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Isolation and characterization of free radical scavenging activities peptides derived from casein

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A peptide having the strong free radical scavenging activities was separated from casein protein hydrolysate by chromatographic analyses such as ion-exchange and gel filtration. SP-II fraction obtained by SP-Sephadex C-25 chromatography showed the most potent superoxide anion scavenging activity (SOSA), and it was further separated into a peptide using an octadecylsilano-high performance liquid chromatography. The amino acid sequence of the peptide was Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL). The concentration of the test compound required to reduce the produced superoxide anion to one-half (IC_{50}) value for SOSA was 79.2 μM using tetrazolium salt 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate method. The IC_{50} value for the 1,1-diphenyl-2-picrylhydrazyl radical and hydroxyl radical scavenging activities were 98 and 251 μM, respectively, based on the electron spin resonance method. We characterized SOSA of the C-terminal sequence using EL, PEL, YPEL, and FYPEL. The activities of preferred sequences were EL > YFYPEL > FYPEL > YPEL > PEL, suggesting that the Glu-Leu sequence is important for the activity. (J. Nutr. Biochem. 11:128–131, 2000) *© Elsevier Science Inc. 2000. All rights reserved.*

Keywords: peptide from casein; free radical scavenging activity; superoxide anion radical; DPPH radical; hydroxyl radical; tetrazolium salt XTT; ESR

Introduction

Free radicals and active oxygen species such as the oxygen molecule behave as double-edged swords. They are important mediators in signal transduction and play a vital role in the production of biologically active and essential compounds. At the same time, however, they are toxic and known to play a causative role in a variety of diseases including cancer and aging. They attack lipids, proteins, and DNA to induce peroxidation, modification, and strand break.[1]

Aerobic organisms are protected from such oxidative stress by various defense systems. The capacity of such protective systems, however, gradually decreases with aging, resulting in disturbances to the normal redox equilibrium established in healthy systems. Therefore, to replenish this aging-induced loss, the body must be provided with a constant supply of phytochemicals by the regular intake of a proper diet.[2]

Some proteins from certain foods were found to have the scavenging activity of active oxygen species.[3] Although the mechanism remains to be elucidated, the primary sequence may contribute to generate the activity. As an example, milk casein was reported to inhibit lipoxygenase-catalyzed lipid autoxidation.[4] Quenching of free radicals by oxidation of amino acid residues in casein was proposed as the explanation. However, free amino acids could not substitute for casein as the antioxidant, suggesting that the primary structure of casein molecules played a role.[4] To clarify whether a primary sequence in milk casein has the radical scavenging activity, we isolated, purified, and identified free radical scavenging activity peptide derived from the peptic digest of casein. Further comparative studies for free radical scavenging activity of synthetic fragment peptides have been performed.

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Received October 14, 1999; accepted November 15, 1999.

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655 Avenue of the Americas, New York, NY 10010

0955-2863/00/$–see front matter
PII S0955-2863(99)00083-2

Materials and methods

Materials

Milk casein was purchased from Oriental Yeast Co. (Tokyo, Japan). Pepsin (from porcine gastric mucosa, EC 3.4.23.1) from Merck Co. (Darmstadt, Germany) and superoxide dismutase (SOD; from bovine erythrocytes, EC 1.15.1.1; 3380 units/mg protein), xanthine oxidase (XOD; from buttermilk, EC 1.2.3.2; 0.3 units/mg), 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT), and 5,5-diethyl-1-pyrroline-N-oxide (DMPO) from Sigma Chemical Co. (St. Louis, MO USA) were used as received. All other reagents were of analytical grade from Nacalai Tesque Co. (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Purification of the peptide from milk casein

Thirty grams of milk casein were homogenized in 100 mL of deionized water. The pH value of the homogenate was adjusted to 2.0 with 2 N HCl, and 0.9 g of pepsin was added. After 20 hours of digestion at 37°C, the hydrolysate was filtered to remove the residue and centrifuged at 20,000 × g for 20 minutes at 4°C. The supernatant (100 mL) was applied to a Dowex 50W column (2.6 × 20 cm, 50–100 mesh, H^+ form; Muromachi Kagaku, Tokyo, Japan). The column was washed thoroughly with deionized water, and the retained peptides were then eluted with 300 mL of 2 N NH_4OH. The peptide fraction was concentrated to 5 mL under vacuum. The 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 30 mL/hr. Each fraction of 7.8 mL was collected. Protein content of each fraction was measured by the Lowry method,[5] using bovine serum albumin as the standard. The peptide fractions (molecular weight range 300–5,000) were pooled and evaporated to dryness to give a peptide powder. Two grams of the peptide powder were dissolved in deionized water and applied to an SP-Sephadex C-25 column (2 × 50 cm, H^+ form; Pharmacia LKB Biotechnology) equilibrated with deionized water. The column was chromatographed using the linear gradient method with 1 L of deionized water to 1 L of 3% NaCl solution at a flow rate of 90 mL/hr, and fractions of 8.6 mL were collected. The peptide fractions were collected and freeze-dried.

Purification of the peptide by reversed-phase high performance liquid chromatography and peptide identification and synthesis

The fraction eluted from SP-Sephadex C-25 was collected and assayed for superoxide anion scavenging activity (SOSA). Among those fractions, a solution (7 mg/25 μL) from the fraction with the highest activity was further isolated and purified by reversed-phase high performance liquid chromatography (HPLC) with a column (4.6 × 250 cm) of Develosil ODS-5 (Nomura Chemical, Ltd., Nagoya, Japan) using a linear gradient of acetonitrile (MeCN) from 0 to 16% in 0.05% trifluoroacetic acid (TFA) for 2 hours at a flow rate of 1.0 mL/min, and the eluent was monitored at 220 nm.

Amino acid analysis of peptide was carried out in 6 N hydrochloric acid containing 0.1% phenol at 110°C for 24 hours using a PICO-TAG™ amino acid analyzer (Waters Ltd., Milford, MA USA). Sequence analysis was done by stepwise Edman degradation using a 477A gas-phase automated sequencer (Applied Biosystems, Inc., Foster City, CA USA) coupled to HPLC, and by identification of the resulting PTH-amino acid compounds.

The molecular formula of each peptide was confirmed by fast atom bombardment mass spectrometry (FAB-MS) obtained with a JEOL DX-300 spectrometer (JEOL Co., Tokyo, Japan).

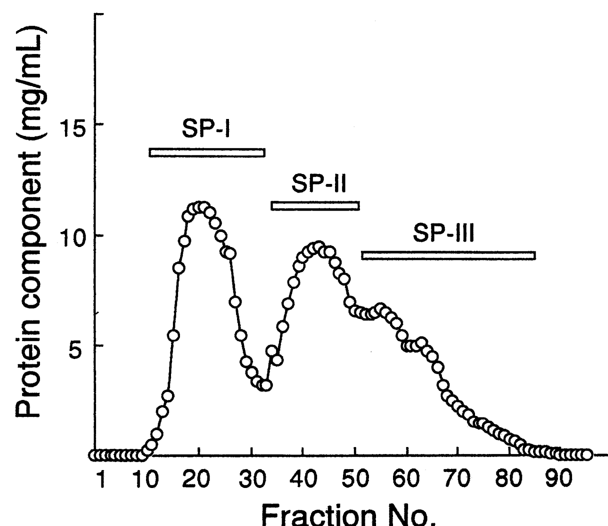

Figure 1 Column chromatogram of casein peptide on SP-Sephadex C-25. The protein component (○) was determined using the Lowry method, and the bovine serum albumin was used as the standard.

Peptides were synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystems, Inc.), followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. The final products were homogeneous on high-resolution reversed-phase HPLC with a Develosil ODS column (4.6 × 150 mm), using gradient of MeCN from 0 to 16.7% in 0.05% TFA for 2 hours at a flow rate of 0.8 mL/min, and the eluent was monitored at 215 nm. The result of amino acid analyses and sequence analyses was in agreement with each expected value.

Assay of SOSA by tetrazolium salt XTT method[6]

Into 2.5 mL of 50 mM sodium carbonate buffer (pH 10.2) were added 0.1 mL each of 3 mM xanthine, 3 mM ethylendaimine-tetraacetic acid (EDTA), 0.75 mM XTT solution, and sample

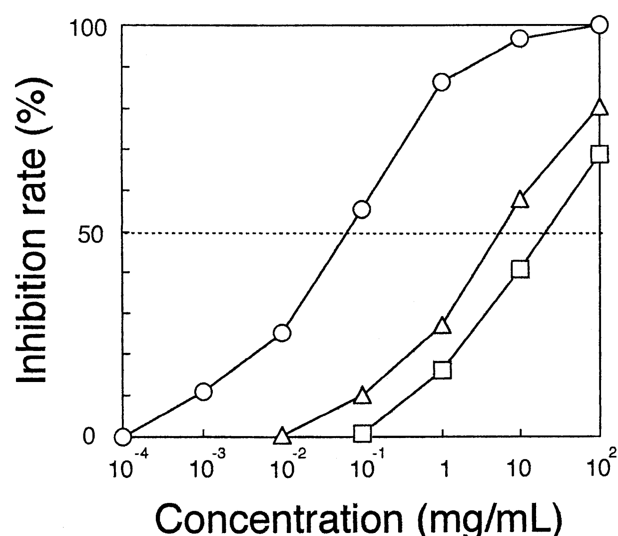

Figure 2 Scavenging of superoxide anion with peptide fractions from SP-Sephadex C-25 chromatography. SP-I, △; SP-II, ○; SP-III, □.

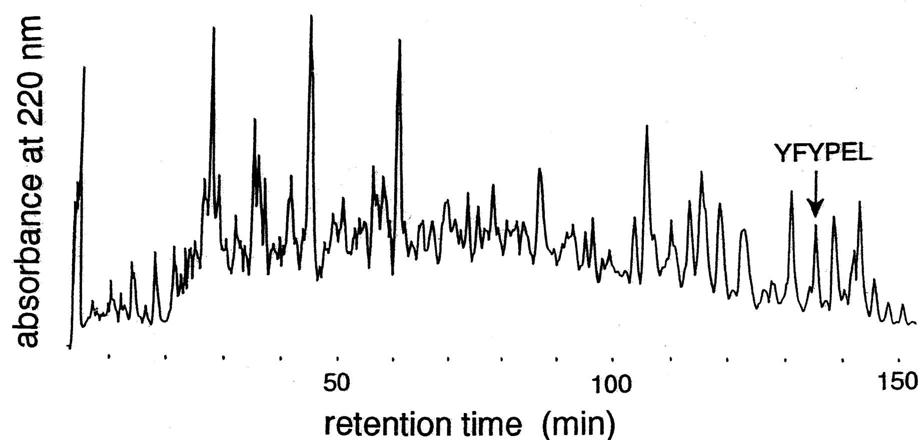

Figure 3 A chromatogram on reversed-phase Develosil ODS-5 column of the active fraction isolated from the SP-Sephadex C-25 column (*Figure 1*). For column conditions, see the text. The peak marked YFYPEL representing the hexapeptide Tyr-Phe-Tyr-Pro-Glu-Leu was found to have free radical scavenging activity.

solution or water. The reaction was initiated by the addition of 56 mU/mL XOD solution (0.1 mL). The absorbance change at 470 nm for 20 minutes was monitored with a UVIDEC 220E spectrophotometer (JASCO, Tokyo, Japan) thermostated at 25°C. The concentration of the test compound required to reduce the produced superoxide anion to one-half (IC_{50}) was calculated.

Assay of 1,1-diphenyl-2-picrylhydrazyl radical and hydroxyl radical scavenging activity by electron spin resonance[7]

The activity of the peptide to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was examined by the spin trapping method using a JES-FR 30 electron spin resonance (ESR) spectrometer (JEOL). One hundred microliters of 150 μM DPPH in ethanol were put in a test tube and stirred after the addition of 100 μL of tested compound solution to initiate the reaction. The reaction mixture was then transferred to a quartz cell used for the measurement of ESR. One minute after initiation of the reaction, DPPH radical was measured, and IC_{50} values (the concentration of the test compounds required to reduce DPPH radical to one-half) were calculated. Conditions for the ESR measurement were as follows: magnetic field, 336 ± 10 mT; response time, 0.1 second; sweeping time, 1 minute (interval of 10 mT).

The activity of the peptide to scavenge the hydroxyl radical produced was examined by the spin trapping method using DMPO as the spin trapping agent. Seventy-five microliters of 1 mM $FeSO_4$ in 0.1 mM sodium phosphate buffer (pH 7.8) containing EDTA, 50 μL of tested compound solution, and 10 μL of 0.9 M DMPO were successively put into a test tube, and stirred after the addition of 75 μL of 10 mM hydrogen peroxide (H_2O_2) to initiate the reaction. The reaction mixture was then transferred to a quartz cell used for the measurement of ESR. One minute after initiation of the reaction, formed DMPO-OH was measured, and IC_{50} values (the concentrations of the test compounds required to reduce the produced hydroxyl radical to one-half) were calculated. The conditions for the ESR measurement were as follows: magnetic field, 336 ± 5 mT; output, 10 mW; modulating, 100 kHz with 0.1 mT; response time, 0.1 second; sweeping time, 1 minute (interval of 10 mT); and amplification ratio, 125.

Results

Peptides having strong SOSA were isolated from peptic hydrolysate of casein using a Dowex 50W (H^+), Sephadex G-25 column as described previously.[8] The fractions having molecular weights ranging from 300 to 5,000 were collected and concentrated to dryness to give a peptide powder. The yield of the peptide powder with SOSA from 30 g of milk casein was 2.68 g. The peptides were fractionated by ion-exchange chromatography on SP-Sephadex C-25 (H^+) to give SP-I (fraction number 10–33), SP-II (34–51), and SP-III (52–85) as shown in *Figure 1*. *Figure 2* shows SOSA of those casein peptide fractions from SP-Sephadex C-25 chromatography. The IC_{50} values of peptide fractions were SP-I, 5.1 mg/mL; SP-II, 0.06 mg/mL; and SP-III, 22.9 mg/mL. The SP-II fraction (average molecular weight, 1,000) showed strongest SOSA using tetrazolium salt XTT method.

Table 1 Superoxide anion radical scavenging activity of synthetic peptides

Peptide	FAB-MS (MH+)	Scavenging activity[1] (IC_{50}, μM)
Tyr-Phe-Tyr-Pro-Glu-Leu	831	79.2
Phe-Tyr-Pro-Glu-Leu	668	127.5
Tyr-Pro-Glu-Leu	521	189.3
Pro-Glu-Leu	358	306.0
Glu-Leu	260	63.1
Tyr+Phe+Pro+Glu+Leu 5 mM each		N.D.[2]

[1] IC_{50} means the concentration of synthetic peptide at 50% of blank absorbance at 550 nm by using tetrazolium salt XTT method.
[2] N.D. means "not detected".

The active fractions were purified further by reversed-phase HPLC (*Figure 3*). From the SP-II fraction, active peptide was obtained eluting at 135 min. Amino acid analysis of the peptide after 6 N HCl hydrolysis found the amino acids L-tyrosine (Tyr), 1.93; L-phenylalanine (Phe), 1.06; L-proline (Pro), 0.86; L-glutamic acid (Glu), 0.92; and L-leucine (Leu), 1.01. The ion peak (MH^+) of the purified peptide was 831 using the FAB-MS. Using protein sequencing, the primary structure of the peptide was found to be Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL).

The SOSA of YFYPEL (IC_{50} = 79.2 μM), which was originally isolated from the hydrolysate of milk casein, was compared with the synthetic fragment-peptides. As shown in *Table 1*, the deletion of the C-terminal Y, YF, and YFY of YFYPEL caused loss of the activity. The highest activity was recognized in EL among the peptides examined. This result suggests that EL played an important role in the activity. When the SOD preparation was assayed as a standard substance using the same method, the IC_{50} was 0.01 μM.

Additionally, the synthetic peptide showed the activity to scavenge hydroxyl radical and DPPH radical. The activities of the peptide to scavenge hydroxyl radical produced in the system of H_2O_2-Fe^{2+} and DPPH radical were 251 μM and 98 μM, respectively. When glutathione (reduced form) and carnosine were assayed as the standard substances based on both methods, the activities to scavenge hydroxyl radical and DPPH radical were 661 μM and 6.12 μM (for glutathione) and 654 μM and 23.3 μM (carnosine), respectively.

Discussion

In the present investigation, we isolated and identified the peptide having the SOSA from the pepsin digest of milk casein. The synthetic peptide with the same amino acid sequence as the isolated one certainly demonstrated the SOSA. Although the SOSA was much lower than that of SOD preparation, the constituent amino acids mixed with the same concentration as the peptide had no activity and thus the characteristic amino acid sequence of the peptide was responsible for the activity. The synthetic peptide also showed the scavenging activity of hydroxyl radical and DPPH radical. Compared with the standard substances such as glutathione and carnosine, the synthetic peptide had higher scavenging activity of hydroxyl radical. Carnosine is known to have hydroxyl radical scavenging activity and thus inhibits autoxidation of various lipids.[9] This property is suitable for carnosine's application as a "natural" food antioxidant. The finding in the present investigation that the peptide from the pepsin digest of milk casein showed a higher scavenging activity of hydroxyl radical than carnosine suggests the usefulness of the casein peptide as the food antioxidant. Actually, Laakso[4] reported that milk casein inhibited lipoxygenase-catalyzed lipid peroxidation and suggested that, as the mechanism, casein might have a radical trapping property. The radical trapping property may be the scavenging activity of hydroxyl radical shown in this article.

Autoxidation of polyunsaturated fatty acids in not only food system but also the biological system is known to be accompanied by the formation of a complex mixture of secondary breakdown products of lipid peroxides including aldehydic compounds.[10] These compounds can further react with biomolecules and cause a number of adverse effects including loss of enzyme activity,[11] mutagenicity and toxicity to mammalian cells,[12] and modification of DNA[13] and low density lipoproteins.[14] Because the casein peptide has SOSA and the scavenging activity of hydroxyl and DPPH radical, it may be expected that it may also function as an antioxidant in vivo. The finding that the dipeptide derived from casein showed highest SOSA among all peptides examined in the present investigation is especially promising because the dipeptides can be directly absorbed via the intestinal dipeptide transporter.[15] Thus, based on the results obtained in the present investigation, it would be of value to examine the activity of the peptide in vivo. Therefore, in an effort to search for more potent radical scavenging activity, comparative studies of the dipeptide analogues are currently underway.

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