Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste

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Abstract

A hendeca-peptide with angiotensin I-converting enzyme (ACE) inhibitory activity was isolated from the pepsin hydrolysate of algae protein waste, a mass-produced industrial by-product of an algae essence from microalgae, *Chlorella vulgaris*. Edman degradation revealed its amino acid sequence to be Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe. Inhibitory kinetics revealed a non-competitive binding mode with IC\textsubscript{50} value against ACE of 29.6 \textmu M, suggesting a potent amount of ACE inhibitory activity compared with other peptides from the microalgae protein hydrolysates which have a reported range between 11.4 and 315.3 \textmu M. In addition, the purified hendeca-peptide completely retained its ACE inhibitory activity at a pH range of 2–10, temperatures of 40–100 °C, as well as after treatments in vitro by a gastrointestinal enzyme, thus indicating its heat- and pH-stability. The combination of the biochemical properties of this isolated hendeca-peptide and a cheap algae protein resource make an attractive alternative for producing a high value product for blood pressure regulation as well as water and fluid balance.

1. Introduction

Hypertension is identified as a cardiovascular risk factor, and is often called a “silent killer” because persons with hypertension are often asymptomatic for years. This disease currently affects 15–20% of all adults (Je, Park, Jung, Park, & Kim, 2005). The renin-angiotensin system (RAS) plays an important role in the regulation of an organism’s water, electrolytes and blood (Rosenthal, 1993); the angiotensin I-converting enzyme (ACE) participates in regulating blood pressure. ACE inhibitors such as enalapril and captopril cause a number of undesirable side effects such as cough, lost of taste, renal impairment, and angioneurotic oedema (Antonios & MacGregor, 1995), there has been a trend towards the development of a natural ACE inhibitors. In recent years, peptides have been shown to possess many physiological functions, including immune-modulation (Horiguchi, Horiguchi, & Suzuki, 2005), antioxidation (Qian, Jung, & Kim, 2008), antihypertension (Donkor, Henriksen, Singh, Vasiljevic, & Shah, 2007), and antimicrobial activities (Jang, Jo, Kang, & Lee, 2008). Among the different groups of bioactive peptides, the ACE inhibitory peptides have received great attention, due to their potential beneficial effects related to hypertension.

A large variety of algae protein resources exists in the ocean, but very few papers report the functional peptides from algae protein hydrolysates (Sato et al., 2002; Suetsuna & Chen, 2001; Suetsuna & Nakano, 2000). Among the known species of algae, *Chlorella vulgaris* has been the most popular edible microalgae with no side effects. Algae essence is an industrial product derived from water extracts of microalgae, and high molecular weight algae protein waste is a by-product of production. More than 100 tons of algae protein wastes are harvested every year in Taiwan, and it is all remade into low economical-value animal feed. However, this by-product might become an important protein source for the selection of novel ACE inhibitory peptides by enzymatic hydrolysis. This is a comparatively cheap protein source in contrast to most ACE inhibitory peptides originating from costly animal proteins and plant proteins. In this study, we screened an ACE inhibitory peptide from algae protein waste digested with commercial enzymes. We also investigated the ACEs inhibitory potency, inhibition mechanism, and stability against temperature, pH, and gastric proteases of the purified peptide from algae protein waste in vitro.

2. Materials and methods

2.1. Materials

Algae protein waste was dried and kept at –20 °C prior to use. Hippuryl-l-histidyl-l-leucine (HHL), ACE obtained from human...
skin, hippuryl acid, and pancreatin from porcine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Flavourzyme Type A and alcalase were purchased from Novo Nordisk A/S (Copenhagen, Denmark) and papain was obtained from Amano (Nagoya, Japan). Pepsin was obtained from Nacalai Tesque (Kyoto, Japan). Sephacryl S-100 HR, Q-sepharose Fast Flow, and the Sephasil peptide C8 column were purchased from Pharmacia Biotech. Co. (Uppsala, Sweden).

2.4. Purification of ACE inhibitory peptides from algae protein waste

2.4.1. Ammonium sulfate fractionation

Ammonium sulfate was added to a concentration of 20% saturation in the supernatant from the pepsin hydrolysates; and precipitated protein was removed by centrifugation (10,000 g, 20 min). The ammonium sulfate concentration was continually raised to 80% saturation in the permeate, stepwise. Each fraction was assessed for ACE inhibitory activity. A strong ACE inhibitory activity fraction was collected and lyophilised for the next step.

2.4.2. Gel filtration chromatography

The 40–80% precipitate was dissolved in distilled water and the solution was fractionated using a Sephacryl S-100 high HR column (φ2.6 × 70 cm), pre-equilibrated with distilled water. The column was eluted with the same buffer, and 6 ml fractions were collected at a flow rate of 1.5 ml/min. Fractions showing ACE inhibitory activity were pooled and lyophilised.

2.4.3. Ion exchange chromatography

A strong ACE inhibitory activity fraction subsequently was loaded onto a Q-sepharose Fast Flow column (φ2.6 × 40 cm), which was pre-equilibrated with 20 mM Tris–HCl buffer solution (pH 7.8), then eluted with a linear gradient of NaCl (0.0–1.0 M) in the same buffer at a flow rate of 1.5 ml/min. Bioactive peptides with antihypertensive activity in the eluent were pooled for further experiments.

2.4.4. Reverse-phase high-performance (RP-HPLC) chromatography

The lyophilised fraction was further purified on an Inertsil ODS-3 C18 semi-prep column (10 × 250 mm). The column was eluted with a linear gradient of acetonitrile (25–40% in 30 min) containing 0.1% TFA at 2 ml/min. The active fraction was re-chromatographed on a Sephasil peptide C8 column (4.6 × 250 mm) at a flow rate of 1.0 ml/min.

2.5. Determination of amino acid sequence

The amino acid sequence of the purified peptide was determined by an automated Edman degradation with an Applied Biosystems Procise 494 protein sequencer (Foster City, CA, USA).

2.6. Stability of ACE inhibitory peptide

The purified peptide solutions were incubated at different temperatures (40, 60, 80, and 100 °C) for 1 h, and then assayed for residual ACE inhibitory activity. The peptide solutions were also incubated at 37 °C, and pH values of 2, 4, 6, 8, and 10, for 1 h. Stability against gastrointestinal protease was also assayed in vitro. 1% (w/w) of ACE inhibitory peptide solution in 0.1 M KCl–HCl (pH 2.0) buffer with pepsin was incubated for 3 h in a water bath at 37 °C; then neutralised to pH 7.8 before heating to boiling for 10 min. The remaining suspension was further digested by a 1% (w/w) porcine pancreatin for 4 h at 37 °C. The sample was boiled for 10 min followed by centrifugation (10,000 g, 10 min) and then assayed for residual ACE inhibitory activity (Wu & Ding, 2002).

2.7. Determination of the inhibition pattern on ACE

Various substrate (HHL) concentrations were co-incubated with purified peptides and the ACE solution, and each reaction mixture was assayed as described in Section 2.3. Standard hippuric acid solution was injected as a reference. The IC50 value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

2.8. Statistical analysis

Results were presented as means of experiments done in triplicate ± standard deviation. The Student’s t-test was used to determine the level of significance. A p value of less than 0.05 was taken as significant.

3. Results and discussion

3.1. Preparation of ACE inhibitory peptides from algae protein waste

Many ACE inhibitory peptides have been discovered from enzymatic hydrolysates of different food proteins, but so far, there has been no research focused on cheaper algae protein waste which consists of over 50% protein content. In this study, the algae protein waste hydrolysates were prepared by means of hydrolysis with commercial proteases including pepsin, papain, alcalase, and flavourzyme. The hydrolysis was necessary in order to release ACE inhibitory peptides from the inactive forms of intact algae protein waste. The results indicated the specificity of the enzymes in the generation of the ACE inhibitory peptides, as shown in Fig. 1. The pepsin treatment released specific peptides from the inactive algae protein, with the highest ACE inhibitory activity and protein yield among the hydrolysates (p < 0.05). Other reports indicate that pep-
sin was capable of producing ACE inhibitory peptides from algae protein (Suetsuna & Chen, 2001; Suetsuna & Nakano, 2000).

3.2. Purification of ACE inhibitory peptide from pepsin hydrolysate of algae protein

The peptides present in pepsin hydrolysates from the algae protein were fractionated with ammonium sulfate, and then separated into four fractions. The 40–60% and 60–80% fraction exhibited higher ACE inhibitory activity than other fractions (Fig. 2a). These two fractions were combined, precipitated, and re-dissolved in a small volume of distilled water, and subsequently purified using column chromatographic methods. Size exclusion chromatography of the ammonium sulfate fraction on a Sephacryl S-100 high HR column resulted in two fractions (designated as A and B). Fraction B was found to possess higher ACE inhibitory activity (Fig. 2b), so it was further subjected to a Q-sepharose Fast Flow column with a linear gradient of NaCl (0.0–1.0 M) (Fig. 3). The bound peptides (B2 fraction), which were eluted at 0.35–0.45 M NaCl concentration, had no ACE inhibitory activity, but the non-adsorption fraction (B1 fraction) expressed strong ACE inhibitory activity. The B1 fraction was pooled, lyophilised, and further separated by RP-HPLC on an Inertsil ODS-3 C18 reverse-phase semi-prep column (10 × 250 mm). Fraction B1a showed the most potent ACE inhibitory activity, and was reloaded on a Sephasil peptide C8 reverse-phase analytical column (4.6 × 250 mm) to attain a purified peptide (P fraction) (Fig. 4). The purified peptide was shown to inhibit ACE in a dose-dependent manner with an IC50 value 29.6 μM (Fig. 5). There have been few studies on ACE inhibitory peptides from algae protein hydrolysates. Suetsuna and Nakano (2000) reported IC50 values of Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly, and Tyr-Asn-Lys-Leu from the peptic digest of wakame, Undaria pinnatifida, were 213, 64.2, 90.5 and 21 μM, respectively. The IC50 values of Ile-Val-Val-Glu, Ala-Phe-Leu, Phe-Ala-Leu, Ala-Glu-Leu and Val-Val-Pro-Pro-Ala from the peptic digest of microalgae, C. vulgaris were 315.3, 63.8, 26.3, 57.1 and 79.5 μM.
to validate the ACE inhibitory activity of the purified peptide, a synthetic hendeca-peptide with the same sequence was synthesized and tested. The synthetic peptide exhibited the same ACE inhibitory activity as the purified peptide from algae protein hydrolysate (data not shown). The result suggests that the purified peptide actually possesses ACE inhibitory activity.

The hendeca-peptide sequence was next subjected to secondary structure prediction to elucidate possible structure-activity correlations (Garnier, Gibrat, & Robson, 1996). The results showed that the hendeca-peptide contains 18.2% extended and 81.8% coiled secondary structure. Elias, Kellerby, and Decker (2008) previously reported that the function of a bioactive peptide was dependent on its amino acid composition; however, the activity of these amino acid residues was also limited by the structure of the polypeptide (Elias et al., 2008). Therefore, the extended and coiled structure in this peptide might contribute to ACE inhibitory activity. In parallel, other structure-activity correlation studies have indicated that ACE binding was strongly influenced by the C-terminal tripeptide sequence of the substrate, and the tripeptide could interact with the subunits S1, S1′, and S2′ of ACE (Ondetti & Cushman, 1982). The ACE preferred substrates containing branched amino acid residues at the N-terminal position, and hydrophobic amino acid residues (aromatic or branched-side chains) at the C-terminal position (Byun & Kim, 2002; Cheung, Wang, Miguel, Emily, & David, 1980). The hydrophilic amino acid residues in the peptide sequence could also affect inhibitory activity by disrupting the access of the peptide to the active site of ACE. The hydrophilic–hydrophobic partitioning in the sequence was also a critical factor in the inhibitory activity (Li et al., 2004). The peptide with comparative low IC50 value had a high content of branched and aromatic amino acids such as Pro, Glu, Val, Phe, and Tyr in its peptide sequence. Thus, it was very likely to have a higher antihypertensive potential (He, Chen, Wu, Sun, & Zhang, 2007). Perhaps the abundance of the above-mentioned amino acids in the purified peptide might account for the exhibited potency of its ACE inhibitory activity.

### 3.4. Stability of the purified peptide

The processing stability of the purified peptide after various pH and temperature treatments was a prerequisite in preparing foods with “functional peptides”. To investigate the pH and heat-stability of the purified peptide, the peptide was subjected to incubation at pH 2–10 and temperature 40–100°C for 1 h and measured for residual activity (data not shown). The results showed that the purified hendeca-peptide completely retained its ACE inhibitory activity (p > 0.05), indicating that the purified peptide was both pH and heat-stable.

Gastrointestinal enzyme incubation in vitro provided an easy process to imitate the fate of these peptides under oral administration. Some ACE inhibitory substances failed to show the hypotensive activity after oral administration in vivo, due to the possible hydrolysis of these peptides by ACE or gastrointestinal proteases (Fujita, Yokoyama, & Yoshikaw, 2000; Li et al., 2004). To evaluate the stability of the purified peptide under gastrointestinal enzymes digestion, the purified peptide was first incubated with various gastrointestinal enzymes, including pepsin and pancreatin, then subjected to ACE inhibitory activity assays and HPLC profile comparisons. The results showed that no apparent change was observed after in vitro incubation with gastrointestinal enzymes (p > 0.05), suggesting that there is resistance of the purified peptide to digestion in the gastrointestinal tract, and that the active sequence of the peptide would not be destroyed by these enzymes. The low susceptibility of the purified peptide to hydrolysis by gastric proteases was similar to that of shorter oligopeptides, as shown by Wu and Ding for small peptides (Wu & Ding, 2002).
3.5. Determination of the inhibition pattern on ACE

To shed light on the inhibition pattern of the ACE by the purified hendeca-peptide, the purified peptide was co-incubated with various substrate (HHL) concentrations and an ACE solution, and the double reciprocal velocity-substrate plot is shown in Fig. 6. The $K_m$ and $V_{max}$ values for the reaction at different concentrations of purified peptides were determined according to Lineweaver–Burk plots. The inhibition mode of the purified peptide was determined to be non-competitive. This represents that the peptide could bind to the ACE enzyme, regardless of whether a substrate molecule was present or absent. It might inhibit the enzyme by causing a conformational change, which prevented the enzyme from converting substrate to product, so the inhibitor worked equally well at low and high concentrations of the substrate. Interestingly, the result was similar to those of the peptide Met-Ile-Phe-Pro-Gly-Ala-Gly-Pro-Glu-Leu from Limanda aspera frame protein (Jung et al., 2006), Leu-Ile-Tyr from human plasma (Nakagomi, Ebisu et al., 2000), and Ile-Phe-Leu and Trp-Leu from fermented soybean food (Nakagomi, Yamada et al., 2000). Although most of these reported peptides acted as competitive inhibitors for ACE, a few peptides inhibited ACE activity in a non-competitive manner (Li et al., 2004). The inhibition site of the non-competitive inhibitor on ACE was not specified, and the precise inhibition mechanism of ACE inhibitory peptide is also not yet clear (Kuba et al., 2003; Li et al., 2004).

4. Conclusion

While there are several reports on the physiological effects of various food sources, no reports to date on algae protein waste-derived peptides from the microalgae, C. vulgaris. The purified hendeca-peptide developed in the study has excellent ACE inhibitory abilities, good pH- and heat-stability, low gastrointestinal enzyme susceptibility, and established safety. In conclusion, the hendeca-peptide, with its combination of important biochemical properties and its easily accessible source, holds promising potential as a candidate in future industrial production of functional peptides for blood level regulation in hypertensive patients.

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References


