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Original Research Article

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

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KEYWORDS

Spirulina platensis

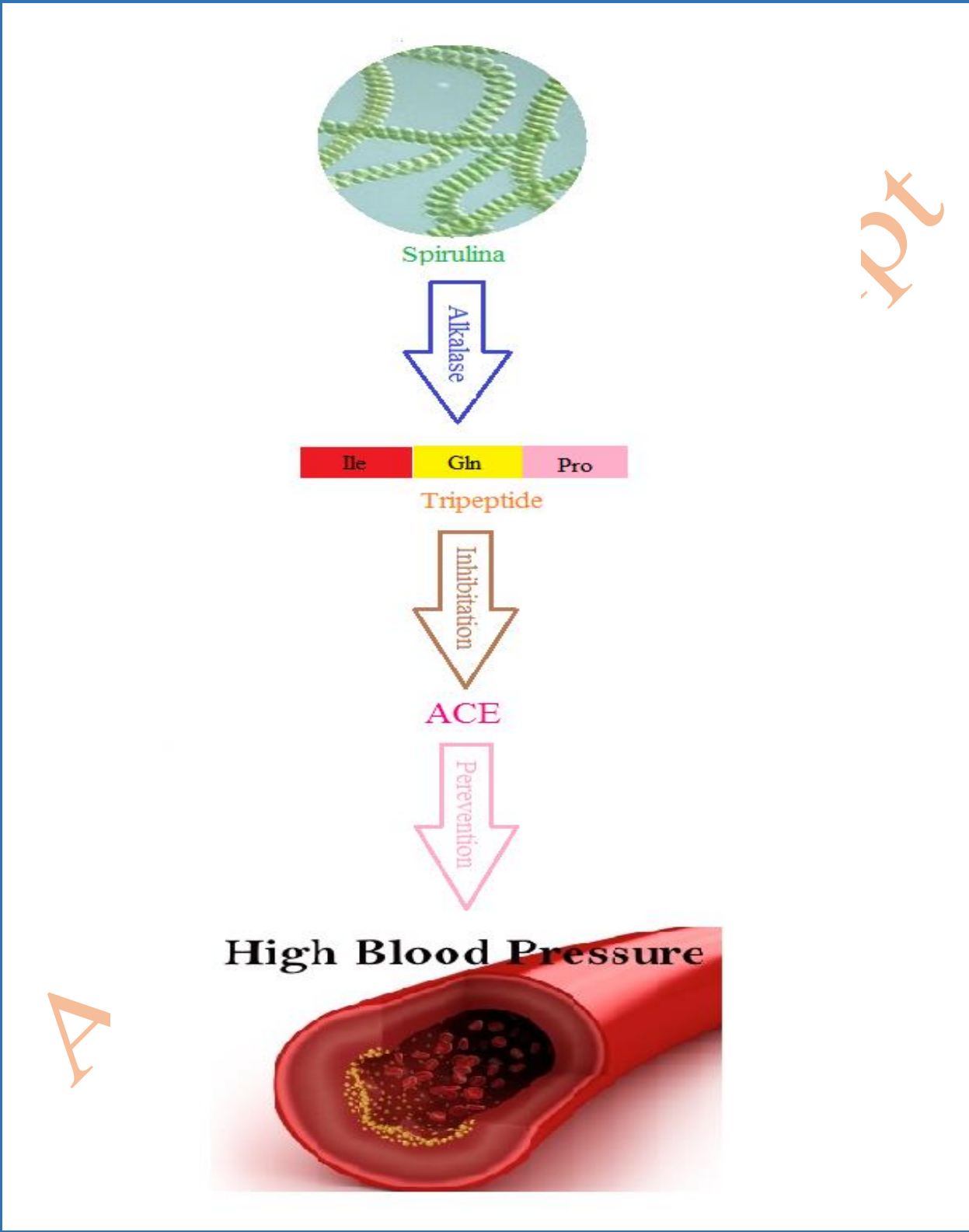
Alkalase

ACE inhibitory peptide

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 activity. This study provides an overview of the biological peptides derived from *Spirulina* and some biological activities with health benefits. In this study peptide Ile-Gln-Pro from *spirulina platensis* was isolated using an alkalase enzyme and investigate its inhibitory effect on the angiotensin I-converting enzyme (ACE). 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, and the K_i value was $5.8 \mu\text{m}$.

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 are 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 disease [6]. A lot of research have shown that spirulina is applied useful anti-virus [7], anti-cancer [8], prevention of obesity and liver [9] and the effects of immunosuppression [10]. According to 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 essential for human health [11] that is interested 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. They found 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 digested with various endo proteases 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 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* dissolved in 160 mL of water and alternately frozen and melted 5 times. Then 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 at 2% w/w protein concentration and 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 lowering the temperature by placing the solution on ice, then centrifuged it for 10 min at 6000 rpm. Supernatant was ultra-filtrated by cellulose acetate filter with pore size of 0.45, 0.2 µm, and kept at 4 °C.

Purification peptide

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

Analysis by thin layer chromatography (TLC)

By the thin layer chromatography (TLC) the bioactive active compounds were identified. The small drop of extracted and the standard of tripeptide (Ile-Gln-Pro) placed on the TLC silica gel plate (Merck) and 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)

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 extracted peptide, a mixture of 0.1 M Tris-HCl (pH 7.0), 50 Mm (millimolar) NaCl, 10 μ MZnCl₂, 0.001 ACE units and 100 μ l of different concentration of inhibited peptide solution was prepared in a 2 mL cuvette and 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 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 was between 3–4 min.

Calculation of IC₅₀ Changes

The IC₅₀ values at various stages were also measured to illustrate the effectiveness of 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

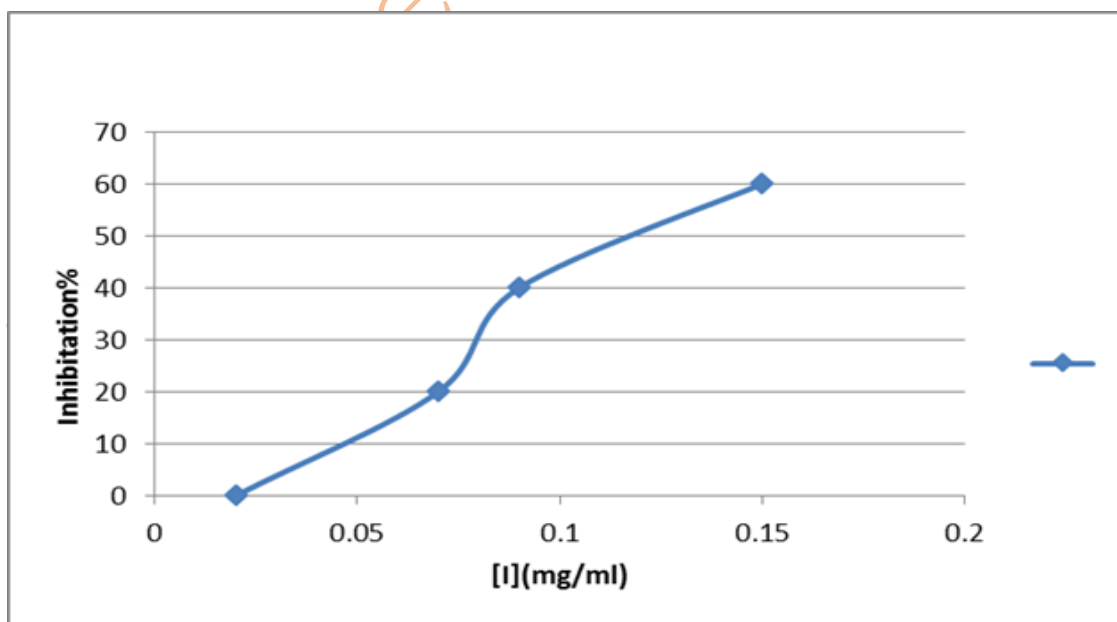


Figure 1. Inhibition curve of ACE inhibitor peptide extracted from spirulina plasticization

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

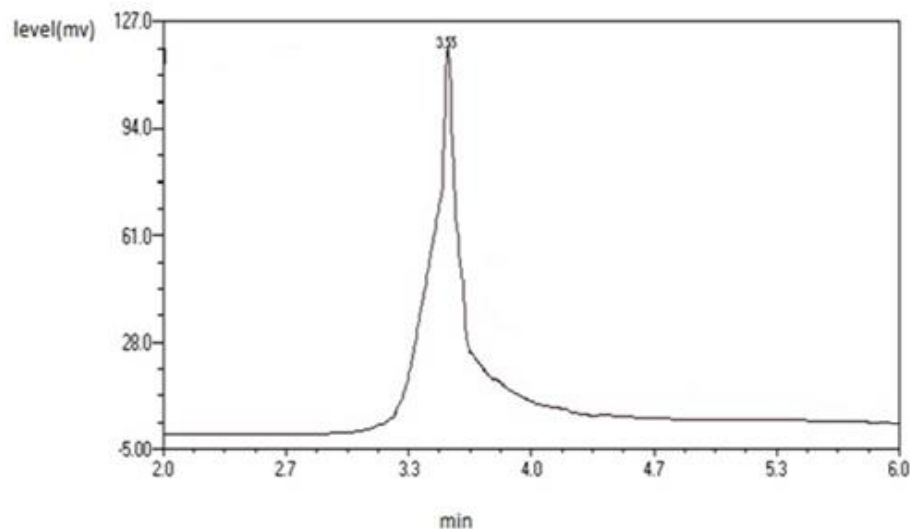


Table 1. Purification Efficiencies of the ACE Inhibitory Peptide at Different Purification Steps

| 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 non-competitive Inhibitor (Figure 3). The K_i rate was determined to be about $5.8 \mu\text{m}$.

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

Using the Fourier transfusion infrared spectrometry (FT-IR) spectroscopy, the functional groups of ACE peptide inhibitory 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) are 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 regulation and metabolism, which their 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 important feature in the production of food and food products can be the identification of specific molecular properties that determine the biological activity of the peptide [21].

Figure 3. Lineweaver-Burk plot of the reciprocal velocity ($1/V_0$) against $1/[S]$ in the presence of Ile-Gln-Pro at concentrations of a) $0 \mu\text{M}$, b) $3 \mu\text{M}$, c) $7 \mu\text{M}$

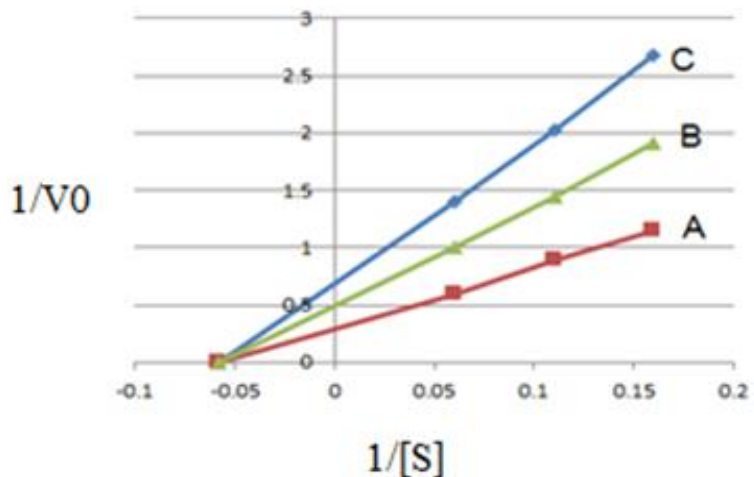


Table 2. Functional groups of the peptide extracted from spirulina

| 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 |

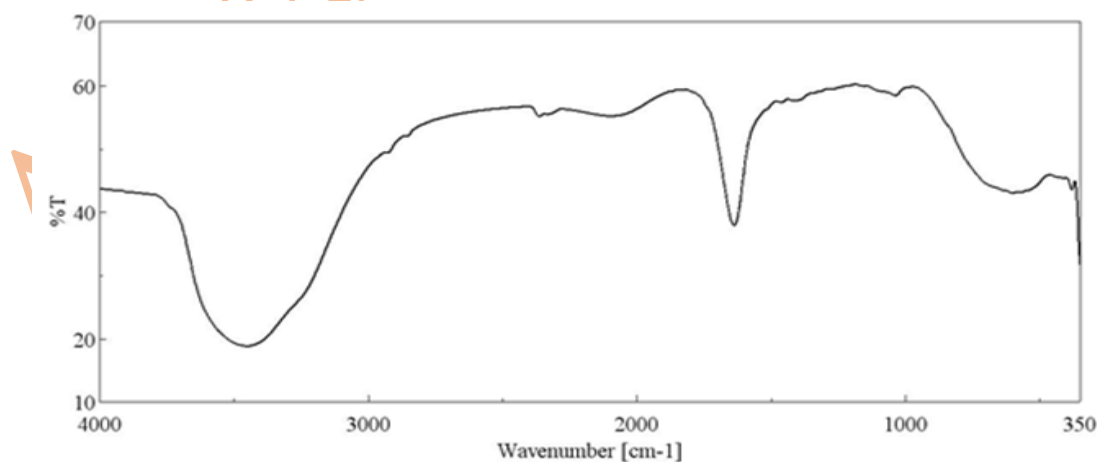


Figure 4. FT-IR spectrum of the peptide extracted from spirulina

Conclusion

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

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Disclosure statement

No potential conflict of interest was reported by the authors.

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