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Original Research Article

Extraction and isolation of anti-hypertensive peptide by alkalase from spirulina platensis

Gholamreza Mahdieh, Mohamad Fazilati, Mahdieh Izadi*

Department of Biochemistry, Payame Noor University, 19395- 4697, Tehran, Iran

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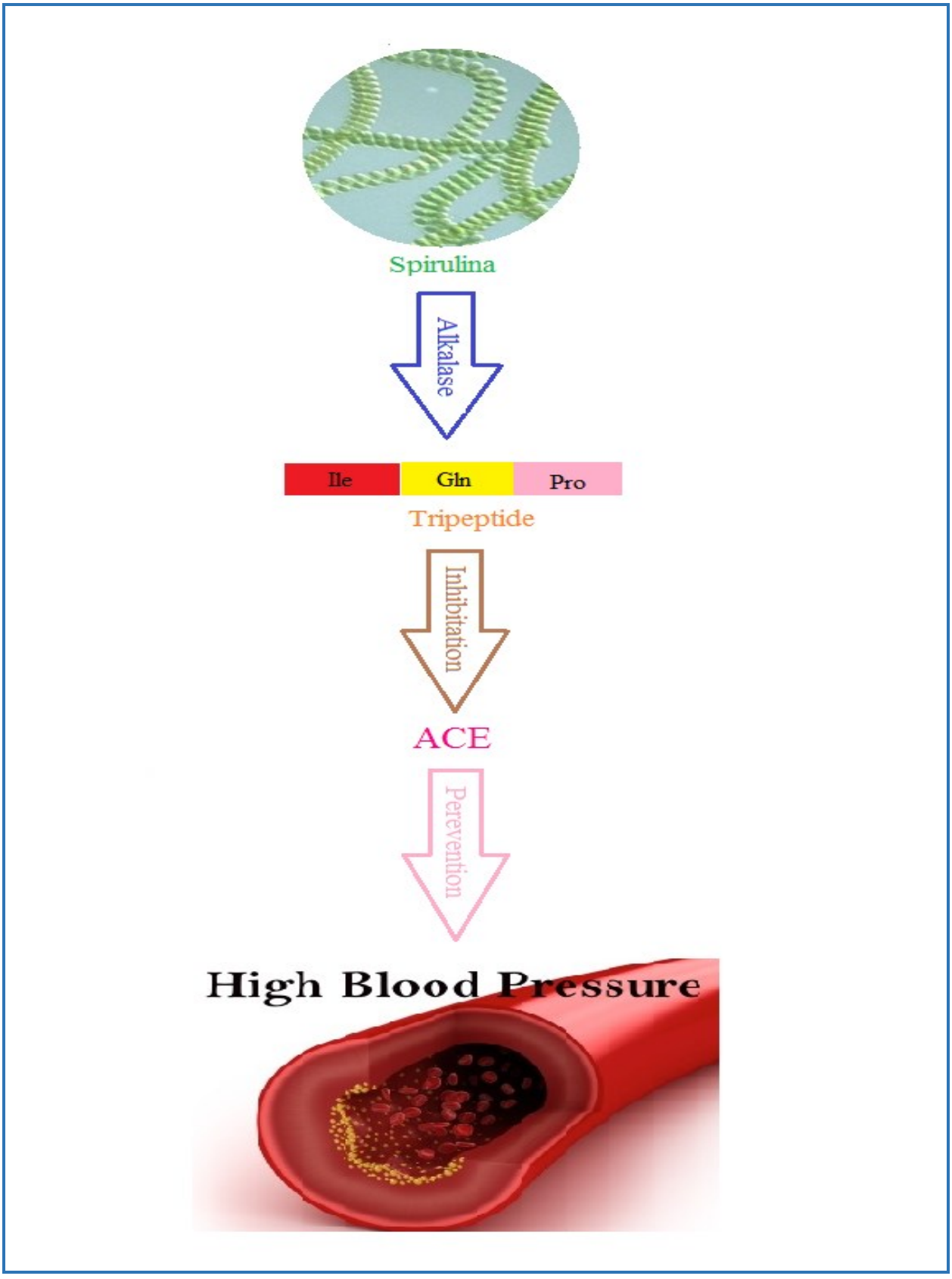
ABSTRACT

Spirulina has attracted a great attention as it contains many nutrients, such as protein, vitamins and minerals. Spirulina contains peptides that have therapeutic and beneficial effects on the human body. Some medicinal properties of the biological peptides of Spirulina platensis include antioxidants, antifungal, antimicrobial, anti-diabetes and anticoagulant activities. This study provides an overview of the biological peptides derived from Spirulina and some other biological activities with health benefits. In this study, peptide Ile-Gln-Pro from spirulina platalsis was isolated using an alkalase enzyme and, then, its inhibitory effect on the angiotensin I-converting enzyme (ACE) was investigated. This peptide was purified by gel filtration chromatography and Reverse-phase high-performance liquid chromatography (RP-HPLC). Enzymatic kinetic studies showed a non-competitive inhibitory activity of this peptide as the Ki value was 5.8 μm.

Corresponding author, email: izadi.mahdieh@yahoo.com (M. Izadi).
Phone: +98913 2318084.

Graphical Abstract



Introduction

Spirulina is a green alga that has been commercialized for many years and has paid more attention to treating various diseases as a human food supplement [1]. This algae with high protein content, γ-linolenic acid, vitamins and minerals is currently sold as nutraceutical supplement. Many studies have demonstrated the health effects of spirulina, including health problems such as diabetes, arthritis, anemia, cardiovascular disease and cancer [2–5]. Spirulina will be useful in various food products to help increase its nutritional qualities and preparations for the treatment of chronic diseases such as diabetes, high blood pressure and heart diseases [6]. A lot of research has shown that spirulina is applied as a useful anti-virus [7] and anti-cancer [8], and also for the prevention of obesity and liver [9] and for its effects of immunosuppression [10]. According to the research on ACE, spirulina has inhibitory activity or antihypertensive effects. About 55 to 77% of the spirulina dry weight contains protein and all amino acids which are essential for human health [11] are likely to separate of bioactive peptides with ACE inhibitor properties from Spirulina. One of the most important causes of cardiovascular disease is high blood pressure [12]. Blood pressure regulates by the angiotensin I converting enzyme (ACE) in the renin-angiotensin system [13]. In some studies, peptides extracted from Spirulina platensis showed antihypertensive activity in hypertensive rats. It was found out that ACE inhibitor peptides extracted from Spirulina platensis showed the lowest IC50 value [14].

Proteins found in plant and animal resources are used to obtain a wide range of bioactive peptides [15]. Peptides and protein hydrolyzates extracted from food sources can be digested with various endo proteases in order to produce oligopeptides. The bioactive peptides have a several biological activities such as the angiotensin converting enzyme (ACE) Inhibitory, anti-thrombotic, surface tension and antioxidant properties [16]. Spirulina has a high potency in treating people with cardiovascular disease by creating better lipid profiles, controlling high blood pressure and enhancing the flexibility of blood vessels [17]. Apart from antihypertensive drugs, diet can also be considered as a risk factor for cardiovascular disease [18]. The bioactive peptides that can be found in foods are safer than chemical drugs and can be used as preventive agents [19]. Captopril 1 is the first oral drug available to inhibit the angiotensin converting enzyme**.** At present, the most suitable candidate for the treatment of hypertension is captopril [20].

Experimental

Materials and methods

Extraction of peptide

20 g dried powder from Spirulina platensis was dissolved in 160 mL of water and alternately frozen and melted 5 times. Then, it was sonicated for 5 min at 40 kHz. The solution was then incubated at 30 °C for 12 h and centrifuged at 6000 rpm for 30 min. The protein content of supernatant was measured using Biuret Protein Assay and cow serum (As a standard). The solution was then mixed with water at the pH of 8.5 and at 2% w/w protein concentration and, then, 2.4 mL of alkalase (~ 2.4 units per mL) was added. The amount of enzyme to the protein substrate was about 0.04% (v/v). The solution was digested for 10 hours at 50 °C for 10 h to enzymatic digestion. This reaction was stopped by adding HCl to pH 4 and also by lowering the temperature specifically by placing the solution on ice, then, it then centrifuged it for 10 min at 6000 rpm. Moreover, Supernatant was ultra-filtrated by cellulose acetate filter with pore size of 0.45, 0.2 μm, and, then, kept at 4 °C.

Purification peptide

The ultrafiltration sample was poured onto the silica gel column (2−10 cm), and, then, washed with HPLC water (Lc-Ms Grade). The obtained fractions were investigated at 214 nm wavelengths by UV-spectrophotometer (Shimadzu, Japan, model UV-2550).

Analysis by thin layer chromatography (TLC)

By noticing the thin layer chromatography (TLC) the bioactive active compounds were identified. The small drop of the extracted and the standard tripeptide (Ile-Gln-Pro) placed on the TLC silica gel plate (Merck) and, then, placed in a tank containing methanol/*n*-hexane (70:30).

Reverse-phase high-performance liquid chromatography (RP-HPLC)

The active fractions were subjected to Reverse-phase high-performance liquid chromatography (SY-8100) with C18 column (COSMOSILMS-II; 4.6/250 mm; Nacalai Tesque Co. Kyoto, Japan) at a flow rate of 1 mL/min. The mobile phase was a mixture of 0.1% trifluoroacetic acid (TFA) in 1% (v/v) acetonitrile (ACN)/99% (v/v) water solution.

Analysis by fourier transfer infrared spectral (FT-IR)

In order to investigate the chemical structure and molecular bonds of compounds, FT-IR spectroscopy was used. The extracted peptid was mixed with KBr and, then, the IR spectral analysis was performed in a Fourier Transmission Infrared Spectrophotometer (JASCO-4200).

ACE inhibitor activity test

In order to measure the inhibition of ACE by the extracted peptide, a mixture of 0.1 M Tris-HCl (pH 7.0), 50 Mm (millimolar) NaCl, 10 μMZnCl$_2$, 0.001 ACE units and 100 µl of different concentration of inhibited peptide solution was prepared in a 2 mL cuvette and, then, their absorbance was determined by UV-spectrophotometer.

Results and discussion

The peptide obtained by alkalysis after ultrafiltration and passing through silica gel showed an ACE inhibitory activity of about 0.087 mg/mL. As shown in Figure 1, ACE inhibition increased with increasing the peptide concentrations.

Reverse-phase high-performance liquid chromatography

Figure 2 demonstrates the total extracted peptide graph in reverse-phase HPLC. The COSMOSILMS-II C18 column and peptide peak time were between 3–4 min.

Calculation of IC50 changes

The IC50 values at various stages were also measured to illustrate the effectiveness of the purification methods. Table 1 shows that the IC50 value was improved.

Evaluation of the inhibition pattern of the ACE inhibitory peptide

Determination of ACE inhibitor peptide inhibition pattern

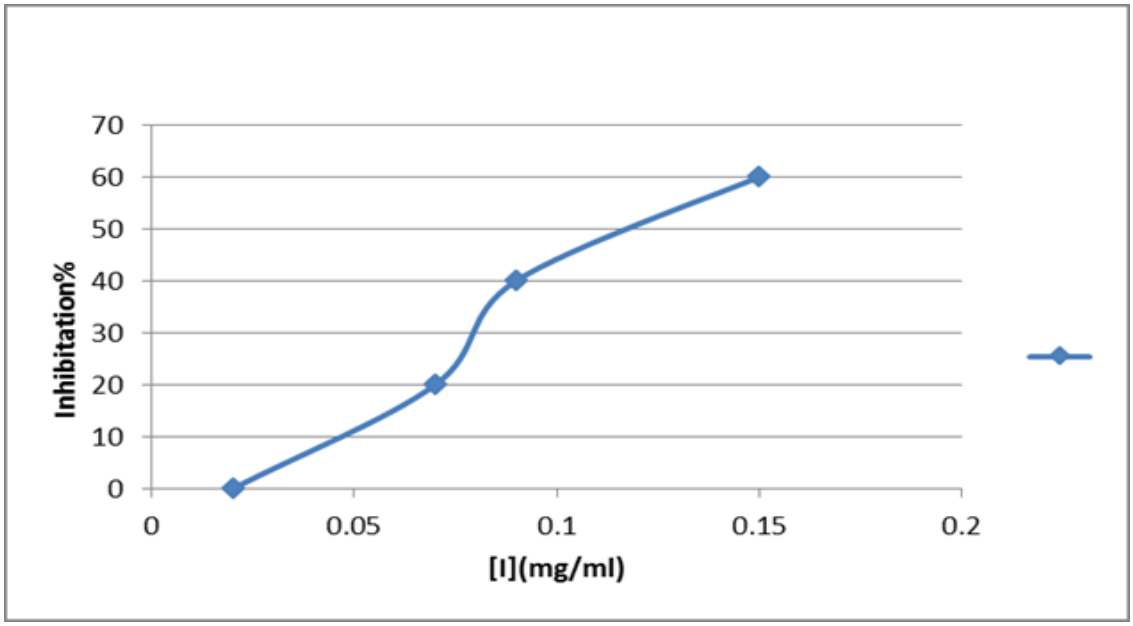


Figure 1. Inhibition curve of ACE inhibitor peptide extracted from spirulina plasticization

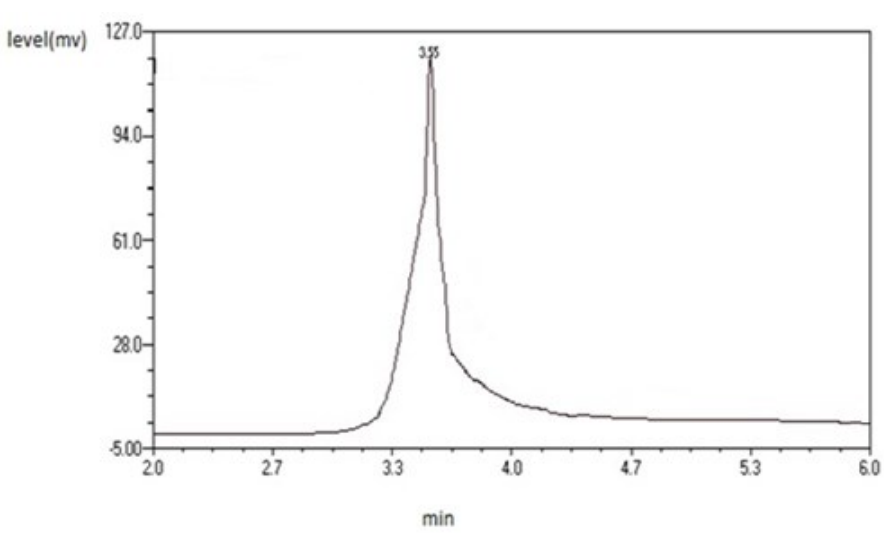


Figure 2. Reverse-phase HPLC of the ACE inhibitory peptide

Table 1. Purification Efficiencies of the ACE Inhibitory Peptide at Different Purification Steps

Purification step	IC50 (mg/mL)
Alcalase digests	0.46
Ultrafiltration	0.23
Silica gel column	0.087
RP-HPLC	0.0039

By kinetic studies, the mechanism of inhibiting ACE-inhibitory peptide, Ile-Gln-Pro, was determinated. The Lineweaver-Burk plot showed that the inhibitory peptide was a non-competitive Inhibitor (Figure 3). The Ki rate was determined to be about 5.8 μm.

Results of analysis by fourier transfer infrared spectral (FT-IR)

Using the Fourier transfusion infrared spectrometry (FT-IR) spectroscopy, the functional groups of ACE inhibitory peptide, extracted from spirulina, were determined (Table 2 and Figure 4).

The ACE inhibitor drugs in the market are Benazepril, Captopril, Enalapril, Fosonopril, Lysine Epilepsis, Zeistrile, Maozyperil, Prindopril, Quinapril, Accupril, Ramipril, and Trandolapril [21]. The Angiotensin converting enzyme inhibitors (ACE) is generally used to treat high blood pressure and its complications are including the acute myocardial infarction, congestive heart failure, and chronic kidney disease [22]. The peptides with biological activity play an important role in the regulation and metabolism, whose potential use as a food supplement and effective food can be

appropriate for improving health and reducing the risk of disease [16]. One of the most important features in the production of food and food products can be the identification of specific molecular properties that determines the biological activity of the peptide [21].

Figure 3. Lineweaver-Burk plot of the reciprocal velocity (1/V0) against 1/ [S] in the presence of Ile-Gln-Pro at concentrations of a) 0 µM, b) 3 µM, c) 7 μM

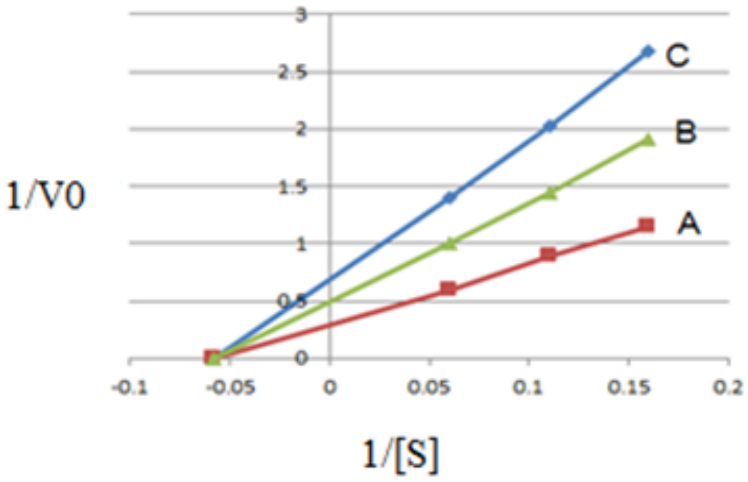


Table 2. Functional groups of the peptide extracted from spirulina

Wavenumber (cm^{-1})	Functional groups
1101.15	C–O Stretching in alcohol groups
1459.85	C=C In aromatic groups
1638.23	C=C in Alkene groups
3458.2	N–H Stretching vibrations presence of amine (proteins) groups, O–H in Alcohol or N–H in amide groups

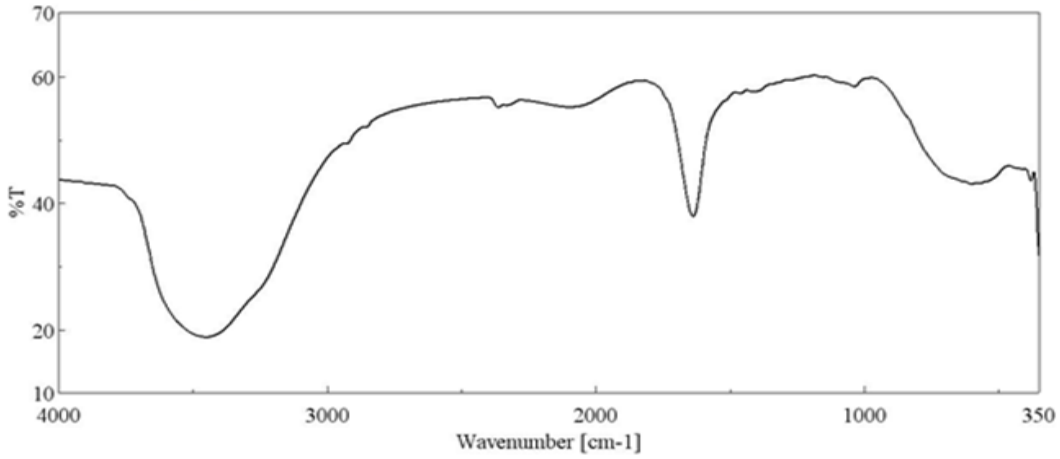


Figure 4. FT-IR spectrum of the peptide extracted from spirulina

Conclusion

In this study, the ACE inhibitory peptide, Ile-Gln-Pro, was extracted from spirulina platensis. The IC50 value of the extracted peptide was about 0.23 mg/mL, which is better than the peptide extracted from the mushroom (IC50:0.31 mg/mL) due to the fact that the peptide had amino acid residues in the carboxy and amine terminals which can inhibit the ACE. With the Lineweaver-Burke plot, it was shown that inhibition of the extracted peptide, (Ile-Gln-Pro), was a non-competitive inhibition. Chemical drugs such as captopril, enalaprilat, and ramiprilat, according to the Lineweaver-Burk plot, are competitive inhibitions. Also, the ACE inhibition curve of the extracted peptide showed that the ACE inhibition increased with enhancing the peptide inhibition concentration.

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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