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Radical scavenging activity of protein from tentacles of jellyfish *Rhopilema esculentum*

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Abstract—In this study, radical scavenging activity of protein from tentacles of jellyfish *Rhopilema esculentum* (*R. esculentum*) was assayed including superoxide anion radical and hydroxyl radical scavenging. The protein samples showed strong scavenging activity on superoxide anion radical and values EC_{50} of full protein (FP), first fraction (FF), second fraction (SF), and 30% $(NH_4)_2SO_4$ precipitate (Fr-1) were 2.65, 7.28, 1.10, and 22.51 μg/mL, respectively, while values EC_{50} of BHA, BHT, and α-tocopherol were 31, 61, and 88 μg/mL, respectively. Also, the protein samples had strong scavenging effect on hydroxyl radical and the values EC_{50} of FP, FF, SF, Fr-1, and Fr-2 were 48.91, 27.72, 1.82, 16.36, and 160.93 μg/mL, but values EC_{50} of Vc and mannitol were 1907 and 4536 μg/mL, respectively. Of the five protein samples, SF had the strongest radical scavenging activity and may have a use as a possible supplement in the food and pharmaceutical industries. The radical scavenging activity was stable at high temperature so that *R. esculentum* may be used as a kind of natural functional food.

1. Introduction

The jellyfish, *Rhopilema esculentum* Kishinouye (*R. esculentum*), a cnidarian of the class Scyphozoa, the order Rhizostomeae, and the family Rhopilema, is distributed widely from the South China Sea, the Yellow Sea to the Bohai Sea and is abundant in late summer to early autumn.[1] Among the several edible species of Scyphozoan jellyfish, it is the most abundant and most important species in the Asian jellyfish fishery which is a multimillion-dollar seafood business.[2] *R. esculentum* contains some nutritional components such as amino acids and fatty acids. Chinese have been eating jellyfish for more than a thousand years and have also regarded it as a treatment for high blood pressure, bronchitis, tracheitis, gastric ulcers, and asthmas.[3,4] Although the content of protein in *R. esculentum* is high to 5–10%,[5] no report on the biological activity of *R. esculentum* protein has yet been made.

Reactive oxygen species (ROS) including the superoxide anion radical ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and nitric oxide ($NO^{\cdot}$) may contribute to a number of pathological events such as aging, cellular injury and DNA degradation when they are generated excessively or when the antioxidant defense systems are depressed.[6] In order to reduce damage of ROS, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) are used. However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis.[7–9] So, in recent years, there has been increasing interesting in finding natural antioxidants, which can protect the human body from free radicals and retard the progress of many chronic disease.[10] A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs.[11] Those natural antioxidants constitute a broad range of compounds including phenolic compounds, nitrogen-containing compounds and carotenoids.[12] However, the study on the radical scavenging activity of protein was comparatively in deficient. Moreover,

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radical scavenging activity of protein from marine invertebrates was never studied before in our knowledge.

In the present study, the radical scavenging activity of protein from tentacles of *R. esculentum* was assayed including superoxide anion radical and hydroxyl radical scavenging, the effect of temperature on radical scavenging activity was also studied.

2. Materials and methods

2.1. Chemicals

Nicotinamide adenine dinucleotide-reduced (NADH), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), and hydroxymethyl aminonethane (Tris) were purchased from Sigma. Sephadex G-100 was purchased from Amersham. All other chemicals and reagents used were of analytical grade.

2.2. Protein preparation

Jellyfish *R. esculentum* were collected in the Aoshan Bay in Qingdao, Shandong Province, China, on August 2003. Tentacles were manually excised in vivo, packed in polythene bags, and frozen immediately at −20 °C. The frozen tentacles were then sonicated in cold (4 °C) phosphate buffer solution (0.01 M, pH 6.0) eight times for 30 s each time at 100 mV. The resultant fluids were clarified by centrifugation (13,000 rpm) for 20 min at 4 °C and used as full protein (FP). Protein concentrations were determined by the method of Bradford,[13] using bovine serum albumin (BSA) as a standard.

2.3. $(NH_4)_2SO_4$ fractionation

The full protein was subjected to 30% $(NH_4)_2SO_4$ saturation by adding solid $(NH_4)_2SO_4$ with gentle stirring at 4 °C. The mixture was left for 2 h complete precipitation to occur, and then centrifuged (13,000 rpm) for 20 min at 4 °C. The precipitate was removed (Fr-1) and the supernatant was subjected to 60% $(NH_4)_2SO_4$ saturation and the whole process was repeated. Both precipitates (Fr-1 and Fr-2) were repeatedly dialyzed in 0.01 M PBS (pH 6.0) to remove $(NH_4)_2SO_4$.

2.4. Sephadex G-100 chromatography

For chromatography of the full protein, the following procedure was used. Sephadex G-100 was allowed to swell in 0.05 M phosphate buffer (pH 6.0) for 2 days at room temperature. Each day the fines were decanted and the buffer replenished. Swollen gel was degassed and poured as a slurry into a buffer solution (0.05 M, pH 6) jacketed column measuring 40 cm in length and 1.0 cm in internal diameter. The column was equilibrated at a flow rate of 25 mL/h with phosphate buffer (0.05 M, pH 6.0). After the gel had settled, column packing was checked using a 2 mg/mL solution of Blue Dextran 2000 (mol. wt = 2×10^6). At the same time the void volume was determined. Samples (1 mL) of full protein from the tentacles were applied to the top of the Sephadex G-100 column, care being taken to prevent disturbance of the bed. Each sample was allowed to drain into the top of the bed and then elution with phosphate buffer (0.05 M, pH 6.0) was commenced. Absorbance at 280 nm of the eluate was determined by an ultraviolet detector. Two major protein fractions appeared in the elution. (FF and SF were the first fraction and second fraction, respectively.)

2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli.[14] Running gels containing 5% acrylamide and stacking gels of 5% acrylamide were used. Samples were diluted 1:1 with sample buffer 0.05 M Tris (pH 6.8), 2% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.04% bromophenol blue and then were boiled for 5 min. Gels were silver stained according to Wray et al.[15] Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) were used as standards for molecular mass determination.

2.6. Superoxide anion radical scavenging assay

The superoxide anion radical scavenging ability of protein was assessed by the method described by Gülçin et al.[10] Superoxide anion radicals are generated in PMS–NADH systems by oxidation of NADH and assayed by the reduction of NBT. In this experiment, superoxide anion radicals were generated in 2.5 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 0.5 mL of NBT (300 μM) solution, 0.5 mL of NADH (468 μM) solution and protein samples. The reaction started by adding 0.5 mL of PMS (60 μM) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

$$\%\ \text{Scavenging effect} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control (without protein samples) and A_1 is the absorbance of mixture contained protein samples.

2.7. Hydroxyl radical assay

The reaction mixture, total volume 4.5 mL, containing protein samples, was incubated with EDTA–Fe^{2+} (220 μM), safranine O (0.23 μM), H_2O_2 (60 μM) in potassium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C.[16] The absorbance of the mixture was measured at 520 nm. Hydroxyl radical bleached the safranine O, so decreased absorbance of the reaction mixture indicated decreased hydroxyl radical scavenging ability.

$$\% \text{ Scavenging effect} = [(A_1 - A_B)/(A_C - A_B)] \times 100,$$

where A_B is the absorbance of the blank (distilled water instead of protein samples), A_C is the absorbance of the control (distilled water instead of H_2O_2), and A_1 is the absorbance of mixture contained protein samples.

2.8. Effect of temperature on radical scavenging activity

Full protein was incubated at 20, 40, 60, and 80 °C for 10, 20, 30, 40, 50, and 60 min. Then the superoxide anion radical and hydroxyl radical scavenging activity were assayed by the method mentioned above.

$$\% \text{ Scavenging ratio} = SE_1 \times 100/SE_0,$$

where SE_1 is the scavenging effect after incubation and SE_0 is the scavenging effect before incubation.

2.9. Statistical analysis

All data were expressed as means ± SD of three parallel measurements. Data were analyzed by student *t*-test and all tests were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. SDS-PAGE

Figure 1 shows the bands of five protein samples. FP was composed of several proteins, eight bands appeared after straining of the SDS-PAGE gel. The molecular weight of these bands was 29, 36, 48, 86, 99, 131, 157, 205 kDa, respectively. For $(NH_4)_2SO_4$ fractionation, four bands exhibited in the Fr-1 lane and one of them appeared in the stacking gels, the molecular weight of other three bands was 131, 157, 205 kDa. The band appeared in the stacking gels was perhaps the tail of other bands because no such band exhibited in the FP lane. Two bands appeared in the Fr-2 lane and the molecular weight was 131, 157 kDa. For Sephadex G-100 chromatography, four bands exhibited in the FF lane and the molecular weight was 99, 131, 157, 205 kDa. To our surprise, there was no band yielded in the SF lane. Other running gels containing 10% and 15% acrylamide were used to analysis the SF, but there was still no band appeared, therefore, the SF was a type of polypeptide possibly.

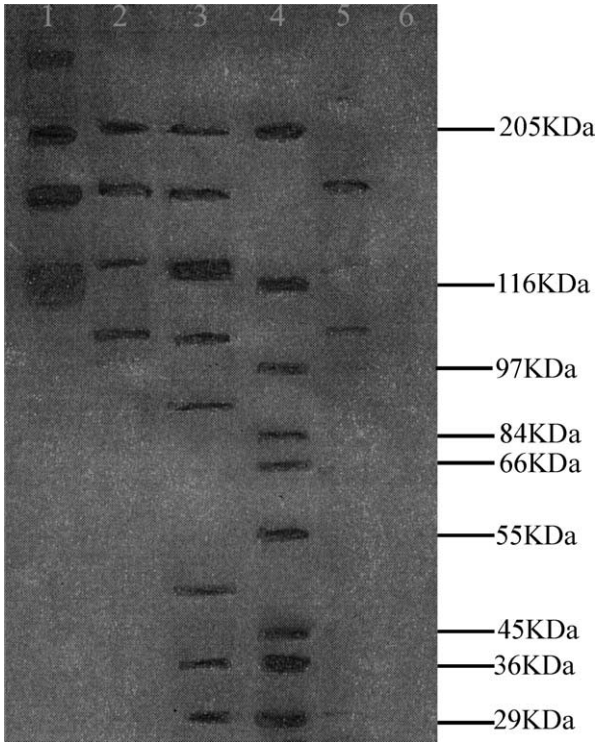

Figure 1. SDS-PAGE of protein samples. Lane 1: Fr-1; lane 2: FF; lane 3: FP; lane 4: marker; lane 5: Fr-2; lane 6: SF.

3.2. Superoxide anion radical scavenging assay

In the PMS/NADH–NBT system, superoxide anion radicals derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion radicals in the reaction mixture.[10,17] Figure 2a shows the % scavenging effect on superoxide anion radicals of FP, FF, SF, and Fr-1. Protein samples had strong superoxide anion radical scavenging activity and the scavenging effect depended on concentration. As shown in Figure 2a, for FP, FF, and SF, at the concentration below 10 μg/mL, the

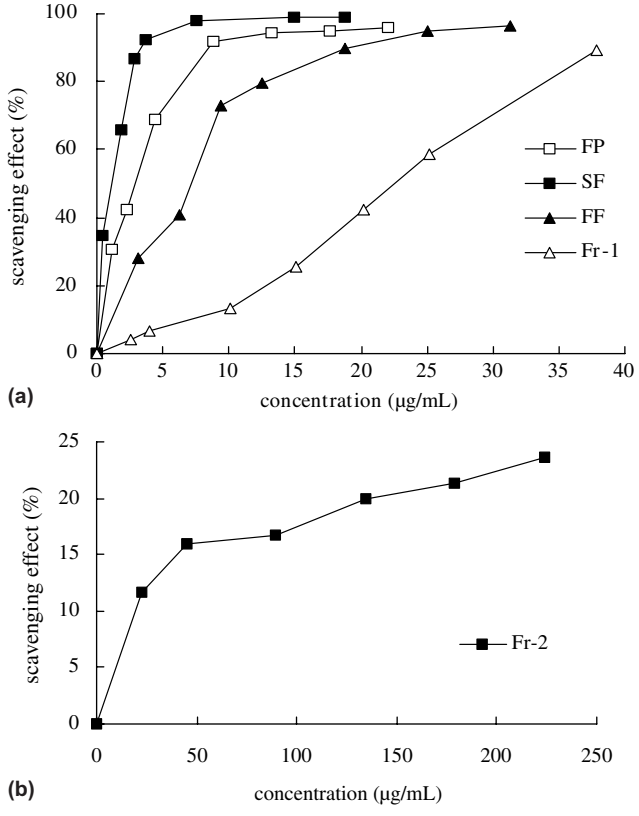

Figure 2. (a) Scavenging effect of FP, FF, SF, and Fr-1 on superoxide anion radical. (b) Scavenging effect of Fr-2 on superoxide anion radical.

scavenging effect increased with the concentration increasing significantly, at the concentration higher than 10 μg/mL, the scavenging effect was above 90% and increased slowly, while for Fr-1, the correlation between scavenging effect and concentration was strong, at the concentration from 2.52–37.83 μg/mL, the scavenging effect was from 4.11% to 89.35%. Figure 2b shows the % scavenging effect on superoxide anion radicals of Fr-2. The scavenging effect of Fr-2 on superoxide anion radicals was only 23.67% at 223.7 μg/mL. Moreover, FP, FF, SF, and Fr-1 exhibited far higher superoxide radical scavenging activity than BHA, BHT, α-tocopherol. The values EC_{50} (Efficient concentration defined as the concentration inhibiting 50% radical generation or scavenging 50% radical generated.) of FP, FF, SF, and Fr-1 were 2.65, 7.28, 1.10, 22.51 μg/mL, respectively, while values EC_{50} of BHA, BHT, α-tocopherol were 31, 61, 88 μg/mL, respectively.[17] Superoxide anion radical scavenging effect of protein samples followed the order: SF > FP > FF > Fr-1 > Fr-2. Of reactive oxygen species, superoxide anion radical is generated first. Although it is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species such as singlet oxygen and hydroxyl radicals.[18] Further, superoxide anion radicals are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursor of hydroxyl radicals.[19] The five protein samples were potential natural antioxidants.

3.3. Hydroxyl radical assay

The hydroxyl radical, known to be generated through the Fenton reaction in this system, was scavenged by protein samples. Figure 3a shows the % scavenging effect on hydroxyl radical of FP, FF, SF, and Fr-1. Figure 3b shows the % scavenging effect on hydroxyl radical of Fr-2. Of the five samples, SF had the strongest hydroxyl radical scavenging activity and it increased sharply with the concentration enhancing. At the concentration of 10 μg/mL, the scavenging effect was 94.49%. FP, FF, Fr-1, and Fr-2 had showed obvious scavenging effect on hydroxyl radical and it was in a concentration-dependent manner. Furthermore, the five samples showed far higher hydroxyl radical scavenging activity than Vc and mannitol. The values EC_{50} of FP, FF, SF, Fr-1, and Fr-2 were 48.91, 27.72, 1.82, 16.36, and 160.93 μg/mL, but values EC_{50} of Vc and mannitol were 1907 and 4536 μg/mL, respectively, as shown in Figure 3c. Among the reactive oxygen species, hydroxyl radical is the most active. Indeed, it is one of the most reactive chemical species known. The hydroxyl radical induces some oxidative damage to biomolecules such as some proteins, DNA, PUFA, nucleic acid, and almost any biological molecule it touches, and this damage causes aging, cancer and several disease.[20]

The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging.[21] For hydroxyl radical, there are two types of antioxidation mechanisms,

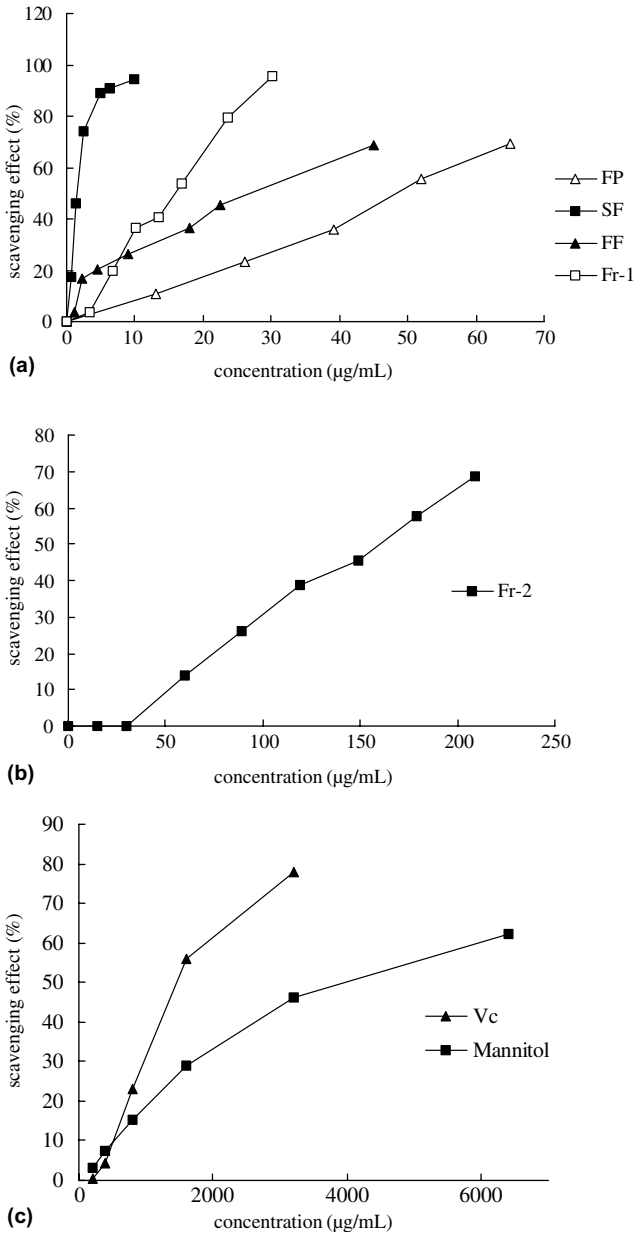

Figure 3. (a) Scavenging effect of FP, FF, SF, and Fr-1 on hydroxyl radical. (b) Scavenging effect of Fr-2 on hydroxyl radical. (c) Scavenging effect of Vc and mannitol on hydroxyl radical.

one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant may ligate to the metal ions, which react with H_2O_2 to give the metal complexes. The metal complex thus formed cannot further react with H_2O_2 to give a hydroxyl radical.[22] Although protein can chelate with metal ion to form metal complex, the radical scavenging activity of proteins from *R. esculentum* cannot be attributed to the chelating ability. The mechanism of protein samples on the hydroxyl radicals need to be further investigated.

3.4. Effect of temperature on radical scavenging activity

Bioactivity of protein is mainly affected by temperature. So effect of temperature on superoxide anion radical and

hydroxyl radical scavenging activity of full protein was studied. Figure 4 shows the scavenging ratio of FP on superoxide anion radical and hydroxyl radical. In general, temperature affected radical scavenging activity, but it did not change largely or lost entirely. For superoxide anion radical, at 80 °C incubated for 10 and 30 min, the scavenging activity increased and the scavenging ratios were 100.68% and 104.00%, respectively. Under other conditions, the scavenging activity decreased and the lowest scavenging ratio was 74.31% at 20 °C incubated for 30 min. The scavenging activity followed the order: 80 > 40 > 60 and 20 °C. Apart from incubation at 40 °C, the scavenging activity changed irregularly with the incubated time going, while incubation at 40 °C, the scavenging activity increased slowly and the scavenging ratio was from 90.67% to 93.70%. For hydroxyl radical, incubated at 40, 20 °C for 10, 20, 30 min and 60 °C for 50, 60 min, the scavenging activity increased and the highest scavenging ratio was 110.57% at 40 °C incubated for 30 min. Under other conditions, the scavenging activity decreased and the lowest scavenging ratio was 82.81% at 80 °C incubated for 30 min. The scavenging activity followed the sequence: 40 > 60 and 20 > 80 °C. Incubation at 40 °C, the scavenging activity increased to the highest at incubation for 30 min then the scavenging ratio decreased slowly to 104.38% at incubation for 60 min; incubation at 60 °C, the scavenging activity increased slowly and the scavenging ratio was from 91.46% to 101.63%. Incubation at 20 and 80 °C, the scavenging activity changed irregularly with the incubated time going.

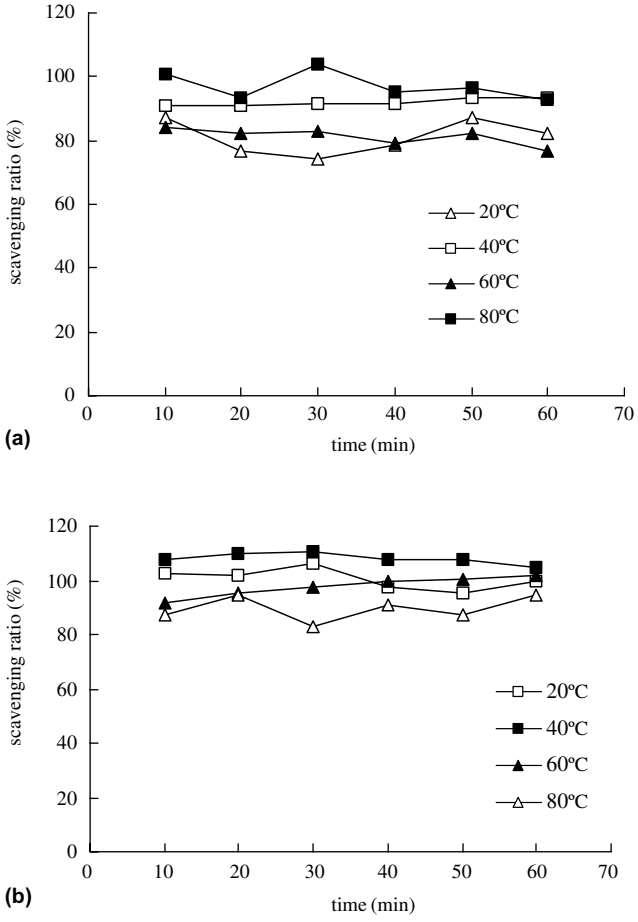

Figure 4. (a) The effect of temperature on superoxide anion radical. (b) The effect of temperature on hydroxyl radical.

Reactive oxygen species can damage the protein. However, some antioxidants in organisms belong to protein and these proteins play an important role in the antioxidant defense system. A dietary deficiency of protein not only impairs the synthesis of antioxidant enzymes but also reduces tissue concentrations of antioxidants, thereby resulting in a compromised antioxidant status.[23,24] Antioxidant activity of protein is mainly attributed to the content of –SH, protein enzymes such as superoxide dismutase, catalase and peroxidase. Protein enzyme lost their activity easily when the temperature is enhanced, for the FP, perhaps the content of –SH contributes to the radical scavenging activity.

4. Conclusion

Of the five protein samples, SF had the strongest radical scavenging activity and may have a use as a possible supplement in the food and pharmaceutical industries. The radical scavenging activity of protein samples may explain that jellyfish have curative effect for bronchitis, tracheitis, gastric ulcers, and asthmas. The radical scavenging activity was stable at high temperatures so that *R. esculentum* may be used as a kind of natural functional food. However, factors affecting and attributing to radical scavenging activity need to be further studied.

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