INTRODUCTION

As well as its primary function of supplying nutrients and energy, protein also has numerous tertiary functions relating to physiological regulation.1 Various physiological activities have been found in the hydrolyzates derived from the proteolytic hydrolysis of various food proteins.2 Among them, a number of functional peptides derived from milk and soybean have already been identified, and may have anti-oxidant properties,3 reduce blood pressure,4 and regulate cholesterol in serum.5 Natural proteins can thus be considered as 'physiologically functional food'.6 Numerous protein sources, including aquatic species, egg-white albumin, soy protein, and fish muscle, have been confirmed to act as anti-oxidants preventing the peroxidation of lipids or fatty acids.7-11 The anti-oxidative activities of amino acids or peptides have thus been investigated to reveal the anti-oxidative mechanism of protein hydrolyzates.

Successful hydrolysis of proteins also depends on proteases. Microbial proteases are usually used for the hydrolysis of proteins. Aspergillus oryzae, which has been used widely in fermented food, is a good source of proteases because it produces more than 10 endo- and exoproteases with a wide range of optimum pH.12

Cooking is crucial to canned tuna processing, resulting in approximately 4% water-soluble protein in cooking juice, including sarcoplasmic proteins and collagen,13 which are then discarded in the waste water.14 Recovering these proteins and utilizing them as foodstuffs is essential to enhancing their value and reducing waste water-treatment costs. Although several methods of recovering fish water-soluble proteins from the waste water of seafood processing plants have been investigated,13,15-17 the recovered proteins are generally used in animal feeds and fertilizers. In addition to preparing tuna cooking juice hydrolyzate using A. oryzae protease, this work

Original Article

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from tuna cooking juice

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ABSTRACT: Protease XXIII, from Aspergillus oryzae, was used to hydrolyze tuna cooking juice at 37°C for up to 6 h. The hydrolyzate obtained at the degree of hydrolysis of 25.68% (after hydrolysis for 2.5 h) displayed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging effect, reaching 82.19%. Six major fractions (A, B, C, D, E, and F) of this hydrolyzate were obtained by Sephadex G-25 column chromatography using a 0.05 M phosphate buffer (pH 6.5) as the mobile phase. All six fractions displayed a scavenging effect for the DPPH radical, but the scavenging effect was only obvious in two fractions (B and C). After the solid content of hydrolyzates was concentrated from one to five times, the scavenging effect of the DPPH radical increased from 17% to 75% for fraction B, and from 13% to 66% for fraction C. Seven anti-oxidative peptides were isolated from the hydrolyzates (mixture of B and C fractions) by reversed-phase HPLC. The peptide sequences comprised four to eight amino acid residues, including Val, Ser, Pro, His, Ala, Asp, Lys, Gly, or Tyr.

KEY WORDS: 1,1-diphenyl-2-picrylhydrazyl radical, protein hydrolyzate, scavenging effect, tuna cooking juice.

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investigated the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity of the hydrolyzate. The biologically active substances created by the proteolytic digestion were also identified.

MATERIALS AND METHODS

Materials

Tuna cooking juice was obtained from a canned tuna processing plant in Chiayi, Taiwan. Tuna meat was steamed (100–105°C) for 3–4 h, after which the hot cooking juice was collected and sealed in 400 g polyethylene bags and then transferred to the laboratory and stored at 4°C overnight. The cooled cooking juice was then filtered through two layers of gauze to remove any floating fats and solids, and the filtrate was collected and stored at −30°C until required for use. The DPPH and protease XXIII derived from A. oryzae were purchased from Sigma Chemical Co. (St Louis, MO, USA), the Sephadex G-25 column was produced by Pharmacia (Uppsala, Sweden), and all other chemicals used were analytical grade products.

Enzymatic hydrolysis

A volume of 2 mL of 0.5% protease solution was added to 50 mL of treated cooking juice, and aliquots were removed at intervals of 0–6 h at 37°C. Digested hydrolyzates were heated in boiling water for 5 min to inactivate the protease. The hydrolyzates were then centrifuged (5000 ×g, 10 min), and the resultant supernatants were freeze-dried and stored at −18°C until required for use. The approximate degree of hydrolysis of the tuna cooking juice was determined by a modification of the method described by Boudrant and Cheftel.

Analysis of free and combined amino acids

Free and combined amino acids were determined following the procedure of Moore and Stein. Protein hydrolyzate (15 mL) was precipitated with 10 mL of 15% trichloroacetic acid (TCA). After setting for 30 min, the hydrolyzate was centrifuged (5000 ×g) at 4°C for 10 min, and this process was repeated three times to extract the precipitate. Next, an equal volume of ether was added to the extracted supernatant to remove the TCA. The supernatant was then freeze-dried after being frozen overnight at −30°C, and added to a 25-mL volumetric flask containing 10 mL of 0.2 M citrate buffer (pH 2.2). Simultaneously, the protein hydrolyzate was also hydrolyzed in 6 N HCl at 110°C for 24 h in a vacuumed sealed tube. Amino acids obtained without HCl hydrolysis were termed ‘free amino acids’, and the differences between the values of amino acids with HCl hydrolysis and free amino acids were termed ‘combined amino acids’. Amino acids were measured using a ninhydrin reagent with an amino acid standard, which was applied using a high-performance amino acid analyzer (Beckman 6300, Fullerton, CA, USA) with the following settings: column, cation exchange resin; analysis temperature, 50–70°C; cuvette path length, 12 mm; citrate buffer rate, 20 mL/h; and ninhydrin rate, 10 mL/h.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical

The DPPH radical, which is organic, stable, and has been proven to be absorptive at 517 nm, is a convenient reagent for the radical scavenging assay, which is independent of any enzyme. When this compound is stabilized by accepting an electron or hydrogen radical, its absorptive characteristics vanish. Unlike laboratory-generated free radicals such as superoxide radical and hydroxyl radical, DPPH has the advantage of being free of additive-induced complications such as metal chelation and enzyme inhibition.

The scavenging effect of the hydrolyzate on the DPPH radical was estimated using a modification of Yen and Wu’s method. A volume of 1 mL of hydrolyzates were first added to a methanolic solution (0.05 mL) of DPPH radical (with a final concentration of 10 mM). The mixture was then shaken vigorously and allowed to stand for 30 min, after which the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (Hitachi U-2000; Hitachi, Tokyo, Japan). The DPPH scavenging effect (%) was calculated as:

\[
\left(\frac{OD_{517 \text{ control}} - OD_{517 \text{ sample}}}{OD_{517 \text{ control}}}\right) \times 100\%.
\]

The control used was 0.1% TFA in deionized water, and the scavenging effect was measured as described earlier. All tests and analyses were repeated three times and the results averaged.

Purification of anti-oxidant peptide with scavenging effect

The anti-oxidant peptide was purified using column chromatography, as described by Astawan et al., but with slight modifications. Approximately 0.27 g of freeze-dried powder (obtained from the protease hydrolyzate) was diluted in 2 mL of dis-
tilled water, fractionated by gel filtration on a Sephadex G-25 column (2.5 cm × 50 cm; Pharmacia, Uppsala, Sweden), and eluted with 0.05 M phosphate buffer (pH 6.5). Each 5 mL fraction was collected at a flow rate of 40 mL/h, and absorbance at 280 nm and the DPPH radical scavenging activity were measured for all fractions.

Samples exhibiting anti-oxidative activity were further purified using high-performance liquid chromatography (HPLC; Hitachi), and 4 mL of the active sample was collected and dried. The residue was dissolved in 0.5 mL of 0.1% TFA, and the substance was then injected into an RP-18(e) column (4 mm i.d × 250 mm length; Merck, Gibbstown, NJ, USA), equilibrated with 0.1% TFA, and eluted using a linear gradient of acetonitrile (0–20%/50 min) in 0.1% TFA at a flow rate of 0.7 mL/min. The eluted active fractions were concentrated using a centrifugal concentrator (VaCO I; Zirbus, Hilfe Gottes, Germany), and then applied to a MICRA NPS RP-18 column (4.6 mm × 33 mm; MICRA, Northbrook, IL, USA). The gradient was then eluted with 0–35% acetonitrile at a flow rate of 0.8 mL/min to obtain purified peptides with a scavenging effect. Each chromatograph was monitored for its absorbance of ultraviolet light at 210 nm. Finally, the amino acid of the peptide was sequenced using a 477-A protein sequencer chromatogram (Biosystems, Foster, CA, USA).

RESULTS AND DISCUSSION

1,1-Diphenyl-2-picrylhydrazyl scavenging effect at different degrees of hydrolysis

Protease XXIII was screened for the hydrolysis of tuna cooking juice. The degree of hydrolysis and the hydrolyzates’ scavenging activities on the DPPH radical were measured over a 6-h period (Fig. 1). The initial DPPH scavenging activity of tuna cooking juice was approximately 18%, and scavenging effect did not increase with increasing degree of hydrolysis, whereby hydrolysis proceeded when the degree of hydrolysis reached 32% at 6 h, and no increase in activity being observed after 2.5 h (degree of hydrolysis was 25.68%). Several investigations on protein hydrolyzates have frequently reported their anti-oxidative properties. The results of these investigations suggest that the anti-oxidative activity of protein hydrolyzates is influenced by the levels of amino acids and peptides, which are primarily influenced by enzyme hydrolysis.

Isolation of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity peptides

To characterize the DPPH radical scavenging activity of peptides derived from tuna cooking juice, the protein was hydrolyzed with protease XXIII for 2.5 h, and the hydrolyzate (AOH) was then separated by gel filtration chromatography. Figure 2 displays the fraction profile for AOH. Although continuous, the profile clearly contained six major sections, which are labeled A (fraction number 13–17), B (18–37), C (38–45), D (46–51), E (52–60), and F (61–73). In the present study, we used the concentrated portion instead of the concentrated

Fig. 1 Time-courses of hydrolysis and the scavenging effect of tuna cooking juice treated with protease XXIII at 1:25(v/v) of enzyme to substrate. The concentration of protein was 4% and that of enzyme was 0.5%. (○) Degree of hydrolysis; (●) scavenging effect.

Fig. 2 Elution profile of protease XXIII hydrolyzate separated by using gel filtration on a Sephadex G-25 column. The column (2.5 cm × 50 cm) was equilibrated and eluted using 0.05 M phosphate buffer (pH 6.5) at a flow rate of 40 mL/h. AOH, mixture of most active peptides, which were obtained from fractions B and C.
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Figure 4 displays the RP-18 column chromatographic pattern and the yields of five fractions (indicated as AOI, AOII, AOIII, AOIV, and AOV) for AOHA. All five fractions were appropriately fractionated, concentrated, and assayed for their DPPH radical scavenging activity. The scavenging effect was measured using 2 μg/mL of fraction AOI, 5 μg/mL of AOII, 23 μg/mL of AOIII, 57 μg/mL of AOIV, and 124 μg/mL of AOV. Fraction AOI displayed an excellent scavenging effect (>80%) at concentrations of approximately 32 μg/mL (Fig. 5). Fraction AOII displayed a scavenging effect of more than 70% at 80 μg/mL, and the scavenging effects of butyl hydroxyanisol (BHA) and L-ascorbic acid were 92% and 80%, respectively, at 100 μg/mL. Furthermore, fractions AOI and AOII clearly contained significantly more anti-oxidant components than AOIII, AOIV, and AOV, and these components could react rapidly with DPPH radicals, thus reducing almost all of the DPPH radical molecules corresponding to available hydroxyl groups.25 These analytical results confirm that peptides are free-radical inhibitors or scavengers, and may be primary anti-oxidants. It has been suggested that the peptides’ anti-oxidant mechanism is that of free radical scavenging.10

Amino acid sequences

Peptides in fractions AOI and AOII, totaling approximately 13 peaks, were collected and analysed (Fig. 6), and it was found that only five peaks (P1, P2, P3, P4, and P5) displayed good scavenging effects for the DPPH radical. The active

peptides for each fraction to compare the scavenging effect. If the scavenging effect did not change with increased number of times concentrated, this indicated that the peptide fractions had a weak scavenging effect. When the solid content of each fraction (48 μg/mL of fraction A, 39 μg/mL of B, 55 μg/mL of C, 69 μg/mL of D, 61 μg/mL of E, and 52 μg/mL of F) was concentrated three times, the scavenging activities of the peptide fractions reached 2.54% (A), 70.43% (B), 47.54% (C), 8.72% (D), 19.87% (E), and 8.48% (F). Fractions B and C (molecular weights ranged from 390 to 1400) had the strongest scavenging effect on the DPPH radicals (Fig. 3). The mixture of active peptides; that is, fractions B and C (marked AOHA), was then collected, concentrated, and purified by reversed-phase HPLC using a 0.1% TFA–acetonitrile system.

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peptides were purified further using a MICRO NPS RP-18 column. P1a, P1b, P2a, and P2b were found to be the active peptides of the P1 and P2 fractions, and P3a, P4a, and P5a were the active peptides derived from the P3, P4, and P5 fractions. The scavenging effects are shown in Table 1.

Table 1 also lists the amino acid sequences of isolated anti-oxidative peptides derived from tuna cooking juice with protease XXIII.

Table 1  Amino acid sequences of isolated anti-oxidative peptides derived from tuna cooking juice with protease XXIII

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Structure</th>
<th>Scavenging effect* (%)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1a</td>
<td>Pro-Ser-His-Asp-Ala-His-Pro-Glu</td>
<td>66</td>
<td>1010</td>
</tr>
<tr>
<td>P1b</td>
<td>Ser-His-Asp-Ala-His-Pro-Glu</td>
<td>71</td>
<td>896</td>
</tr>
<tr>
<td>P2a</td>
<td>Val-Asp-His-Asp-His-Pro-Glu</td>
<td>77</td>
<td>953</td>
</tr>
<tr>
<td>P2b</td>
<td>Pro-Lys-Ala-Val-His-Glu</td>
<td>74</td>
<td>766</td>
</tr>
<tr>
<td>P3a</td>
<td>Pro-Ala-Gly-Tyr</td>
<td>75</td>
<td>457</td>
</tr>
<tr>
<td>P4a</td>
<td>Pro-His-His-Ala-Asp-Ser</td>
<td>81</td>
<td>751</td>
</tr>
<tr>
<td>P5a</td>
<td>Val-Asp-Tyr-Pro</td>
<td>74</td>
<td>544</td>
</tr>
</tbody>
</table>

*The solid content of P1a was 57 μg/mL, P1b was 54 μg/mL, P2a was 61 μg/mL, P2b was 68 μg/mL, P3a was 87 μg/mL, P4a was 95 μg/mL, and P5a was 89 μg/mL.

Fig. 6 Anti-oxidative activity (as measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity) of various peptide fractions obtained from AOH hydrolyzates by high-performance liquid chromatography. (●) DPPH scavenging effect (%) = [(OD<sub>517 control</sub> - OD<sub>517 sample</sub>) / OD<sub>517 control</sub>]<sup>-1</sup> × 100%. Control, 0.1% trifluoroacetic acid in deionized water.

The anti-oxidative activity fractions of P1, P2, P3, P4, and P5 were concentrated using a centrifugal concentrator, and then applied to a MICRA NPS RP-18 column (4.6 mm × 33 mm), and the gradient eluted with 0–35% acetonitrile at a flow rate of 0.8 mL/min.

Several amino acids, such as Tyr, Met, His, Lys, and Trp, are generally accepted as being anti-oxidants despite their occasionally pro-oxidative effects.26 Dipeptides consisting of Ala, Tyr, His, and Met at the N-terminus on linoleic acid have been investigated previously by Kawashima et al.,27 revealing that the dipeptides displayed greater anti-oxidative activities than the constituent amino acid mixtures in an aqueous solution.

The anti-oxidative activity of histidine-containing peptides has been described elsewhere,28 and can be attributed to the chelating and lipid radical-trapping abilities of the imidazole ring. The anti-oxidative activities of histidine-containing peptides exceeded that of histidine itself, a phenomenon that was partly the result of the increased hydrophobicity of the peptides, which increased the interaction between the peptides and fatty acids.3 As proven by the present study, the sequence of five DPPH radical scavenging-activity peptides contained histidine residues. Meanwhile, the remainder of the peptides contained tyrosine residue, which is a significant source of hydrogen. Furthermore, all the anti-oxidative peptides derived from the tuna cooking juice contained proline residue. Interestingly, prolyl polypeptides are sensitive to oxygen.29 Uchida and colleagues have demonstrated that prolyl polypeptides are sensitive to oxidation by Cu(II)/H₂O₂ and generate mainly α-aminobutyric acid, hydroxyproline, aspartic acid, and glutamic acid after hydrolysis of the oxidized substrates.

CONCLUSIONS

The present study has demonstrated clearly that protein hydrolyzates of tuna cooking juice, and the fractions derived from them after separation via a column chromatographic procedure, possess strong DPPH radical-scavenging properties that are comparable to those of BHA and L-ascorbic acid at the same concentration. These effects are attributed to the variable hydrogen-donating
properties of the active peptides present in the hydrolyzates/fractions. Hydrolyzates from tuna cooking juice, and the fractions derived from them, can be incorporated into lipid-containing foods as chain-breaking anti-oxidants to minimize free radical-mediated lipid peroxidation. These hydrolyzates can also be used as alternatives to conventional drugs for treating human diseases associated with free radical-mediated tissue damage. However, such usage must be adequately justified by animal and clinical studies, creating a need for further research.

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