

Antioxidant Activity of Protein Fractions Derived from *Acrochaetium sp.* (Rhodophyta) Enzymatic Hydrolysates

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ABSTRACT

Natural antioxidants are helpful in the prevention of human diseases. The objective of this study is to isolate the potential protein fractions from *Acrochaetium sp.* as an antioxidant. Fractions were obtained by proteolytic digestion using α -chymotrypsin, pepsin, trypsin, thermolysin individually and in combination of two enzymes, then centrifuged using 3 kDa molecular weight cut-off (MWCO) ultrafiltration membrane and fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). The 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH) assay was used to measure the antioxidant activity. Result showed that thermolysin hydrolysate and the combination of trypsin-thermolysin hydrolysates had the highest antioxidant activity compared to the other hydrolysates with IC_{50} value of 1.48 ± 0.92 mg/mL and 1.37 ± 0.84 mg/mL after fractionated using 3 kDa MWCO ultrafiltration membrane. Fractionation using RP-HPLC resulted fraction 7 obtained from thermolysin hydrolysates showed the highest antioxidant activity with IC_{50} value 0.58 ± 0.56 mg/mL and fraction I obtained from trypsin-thermolysin hydrolysates showed the highest antioxidant activity with IC_{50} value 0.38 ± 0.33 mg/mL. The protein fractions from *Acrochaetium sp.* hydrolysates as antioxidant still has not been reported previously, therefore it can indicated as a potential therapeutic source for reducing oxidative stress.

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INTRODUCTION

The key cause of the pathogenic disorders and various chronic diseases is oxidation. The oxidative reaction is not only deteriorates the quality of food products, but also lead to various chronic diseases such as hypertension, cancer and Parkinson's disease. Cellular damage is caused by the high level of oxidative stress due to significant imbalance between the antioxidant defense system and free radicals [1, 2]. Free radicals attacks on protein, lipids and nucleic acids which lead to weakening of the antioxidant enzymes and lipid peroxidation [3]. The easiest way to prevent these diseases from human body is consume vegetables, seed, and fruits to increase the antioxidant capacity in human body. An antioxidant is a substance which inhibits oxidation of the substrate at low concentration compared to that of an oxidizable substrate [4]. Antioxidants are widely applied to medicine, chemical industries, and important food additive which are mainly used to prevent the oxidation of

fats and also avoid nutrition of food damaging, browning and fading by capture and neutralize the free radicals [5].

Currently, synthetic antioxidants such as butyl hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG) are added to food products to retard lipid oxidation, thus inhibit the generation of reactive oxygen species (ROS). The synthetic antioxidants must be used under strict regulation due to their potential health hazards and when compared to natural antioxidants, natural antioxidants are more favored in the present life because of their pure nature, high security, non-toxicity and have strong antioxidant capacity [6, 7]. Therefore, there is an interest in developing natural antioxidants.

Recently, more studies have been carried out to find antioxidant in various natural products, such as seed of pea [8], chickpea [9], peanuts kernels [10] and corn [11]. Several studies about marine organism as antioxidant also have been carried out, such as yellow stripe trevally [12], muscle of ornate threadfin bream [13], pacific hake [14], aquatic species [15], capelin [16], muscle proteins of harp seal [17] and rhodophyta [18].

Marine algae are sustainable resources in marine ecosystems and mostly used as a source of food and medicine. Algae biomass has been used for centuries as food and medicine. Major compounds in algae are polysaccharides, phenolic and phlorotannins, protein, peptides and essential amino acids, lipids, terpenoids and steroids, vitamins and minerals [19, 20]. Algal biomass and algae-derived compounds have a very wide range of potential applications for nutrition and health products. Some algae are considered as rich sources of natural antioxidants. Macroalgae have received much attention as potential natural antioxidants and there has been very limited information on antioxidant activity of macroalgae [21]. Among macroalgae, the antioxidant activity of *Acrochaetium sp.* used in this study is rarely reported. *Acrochaetium sp.* is a rhodophyta which distribute in Taiwan, South America, Atlantic Islands, Indonesia and Africa [22].

Currently, there is attention to the function and bioactivities of protein and its hydrolysates from food sources that may be used as an alternative source in the prevention of some diseases. Besides, food proteins have been known as bio-molecule that plays an important role in human improvement with their well-known nutritional values [23]. Peptides derived from food proteins can be a great source of antioxidants due to its aromatic rings, excessive donor electrons and appropriate hydrophobic character [24]. Enzymatic hydrolysis is the most reliable and an effective method to produce peptides with functional properties [25].

In this study, *Acrochaetium sp.* protein isolate was hydrolyzed using single (α -chymotrypsin, pepsin, trypsin, thermolysin) and in combination enzymatic processes. The aims of this study were to generate *Acrochaetium sp.* protein hydrolysates, fractionate the hydrolysates using RP-HPLC and evaluate the potential antioxidant activity of these samples using DPPH assay.

MATERIAL AND METHODS

Sample Preparation

Salt, sediment, and organic debris from *Acrochaetium sp.* were removed using fresh water. Algae were carefully rinsed with freshwater and dried at 40 °C for 2 h and ground to obtain a powder with a particle size lower than 1 mm and finally stored at 4 °C in plastic bags for further analysis.

Protein extraction, digestion, and ultrafiltration

The dried powder of *Acrochaetium sp.* was dissolved in 20% of trichloroacetic acid (TCA) for 12 h at 4 °C. The TCA was removed using acetone and the pellet was lyophilized. The dried protein then was hydrolyzed by α -chymotrypsin (37 °C), pepsin (37 °C), thermolysin (60 °C) and trypsin (37 °C) for 16 h. *Acrochaetium sp.* was also digested by various combinations of enzymes, for each enzyme was incubated for 3 h. The reaction was stopped by heating the mixture and then fractionated into < 3 kDa MWCO. The filtrate was collected and lyophilized for further analysis.

Fractionation of *Acrochaetium sp.* Protein Hydrolysate by RP-HPLC

Acrochaetium sp. protein hydrolysate was eluted by 5% acetonitrile (ACN) and 0.2% FA in deionized water and fractionated by reverse-phase high performance liquid chromatography (RP-HPLC, Hitachi Chromaster, Tokyo, Japan). The mobile phase of buffer A (5% ACN and 0.1% TFA in deionized water) and buffer B (95% ACN and 0.1% TFA in deionized water). Twenty microliters of < 3 kDa hydrolysates was loaded at a flow rate of 1 mL/min. Absorbance of the fractions was monitored at 214 nm.

DPPH Radical Scavenging Assay

DPPH radical scavenging assay was measured according to Yu et al. [26]. Fresh DPPH solutions (0.1 mM DPPH in purified ethanol) were prepared daily. The samples, which comprised 100 µl samples with 100 µl of DPPH solution in a 96-well plate, was mixed and incubated for 30 min in the dark at room temperature. The absorbance was measured by using ELISA at 517 nm (A_s). Ethanol was used as the blank (A_b), and distilled water was used as the control (A_c). The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_s - A_c}{A_b} \right] \times 100\%$$

where A_b is the absorbance of the blank, A_s is the absorbance of the sample solution, and A_c is the absorbance of the control.

Statistical Analysis

Data was expressed as the mean \pm standard deviation (mean \pm SD). The analysis was done by using one way ANOVA in SPSS 16.0 (Chicago, SPSS Inc.) followed by post-hoc Duncan's test and accepted at the $P < 0.05$ level to identify the significant differences among treatments.

RESULTS AND DISCUSSION

Generation of free radicals and lipid peroxidation often occur in biological and food systems. In biological systems, antioxidants as part of the defense mechanism can prevent oxidative damage [27] and free radical generation by pro-oxidative from environment such as air pollutant, ultraviolet radiation, and cigarette smoke [28]. Recently, there is increased interest in naturally bioactive compounds as alternatives to synthetic substances, even these naturally compounds often show lower activity than the synthetic substances, but they are nontoxic and do not leave any residues [20]. As reported by Margaret et al. [29], bioactive peptides can be released by enzymatic proteolysis of food proteins, therefore pancreatic enzymes; chymotrypsin and trypsin have been used for derivation of bioactive peptides.

Enzymatic hydrolysis is the most effective method to produce peptides with functional properties; in this study we used several proteases individually and in combination. As shown in Figure 1, thermolytic hydrolysate of *Acrochaetium sp.* possessed the highest scavenging of DPPH radicals than other proteases (57.40%). These results are consistent with the previous studies suggested that thermolysin is specifically catalyzes peptide bond containing hydrophobic and aromatic amino acid, which potential as antioxidant peptide [30]. In this study, we also used the combination of two enzymes and resulted the combination of thermolysin-trypsin had the highest scavenging of DPPH radicals compared with other combination of different enzymes, as shown in the Figure 2. Besides thermolysin catalyzes peptide bond containing hydrophobic and aromatic amino acid, the using of trypsin also contribute the releasing of amino acids (2-20 residues) which formed antioxidant peptides and immobile in parent protein [24].

Bioactivity of protein hydrolysates is mainly affected by the molecular weight of the peptides. The molecular weight of hydrolyzed protein is one of an important factor in producing protein hydrolysates [31]. The thermolysin hydrolysate and thermolysin-trypsin hydrolysate was fractionated by ultrafiltration with molecular weight cut-off (MWCO) membranes of < 3 kDa. The IC_{50} values of the thermolysin hydrolysate were 1.83 ± 0.95 mg/mL (> 3 kDa) and 1.48 ± 0.92 mg/mL (< 3 kDa) (Figure 3). The thermolysin-trypsin hydrolysate showed the IC_{50} values of 1.70 ± 1.03 mg/mL (> 3 kDa) and 1.37 ± 0.84 mg/mL (< 3 kDa) (Figure 4). Ultrafiltration membrane system was used to separate the hydrolysates into defined molecular weight ranges. It holds well in purification of simple peptides from various crude protein hydrolysates [32, 33]. The isolated peptide fractions showed higher antioxidant activity than the hydrolysate [34]. This indicated that the peptide generation plays an important part in antioxidant potential of proteins. Purification step will affect the IC_{50} value, it indicated that the lower and more purified molecule has higher inhibition rate, more purified the molecule, and the IC_{50} will be decreased.

RP-HPLC involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. RP-HPLC (detected at 214 nm under an UV-vis detector) was further used to fractionate the antioxidant peptides and the *Acrochaetium sp.* was separated into 12 fractions (fraction 1-12 for the thermolysin hydrolysate and fraction A-L for the thermolysin-trypsin hydrolysate) as shown in the Figure 5

and 7. Each fraction was collected; freeze dried, and determined its antioxidant activity. As shown in Figure 6, fraction 7 exhibited the highest DPPH free radical scavenging activity with the inhibition (57.29%) and among 12 fractions for thermolysin-trypsin hydrolysate of *Acrochaetium sp.*, fraction I showed the highest DPPH free radical scavenging activity (64.54%) (Figure 8). Furthermore, the IC_{50} value was tested for fraction 7 and fraction I. Fraction 7 from *Acrochaetium sp.* hydrolysate using thermolysin had IC_{50} value of 0.58 ± 0.56 mg/mL and in the other hand; fraction I from *Acrochaetium sp.* hydrolysate using thermolysin-trypsin had IC_{50} value of 0.38 ± 0.33 mg/mL (Figure 9). These results showed higher antioxidant activity compared by the other marine organisms, such as *Theragra chalcogramma* (1.3 mg/mL) [35], *Thunnus tonggol* (5 mg/mL) [36], *Gadus morhua* (2.5 mg/mL) [37], and *Navodon septentrionalis* (10 mg/mL) [38].

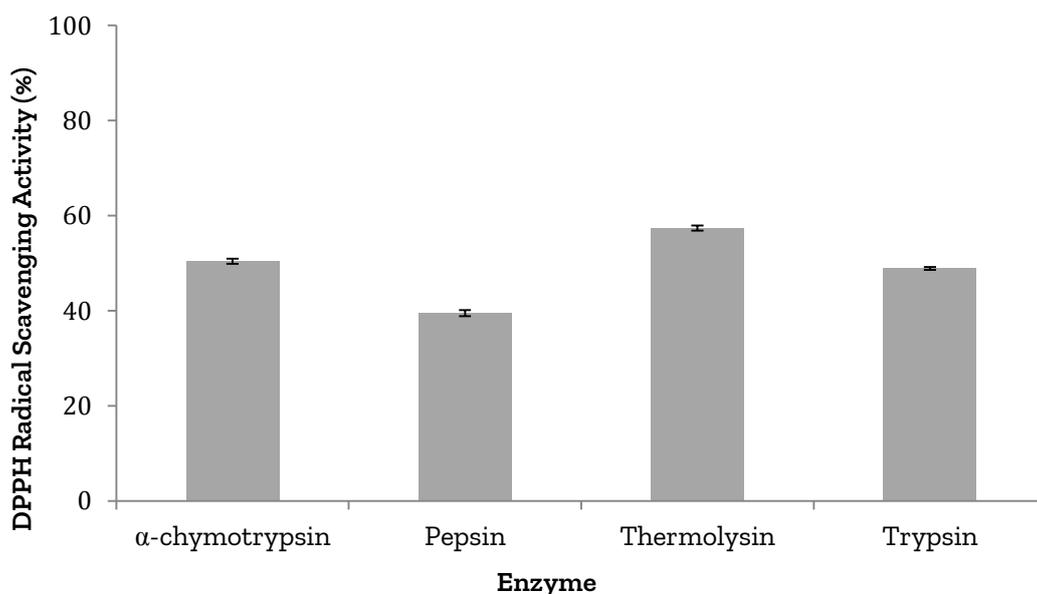


Figure 1. DPPH radical scavenging activity (%) of *Acrochaetium sp.* enzymatic hydrolysate using different single enzyme.

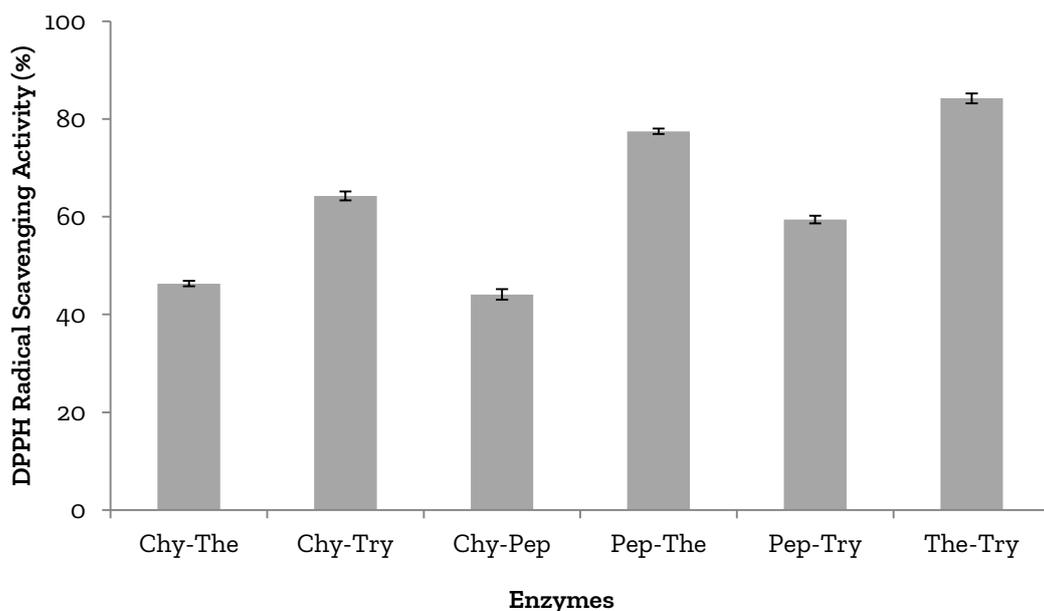


Figure 2. DPPH radical scavenging activity (%) of *Acrochaetium sp.* enzymatic hydrolysate using different combination of two enzymes (Chy: α -chymotrypsin; Pep: Pepsin; The: Thermolysin; Try: Trypsin).

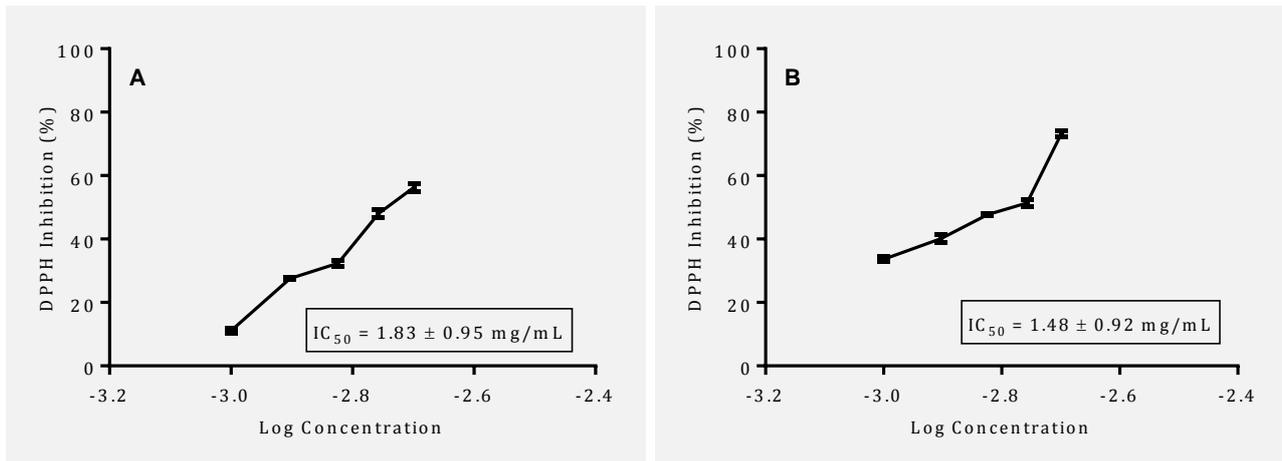


Figure 3. (A) IC_{50} value of *Acrochaetium sp.* hydrolysate using thermolysin (> 3 kDa) and (B) (<3 kDa).

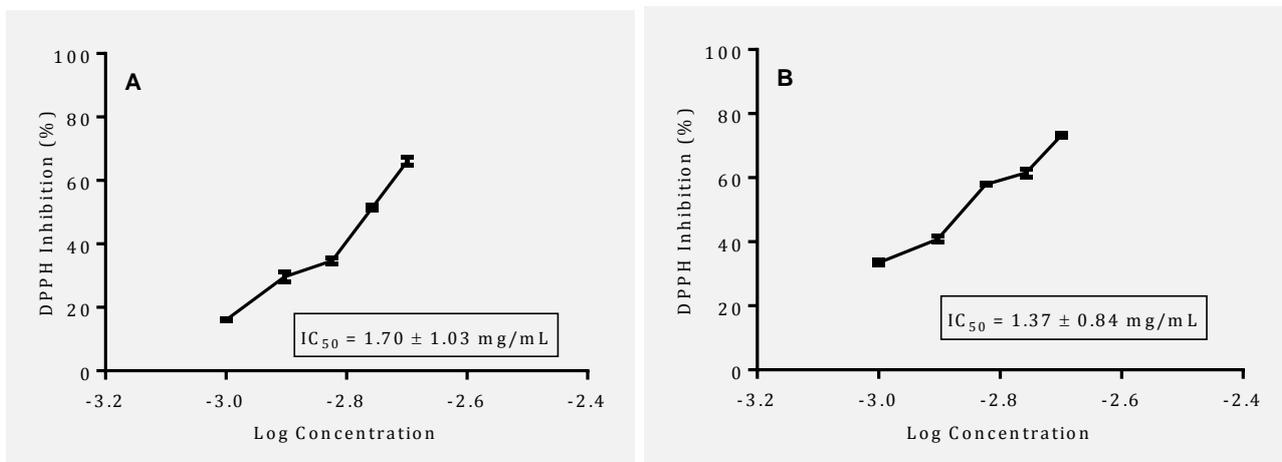


Figure 4. (A) IC_{50} value of *Acrochaetium sp.* hydrolysate using thermolysin-trypsin (> 3 kDa) and (B) (<3 kDa).

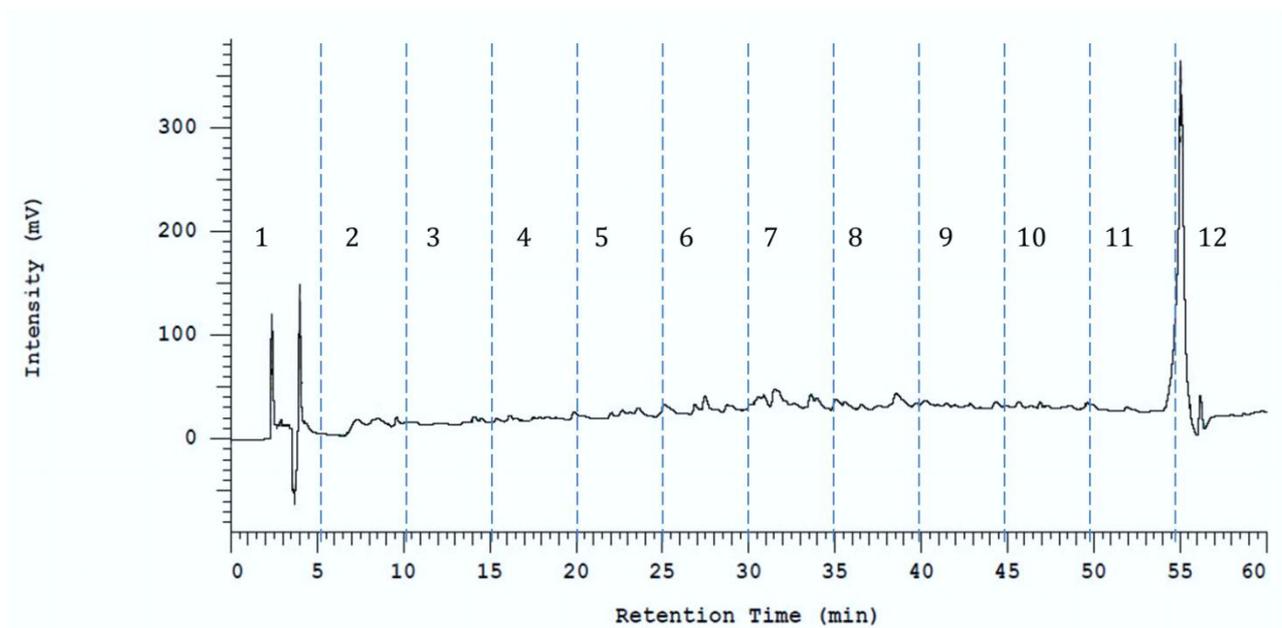


Figure 5. RP chromatogram of thermolysin hydrolysate of *Acrochaetium sp.*

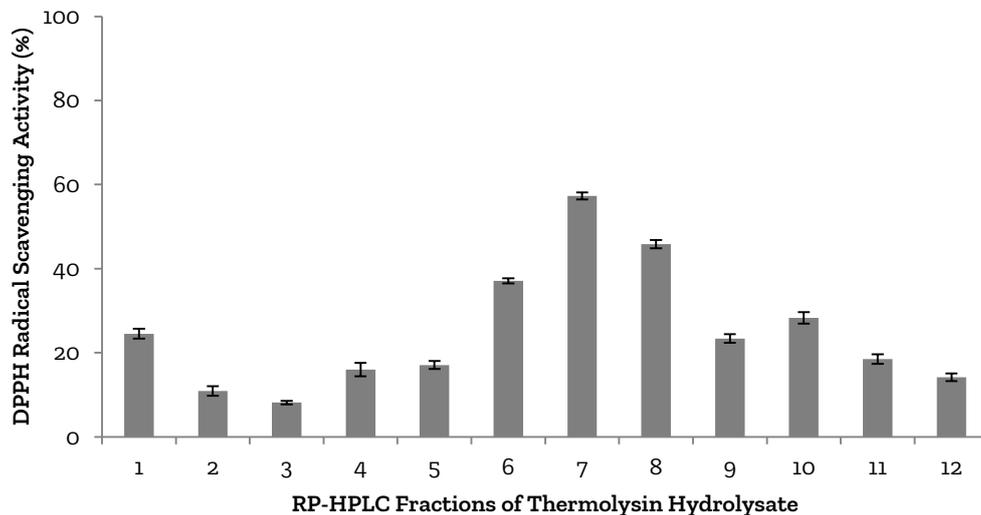


Figure 6. DPPH radical scavenging activity (%) of *Acrochaetium* sp. fractions using thermolysin.

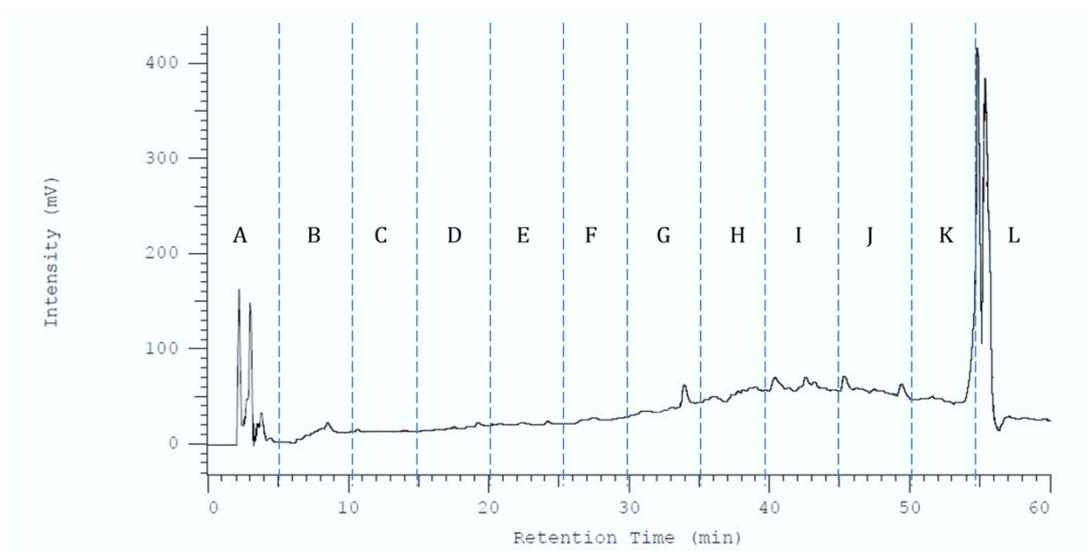


Figure 7. RP chromatogram of thermolysin-trypsin hydrolysate of *Acrochaetium* sp.

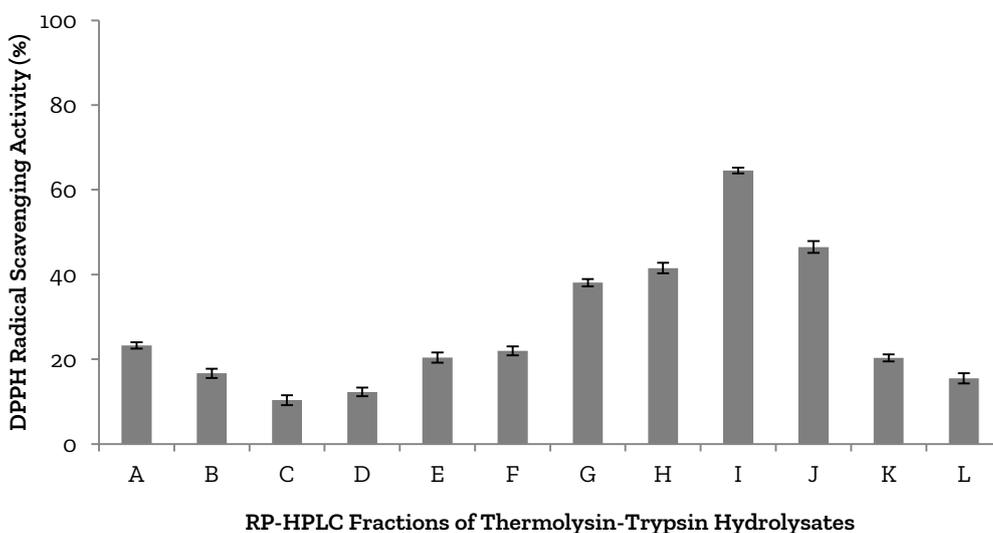
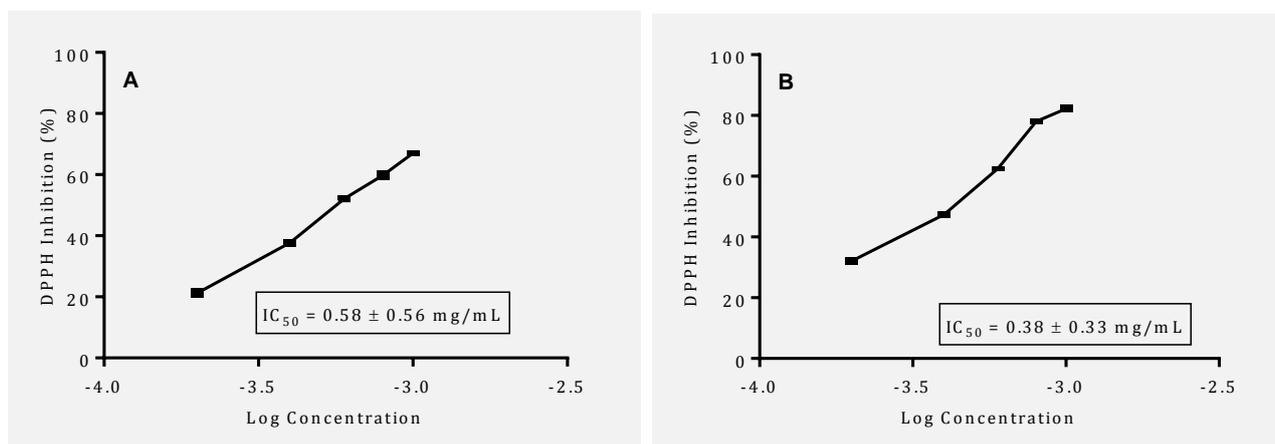


Figure 8. DPPH radical scavenging activity (%) of *Acrochaetium* sp. fractions using thermolysin-trypsin.



CONCLUSION

The bioactivities of *Acrochaetium* sp. as antioxidant used in this study is rarely reported. Peptide fractions showing highly antioxidant, and it obtained from the enzymatic hydrolysates using thermolysin and thermolysin-trypsin, respectively. Fraction obtained by ultrafiltration showed an antioxidant higher than the whole hydrolysate. Fractionation using RP-HPLC resulted fraction 7 from *Acrochaetium* sp. hydrolysate using thermolysin had IC_{50} value of 0.58 ± 0.56 mg/mL and fraction I from *Acrochaetium* sp. hydrolysate using the combination of thermolysin-trypsin had IC_{50} value of 0.38 ± 0.33 mg/mL. Due to increasing concerns about the safety antioxidants, *Acrochaetium* sp. protein hydrolysates represent a novel source of natural antioxidant hydrolysates and antioxidant peptides. Further works such as the identification of the peptide from the fraction using LC-MS/MS, simulated gastrointestinal simulation and antioxidant activity are also suggested.

DECLARATIONS

Authors' Contributions

All authors contributed equally to this work.

Competing interests

The authors declare that they have no competing interests that might have influenced the performance or presentation of the work described in this manuscript.

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