Note

Isolation and Characterization of Peptides with Antioxidant Activity Derived from Wheat Gluten

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Peptides having potent antioxidant activity were separated from the hydrolysate of wheat gluten by ion-exchange and gel filtration chromatography. Peptides obtained by SP Sephadex C-25 chromatography of the most active fraction (WP-3) were further separated using reversed-phase high performance liquid chromatography. The amino acid sequences of these peptides were Leu-Gln-Pro-Gly-Gln-Gly-Gln-Gln-Gly and Ala-Gln-Ile-Pro-Gln-Gln.

Keywords: wheat gluten, hydrolysate, peptide, antioxidant activity

The disease-preventing potential of naturally occurring substances in the diet is a main area of scientific interest. Antioxidants have attracted great attention for disease preventive effects because of lipid peroxidation which can lead to destabilization and disintegration of cell membranes, to many age-related diseases, to aging, and to cancer (Mcbrien & Slater, 1982). Recently, the involvement of free radicals and other oxidants in aging and in several diseases has been investigated in detail. Much physiological damage may be directly imputable to the hydroxyl radical, because it is highly reactive, and so many physiological reactions and disintegration of cell membranes, to many age-related diseases, to aging, and to cancer (Mcbrien & Slater, 1982). Recently, the involvement of free radicals and other oxidants in aging and in several diseases has been investigated in detail. Much physiological damage may be directly imputable to the hydroxyl radical, because it is highly reactive, and so many hydroxyl radicals produced in vivo react at or close to their site of formation. The biochemical literature is full of claims that reactive species are involved in different diseases (Southorn & Powis, 1988).

Many proteins have been reported to have antioxidant activities against the peroxidation of lipids or fatty acids upon hydrolysis (Rival et al., 2001, Suetsuna, 2000; Chen et al., 1998; Hattori et al., 1998; Bishop & Henick, 1972). Gluten has a unique amino acid composition, with Glu/Gln and Pro accounting for more than 50% of the amino acid residues. About 30% of gluten’s amino acid residues are hydrophobic, and the residues contribute greatly to its ability to form protein aggregates by hydrophobic interactions and to bind nonpolar substances (Fennema, 1996). Since wheat gluten had strong affinity for oil and other nonpolar substances, we speculated that gluten’s hydrolysate would have some protective effect on the degradation of fatty acid. In this study, we examined the antioxidant effects of peptic hydrolysate of wheat gluten, and the amino acid sequences of antioxidant peptides were determined.

Materials and methods

Materials Wheat gluten was purchased from Oriental Yeast (Tokyo). Pepsin (from porcine gastric mucosa, EC 3.4.23.1) was obtained from Merck (Darmstadt, Germany).

Linoleic acid (99.9%) and d-α-tocopherol from Sigma Chemical (St. Louis, MO) were used as received. All other reagents were of analytical grade from Nacalai Tesque (Kyoto).

Purification of peptides from wheat gluten Ten grams of wheat gluten was put in 100 ml of deionized water overnight and homogenized in 1 l of deionized water. The pH value of the homogenate was adjusted to 2.0 with 2 N HCl, and 0.3 g of pepsin was added. After 20 h of digestion at 37°C, the hydrolysate was filtered to remove the residue and centrifuged at 20,000 g for 20 min at 4°C. The supernatant was applied to a Dowex 50W column (4.5×20 cm, 50–100 mesh, H+ form). The column was washed thoroughly with deionized water, and the retained peptides were then eluted with 500 ml of 2 N NH₄OH. The peptide fraction was concentrated to 25 ml under a vacuum. Five milliliters of concentrate was applied to a Sephadex G-25 column (2.6×140 cm, Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with deionized water, and gel-filtrated at a flow rate of 60 ml/h. Fractions of 7.2 ml were collected. The peptide content of each fraction was measured by the Lowry method, using bovine serum albumin as the standard (Lowry et al., 1951). The molecular weight of the peptides was estimated with gel filtration using the retention volume relative to the standards. The peptide fractions were concentrated to dryness to give peptide powder. Two grams of the peptide powder was dissolved in 100 ml of deionized water and applied to an SP Sephadex C-25 column (2×50 cm, Pharmacia, H⁺ form) equilibrated with deionized water. The column was chromatographed using a linear gradient method with 1 l of deionized water to 1 l of 3% NaCl solution at a flow rate of 60 ml/h, and fractions of 7.2 ml were collected. The active fractions were collected and freeze-dried.

Assay of antioxidant activity WP fractions were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) and 1.0 ml of 50 mM linoleic acid in ethanol (99.5%) were mixed in test tubes (20×125 mm). The tubes were sealed tightly with silicon rubber caps and kept at 60°C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a microsyringe for measurement of oxidation using the ferric thiocyanate method.
with slight modification (Mitsuda et al., 1966). To 0.1 ml of the reaction mixture was added 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride solution in 3.5% HCl. After 5 min, the absorbance of the colored solution at 500 nm was measured in a 1-cm cuvette with a Hitachi Model U-2000 spectrophotometer. The number of days taken to attain an absorbance of 0.3 was defined as the induction period, referring to the antioxidant activity of the samples.

Purification of the peptides by reversed-phase HPLC
The most potent fraction eluted from SP Sephadex C-25 was further isolated and purified by reversed-phase HPLC with a Hypersil ODS-2 column (4.6x250 mm, Life Sciences International, Ltd., UK) using a linear gradient of acetonitrile from 0% to 15% in 0.05% trifluoroacetic acid (TFA) for 180 min at a flow rate of 1.0 ml/min; the elute was monitored at 220 nm. The active fractions were concentrated to 0.5 ml using a centrifugal evaporator. The antioxidant peptides were rechromatographed on a Hypersil ODS-2 column (4.6x150 mm) using a linear gradient of acetonitrile in 0.05% TFA at a flow rate of 0.5 ml/min.

Peptide identification and synthesis
Peptides were hydrolyzed in 6 n hydrochloric acid containing 0.1% phenol at 110°C for 24 h, and the hydrolysate was analyzed by a PICO-TAG™ amino acid analyzer (Waters, Milford, MA). Sequence analysis was done by stepwise Edman’s degradation using a 477A gas-phase automatic sequencer (Applied Biosystem, CA) coupled to HPLC, for identification of the resulting PTH-amino acid compounds. The molecular mass of each peptide was confirmed from its fast atom bombardment mass spectrum (FAB-MS) obtained with a JEOL DX-300 spectrometer (Nippon Denshi, Japan).

Peptides were synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystem) followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. The synthesized peptides were purified by HPLC on a column of Aquapore™ Prep-10 (Applied Biosystem) with a gradient of acetonitrile from 3% to 10% in 0.05% TFA for 30 min at a flow rate of 0.5 ml/min.

Results
Isolation of peptides from wheat gluten
Peptides having potent antioxidant activity were isolated from the peptic hydrolysate of wheat gluten using an Dowex 50W(H⁺) and Sephadex G-25 column as shown in Fig. 1. The fractions having molecular weights of 300 to 5000 were collected and concentrated to dryness to give a peptide powder. The yield of the peptide powder from 10 g (dry weight) of wheat gluten was 6.5 g. The peptides were fractionated by ion-exchange chromatography on SP Sephadex C-25 (H⁺) to give WP-1 (fraction number, 16 to 33; weight, 235 mg), WP-2 (34 to 53, 96 mg) and WP-3 (54 to 80, 90 mg) as shown in Fig. 2.

Antioxidant activity of the peptide fractions
Figure 3 shows the antioxidant activity of wheat peptide fractions from SP-Sephadex C-25 chromatography at various concentrations. The WP-3 fraction showed stronger antioxidant activity than α-tocopherol with an induction period of 7.5 days. The WP-3 fraction was dissolved in distilled water and applied to an ODS column.

Amino acid sequences of the active peptides
The active fractions were purified further by reversed-phase HPLC. Figure 4 shows a preparative HPLC chromatogram of the fraction. Although approximately 100 peaks were detected by chromatography, two peaks of potent antioxidant peptides were obtained from wheat gluten. Retention times were 48.5 and 56.1, respectively. Analysis of each peptide after 6 n HCl hydrolysis revealed the amino acids listed in Table 1. The ion peak (MH⁺) of each purified peptide is the theoretical value in the FAB-MS. Using protein sequencing, primary structures of the antioxidant peptides were found to be Ala-Gln-Ile-Pro-Gln-Gln and Leu-Gln-Pro-Gly-Gln-Gly-Gln-Gly-Gln respectively. The antioxidant peptides contained glutamine and a proline residue within their sequences. These amino acid sequences are found in the primary structure of gliadin or glutenin. The antioxidant activity of the
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synthetic peptides (LQPGQGQQG and AQIPQQ) was determined by the method described above. The induction period was 16 and 14 days for LQPGQGQQG and AQIPQQ, respectively.

Discussion

Many proteins have been shown to have antioxidant activity against the preoxidation of lipids or fatty acids, and the mechanisms have been suggested to be an increase in the surface area of linoleic acid due to the adsorption by protein molecules which might cause the catalytic effect (Nelson & Potter, 1979). The antioxidant activities of both amino acids and peptides have been investigated to gain insight into the antioxidant mechanism of protein hydrolysates. The amino acids Tyr, Met, His, Lys, and Trp are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases (Marcuse, 1960).

Kawashima et al. (1979) reported on the synergistic ternary antioxidant compositions of tocopherol, partial protein hydrolysate, and an organic acid. They investigated the effects of many synthetic peptides on lipid oxidation and found that some peptides having branched-chain amino acids (Val, Leu, Ile) showed antioxidant properties. Those dipeptides consisting of Ala, Tyr, His, and Met at the N-terminal showed higher antioxidant activities than the constituent amino acid mixtures in an aqueous system. On the correlation between structure and activity of the antioxidant peptides, Chen et al. (1998) reported that peptides with

![Fig. 3. Antioxidant activities of wheat gluten peptide fractionated using SP-Sephadex C-25 column chromatography. The antioxidant activity corresponds to days of the induction period. The control assay was performed using 10 μM d-α-tocopherol.](image)

Table 1. Analytical data for purified peptides from the WP-3 fraction.

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Amino acid composition</th>
<th>FAB-MS (MH⁺)</th>
<th>Induction period (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Gln-Pro-Gly-Gln-Gly-Gln-Gly</td>
<td>Leu, 1.08; Glu, 3.41; Pro, 0.87; Gly, 2.97</td>
<td>912</td>
<td>16</td>
</tr>
<tr>
<td>Ala-Gln-Ile-Pro-Gln-Gln-Gln</td>
<td>Ala, 0.97; Glu, 2.69; Ile, 1.13; Pro, 0.95</td>
<td>684</td>
<td>14</td>
</tr>
</tbody>
</table>

*Each peptide was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 h.

*Antioxidant activity of synthetic peptide corresponds to days of the induction period using the ferric thiocyanate method. The peptide concentration was 5.0×10⁻⁶ M. Data represent the mean of three replications.
highly potent inhibitory activity have hydrophobic amino acids, Val or Leu, at the N-terminal position, and Pro, His, or Tyr in the sequences. In this study, WP-3 was the strongest antioxidant among the three fractions isolated by SP Sephadex C-25 column. We did analyze the antioxidant activity of WP-1 and WP-2 fraction, but were unable to get good results. However, only 2 fractions were seen in further isolation by HPLC. From these data, we suggested that the antioxidant activity of wheat gluten hydrolysate might come from the synergy of the composition or structure of the peptides.

Recently the antioxidant activity of carnosine, a histidine containing dipeptide, was extensively reviewed (Decker et al., 2001; Klebanov et al., 1998). The mechanism of the antioxidant effect of carnosine is chelate with a metal ion such as copper. Although the structure-activity relationship of antioxidant His-containing peptides has not yet been well defined, the activity must be attributed to the hydrogen-donating ability, lipid peroxyl radical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan & Decker, 1994). Furthermore, Tsuge et al. (1991) reported the isolation of a potent antioxidant peptide, Ala-His-Lys, from the hydrolysate of egg white albumin, in which neither His-Lys nor a constituent amino acid mixture had any activity, but Ala-His was as potent as the parent peptide. When the constituent amino acids were mixed at the same concentration as the peptides, no activity was observed. Thus, the characteristic amino acid sequences of peptides are required to express the antioxidant effects. In this study, peptides had either of the hydrophobic amino acids, alanine or leucine, at the N-terminal position, and Pro, His, or Tyr in the sequences. This may be because the oxidation of linoleic acid is inhibited by quenching of the peroxyl radicals.

References


Mitsuda, H., Yasumoto, K. and Iwami, K. (1966). Antioxidative effect of amino acids, alanine or leucine, at the N-terminal position, and Pro, His, or Tyr in the sequences. This may be because the oxidation of linoleic acid is inhibited by quenching of the peroxyl radicals.

References


