at substrate concentrations of 4 μM , 1.67 μM , and 1.05 μM

Molecular weight of the extracted peptide was estimated using mass spectrometry (GC-MS) at a rate of 356 D in accordance with the tripeptide standard. (Figure 6) shows the results of the mass spectrometry analysis.

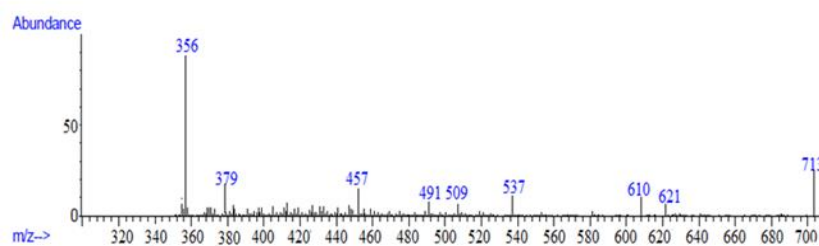


Figure 6. GC-MS chart of the tripeptide

The amount of DPPH radicals scavenging by tripeptide was calculated by measuring the RSA percentage. In this study, percentage of the RSA was 23.22. Figure 7 depicts the antioxidant activity of the tripeptide.

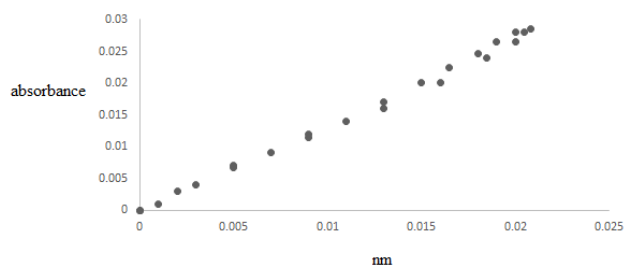


Figure 7. The antioxidant activity chart of the tripeptide

Conclusion

In this study, peptide Ile-Gln-Pro was extracted from spirulina platensis. The peptide extracted with a concentration of 6.23 mg/mL and a purity of 75.72%. Then its inhibitory effect on angiotensin I-converting enzyme (ACE) and its anti-oxidant properties by DDPH (2,2-diphenyl-1-picrylhydrazyl) assay were investigated. Both the Lineweaver-Burk plot and the Dixon plot revealed that the inhibition of extracted peptide Ile-Gln-Pro was non-competitive inhibition. This was due to the slow-tight binding of these inhibitors at the active sites of ACE. The results revealed that the peptides with biological activity play a significant role in regulation and metabolism, indicating that their potential use as beverage supplement and functional food suitable for promoting health and reducing the risk of disease.

Acknowledgments

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Conflict of Interest

We have no conflicts of interest to disclose.

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