JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Properties of Casein Calcium Peptides and Their Effects on Lipid Oxidation in Beef Homogenates

SENJI SAKANAKA,*,† YUMI TACHIBANA,† NORIYUKI ISHIHARA,‡ AND LEKH RAJ JUNEJA‡

School of Bioresources, Hiroshima Prefectural University, 562 Nanatsuka, Shobara, Hiroshima 727-0023, Japan, and Central Research Laboratories, Taiyo Kagaku Co., Ltd., 1-3 Takaramachi, Yokkaichi, Mie 510-0844, Japan

The antioxidant activity of casein calcium peptides in several in vitro assay systems was investigated. Casein calcium peptides were prepared by the microbial enzymic hydrolysis of casein calcium. The main peak of the molecular mass distribution of the peptides was about 3 kDa. Casein calcium peptides showed strong antioxidant activity with the β-carotene bleaching method, and they also showed scavenging activity against radicals such as superoxide radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and hydroxyl radicals. Antioxidant activity was increased with an increasing peptide concentration. Casein calcium peptides also showed strong antioxidant activity against lipid oxidation in ground beef homogenates. These results suggest that casein calcium peptides are a suitable natural antioxidant that prevents the lipid oxidation of meat and related food ingredients.

KEYWORDS: Casein calcium peptides; antioxidant; radical scavenger; ground beef

INTRODUCTION

Lipid oxidation is one of the major factors in deterioration during the storage and processing of foods, because it can lead to the development of unpleasant rancid or off-flavors as well as potentially toxic reaction products (*1*, *2*). Therefore, the control of lipid oxidation in food products is desirable, and the benefits of antioxidants in food storage have been studied by many researchers (*3*−*5*). Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate have been widely used in food products to retard lipid oxidation. However, the demand for natural antioxidants has recently increased because of the toxicity and carcinogenicity of synthetic antioxidants (*6*, *7*).

Many antioxidative substances have been and are being isolated from natural materials, including foods. The antioxidative action and structure of these compounds have been reported by many researchers, and some of these compounds are as effective as synthetic antioxidants in a model system (*8*−*10*). Amino acids and proteins have been reported as water-soluble antioxidants because of the chelating effect on metal ions (*11*, *12*). Furthermore, some protein hydrolysates from animal and plant sources have been found to possess antioxidant activity (*13*, *14*). Use of these antioxidants has been investigated primarily for the prevention of lipid oxidation in foods.

Caseins, accounting for about 80% of milk proteins, are phosphoproteins, α-, β-, and κ-, which differ from each other in their phosphate content (10, 5, and 1 mol per casein mole, respectively). This phosphate could confer antioxidant activity on casein molecules. Caseins are widely used as a functional and nutritional ingredient in food products. Casein hydrolysates are obtained by in vitro enzymatic digestion with hydrolysates both water soluble and high in nutritional value. Casein hydrolysates obtained by the proteases, trypsin, and chymotrypsin contain more than 200 peptides of different sizes (*15*). The hydrolysates are better absorbed than a mixture of free amino acids, which may be due to the size and nature of the peptides during the digestive process (*16*, *17*). It is reported that milk protein (*18*) and casein (*19*) have antioxidant activity, but there are few significant studies on the use of caseinophosphopeptides as antioxidants (*20*). Caseinophosphopeptides, as has been reported, are obtained by decomposing casein sodium by trypsin digestion. On the other hand, casein calcium peptides have been reported in intestinal absorption in rats (*21*), but their antioxidant activity has not been described.

In the present study, we investigated the in vitro antioxidant activity of casein calcium peptides. The aims of this study were to characterize casein calcium peptides derived from casein calcium; measure the antioxidant activity of casein calcium peptides by various assay methods such as β-carotene bleaching, superoxide radicals scavenging, 1,1-diphenyl-2-picrylhydrazyl radicals scavenging, and hydroxyl radicals scavenging; and investigate the inhibitory effect of casein calcium peptides on lipid oxidation in ground beef homogenates.

MATERIALS AND METHODS

Materials. Linoleic acid, β-carotene, Tween 40, nitroblue tetrazolium salt, xanthine, 1,1-diphenyl-2-picrylhydrazyl, 2-deoxy-D-ribose, xanthine oxidase (from buttermilk, 0.049 U/mL), sodium dodecyl sulfate (SDS),

* To whom correspondence should be addressed. Tel: +81 824 74 1779; fax: +81 824 74 1779; e-mail: sakanaka@bio.hiroshima-pu.ac.jp.
† Hiroshima Prefectural University.
‡ Taiyo Kagaku Co., Ltd.

10.1021/jf0487699 CCC: $30.25
Published on Web 12/21/2004

trichloroacetic acid, 2-thiobarbituric acid, HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), and 1,1,3,3-tetramethoxypropane were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ground beef (containing 15.8% fat) was purchased from a local supermarket. All other reagents were of analytical grade.

Preparation of and Analytical Methods for Casein Calcium Peptides. Casein calcium peptides were prepared by the hydrolysis of casein calcium using proteolytic enzyme from *Aspergillus* sp. (EC 3.4.21.63; Amano Enzyme Inc., Nagoya, Japan). The casein calcium was dissolved in water containing 10% ethanol at a concentration of about 13% and hydrolyzed by protease (0.4:100 enzyme/substrate) at 50 °C while the pH was maintained at 6.0 by the addition of 3 M NaOH and 3 M HCl. The hydrolysis reaction was stopped after 20 h by heating at 90 °C for 10 min. The soluble fraction was then filtered using filtration paper (No. 2, Toyo Roshi Co., Tokyo, Japan) with precoat layer of Celite (Sigma Chemical Co., St. Louis, MO) and spray-dried. The powder was used as casein calcium peptides.

The amino acid content of the casein calcium peptides was determined using the method described previously (*22*). The molecular mass distribution of the casein calcium peptides was estimated by a Superdex Peptide HR 10/30 column (10 mm i.d. × 300 mm; Amersham Biosciences Corp., Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.5 mL/min. The elution profiles were monitored with absorption at 220 nm. Standard materials of known molecular weight (bovine serum albumin, 66 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa; bovine lung aprotinin, 6.7 kDa; bradykinin, 1.06 kDa; aspartame, 0.294 kDa) were also separated on the column under similar analytical conditions. The molecular weight was calculated by comparison with standard materials.

Determination of Anti-autoxidant Activity Using the β-Carotene Bleaching Method. Anti-autooxidant activity was assayed using the β-carotene bleaching method (*23*−*25*). β-Carotene (2 mg) was dissolved in 20 mL of chloroform. A 4-mL aliquot of the solution was added to a conical flask with 40 mg linoleic acid and 400 mg Tween 40. The chloroform was removed using a rotary evaporator at 50 °C. Distilled water (100 mL) was added to the β-carotene emulsion and mixed, and aliquots (3 mL) of the β-carotene emulsion and 0.2 mL of the sample solution (casein calcium peptides in distilled water) were placed to give a final concentration of 0.0125, 0.025, 0.05, 0.1, and 0.2% of casein calcium peptides in capped culture tubes and mixed well. The tubes were immediately placed in a water bath and incubated at 50 °C. Oxidation of the β-carotene emulsion was monitored taking absorbance at 20-min intervals at 470 nm for 100 min. A control consisted of 0.2 mL of distilled water instead of the sample solution.

Superoxide Radical-Scavenging Activity. Superoxide radicals were generated in vitro by xanthine oxidase. The scavenging activity of the casein calcium peptides was determined using the nitroblue tetrazolium (NBT) reduction method. In this method, O_2^- reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants can inhibit the blue NBT formation (*26*, *27*). The capacity of the samples to scavenge the superoxide radicals was assayed as follows: The reaction mixture contained 0.5 mL of 0.8 mM xanthine in 0.1 mM phosphate buffer (pH 8.0), 0.48 mM NBT in 0.1 mM phosphate buffer (pH 8.0), and 0.1 mL of the sample solution (casein calcium peptides in 0.1 mM phosphate buffer, pH 8.0) to give a final concentration of 0.03125, 0.0625, 0.125, 0.25, and 0.5% of casein calcium peptides. After heating to 37 °C for 5 min, the reaction was initiated by adding 1.0 mL of XOD (0.049 U/mL) and was carried out at 37 °C for 20 min; the reaction was stopped by adding 2.0 mL of 69 mM SDS. The absorbance of the reaction mixture was measured at 560 nm.

The results were calculated as the percentage inhibition according to the formula

$$\% \text{ inhibition} = [\{(C - CB) - (S - SB)\}/(C - CB)] \times 100$$

where S, SB, C, and CB are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

DPPH Radical-Scavenging Activity. The assay mixture contained 0.3 mL of the 1.0 mM DPPH radical solution, 2.4 mL of ethanol, and 0.3 mL of the sample solution (casein calcium peptides in distilled water) to give a final concentration of 0.0625, 0.125, 0.25, 0.5, and 1.0% of casein calcium peptides. The solution was rapidly mixed, and after standing for 30 min at room temperature, the absorbance of the mixture was measured at 517 nm (*9*). The results were calculated as the percentage inhibition according to the formula

$$\% \text{ inhibition} = [\{(C - CB) - (S - SB)\}/(C - CB)] \times 100$$

where S, SB, C, and CB are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

Hydroxyl Radical-Scavenging Activity. The effect of hydroxyl radicals was assayed using the 2-deoxyribose oxidation method (*28*, *29*). 2-Deoxyribose is oxidized by hydroxyl radicals formed by the Fenton reaction and degrades to malondialdehyde (*30*, *31*). The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM hydrogen peroxide, 0.525 mL of distilled water, and 0.075 mL of the sample solution (casein calcium peptides in distilled water) to give a final concentration of 0.03125, 0.0625, 0.125, 0.25, and 0.5% of casein calcium peptides in a tube. The reaction was initiated by the addition of hydrogen peroxide. After incubation at 37 °C for 4 h, the reaction was quenched through addition of 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice, and absorbance measured at 520 nm. Hydroxyl radical-scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radicals. The results were calculated as the percentage inhibition according to the formula

$$\% \text{ inhibition} = [\{(C - CB) - (S - SB)\}/(C - CB)] \times 100$$

where S, SB, C, and CB are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

Antioxidant Activity against Lipid Peroxidation in Ground Beef Homogenates. Ground beef (5 g) was homogenized in 25 mL of 50 mM HEPES buffer (pH 7.0) using a homogenizer (Nihon Seiki Seisakusyo Co., Tokyo, Japan) for 5 min. Buffered systems have been widely used to study oxidation−reduction reactions in meat systems (*32*). The test medium contained 0.8 mL of beef homogenate and 0.2 mL of either the HEPES buffer or one of the sample solutions (casein calcium peptides in HEPES buffer) to give a final concentration of 0.25, 0.5, 1.0, 2.0, and 4.0% of casein calcium peptides and was incubated at 37 °C for 60 min. After incubation, the mixture was tested for the formation of thiobarbituric acid reactive substances (TBARS).

TBARS were determined by a modified procedure as shown below (*32*−*34*). On the day of use, a trichloroacetic acid (TCA/TBA) stock solution was prepared consisting of 15% TCA (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. After mild heating and agitation to dissolve the components, 3 mL of 2% butylated hydroxytoluene (BHT) in absolute ethanol was added per 100 mL of the TCA/TBA stock solution. At appropriate intervals, 1.0 mL of aliquot of the test medium was added to the TCA/TBA stock solution in a test tube and immediately mixed thoroughly with a Vortex mixer. The sample was then heated in a boiling water bath for 10 min and cooled to room temperature, and it was centrifuged at 1710*g* for 10 min. The absorbance of the supernatant was measured at 532 nm using a UNIDEC-50 spectrophotometer (JASCO Corp., Tokyo, Japan). TBARS were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxypropane (TEP).

Statistical Analysis. Values represent means of triplicate analysis and are given with standard deviations. Differences among experimental data were analyzed by Tukey's studentized rang test, and those at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Characteristics of Casein Calcium Peptides. Figure 1 shows the gel permeation chromatography profiles of casein calcium peptides. The main peak of the molecular mass distribution of casein calcium peptides was about 3 kDa, and

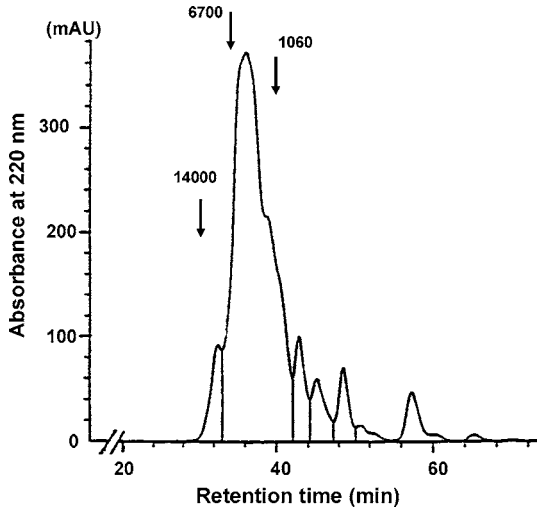

Figure 1. Gel filtration patterns of casein calcium peptides. The arrows indicate the elution times of molecular mass markers.

Table 1. Amino Acid Composition of Casein Calcium Peptides and Casein Calcium[a]

amino acid	casein calcium peptides	casein calcium	amino acid	casein calcium peptides	casein calcium
Asp	6.9	6.7	Ile	5.1	5.4
Thr	4.9	4.7	Leu	9.0	9.1
Ser	7.0	6.9	Tyr	3.8	3.9
Glu	18.4	18.4	Phe	3.8	4.0
Gly	3.2	3.0	Lys	7.4	7.2
Ala	4.3	4.2	His	2.6	2.5
Cys	0.4	0.3	Arg	2.3	2.4
Val	7.1	7.1	Pro	11.5	11.9
Met	2.3	2.4			

[a] Mol (%).

area % of the main peak was 75.2% in the chromatogram. The amino acid compositions of casein calcium peptides and casein calcium were almost the same (**Table 1**).

Antioxidant Effect with the β-Carotene Bleaching Method. Casein calcium peptides were analyzed for their antioxidant activity by the β-carotene bleaching assay. The antioxidant assay using discoloration of β-carotene is widely used, because β-carotene is extremely susceptible to free-radical mediated oxidation. β-Carotene has 11 pairs of double bonds and they are extremely sensitive to oxidation, and β-carotene is decolorized easily with the oxidation of linoleic acid (*35*, *36*). The decrease in the absorbance of β-carotene with or without casein calcium peptides was recorded as a function of time (**Figure 2**). Casein calcium peptides strongly suppressed discoloration of β-carotene compared with the control. The absorbance of the control dropped at a faster rate to 0.4−0.5 after 60 min, whereas at 0.2% casein calcium peptides, the rate was slower, maintaining an absorbance of 0.8 after 60 min. Thus, it is apparent that casein calcium peptides have a strong effect against the discoloration of β-carotene.

Double bonds in β-carotene and unsaturated fatty acids are attacked by radicals. Antioxidants suppress the degradation of double bonds as radical scavengers. In this result, it would be suggested that casein calcium peptides delayed the degradation of β-carotene by acting as antioxidants.

Superoxide Anion Scavenging Activity. Superoxide radicals have been observed to kill cells, inactivate enzymes, and degrade DNA, cell membranes, and polysaccharides (*37*). These radicals

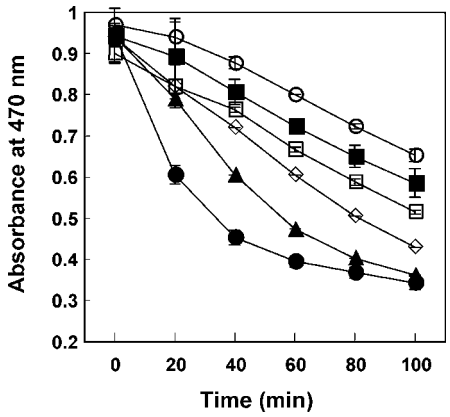

Figure 2. Antioxidant activity of casein calcium peptides measured using the β-carotene bleaching method: control, ●; 0.2%, ○; 0.1%, ■; 0.05%, □; 0.025%, ◇; 0.0125%, ▲. Data represent the mean ± SD of three determinations.

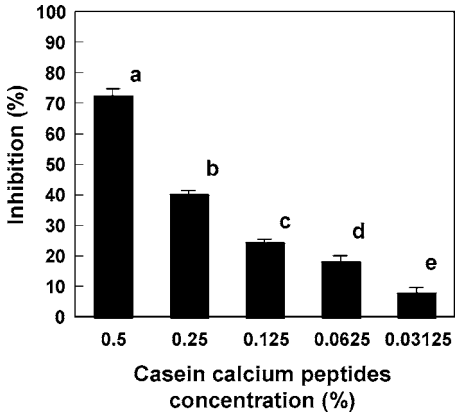

Figure 3. Superoxide radical-scavenging activity of casein calcium peptides. Data represent the mean ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

may also play an important role in the peroxidation of unsaturated fatty acids and possibly other susceptible substances (*38*). Therefore, studying the scavenging effects of casein calcium peptides on superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity.

The superoxide-scavenging activity of casein calcium peptides was measured using the xanthine−xanthine oxidase system, and the results are indicated as the inhibition rate of superoxide activity. Casein calcium peptides exhibited superoxide-scavenging activity, and 0.5% of casein calcium peptides showed more than 70% inhibition. These activities were dose-dependent (**Figure 3**). These results show that casein calcium peptides have strong superoxide radical-scavenging effects.

DPPH Radical-Scavenging Activity. DPPH is a free-radical compound and has been widely used to test the free-radical-scavenging ability of various samples (*39*−*41*). **Figure 4** shows the scavenging activity of casein calcium peptides on DPPH radicals at various concentrations. The scavenging activity of casein calcium peptides on DPPH radicals increased with increasing concentrations (0 to 1%). According to **Figure 4**, casein calcium peptides (1.0%) exhibited 64.4% scavenging activity. The results show that casein calcium peptides are also free-radical scavengers, particularly of the peroxyl radical, which is the major propagator of the oxidation chain of fat, thereby terminating the chain reaction (*42*, *43*).

Hydroxyl Radical-Scavenging Activity. Among the oxygen radicals, the hydroxyl radical is the most reactive and severely

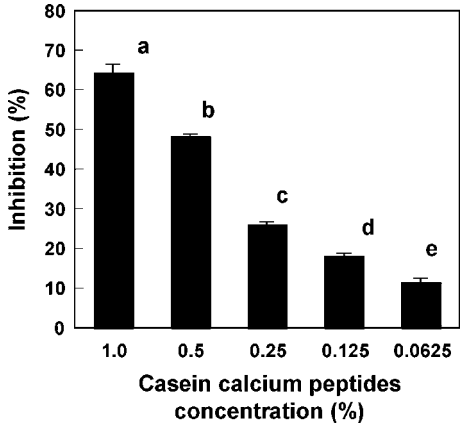

Figure 4. DPPH radical-scavenging activity of casein calcium peptides. Data represent the mean ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

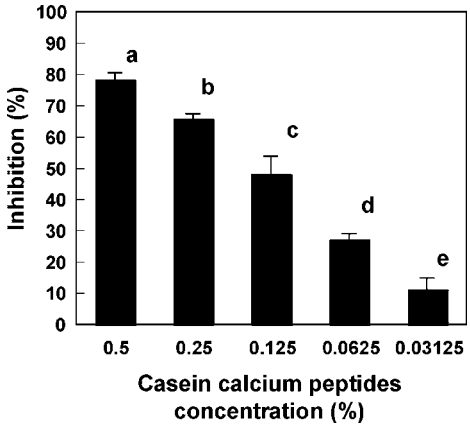

Figure 5. Hydroxyl radical-scavenging activity of casein calcium peptides. Data represent the mean ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

damages adjacent biomolecules. The scavenging effect against hydroxyl radicals was investigated by using the Fenton reaction. **Figure 5** shows the hydroxyl radical-scavenging effects of casein calcium peptides using the 2-deoxyribose oxidation method. The results are shown as the inhibition rate. Casein calcium peptides showed hydroxyl radical-scavenging activity, and its activity increased with an increasing concentration of casein calcium peptides. A 0.5% concentration of casein calcium peptides exhibited 78.4% scavenging activity.

Antioxidant Effects in Ground Beef Homogenates. As mentioned earlier, casein calcium peptides showed antioxidant activity with four different in vitro methods. In addition, their activity was tested using a meat model system. The lipid contents of the ground beef was 15.8%. Casein calcium peptides were added to the homogenates at different concentrations, and lipid oxidation in the homogenates was evaluated. The reaction was measured by monitoring TBARS, and the results are shown in **Figure 6**. In ground beef (20% w/v) homogenized with 50 mM HEPES buffer at pH 7.0, casein calcium peptides effectively inhibited the formation of TBARS in a dose-dependent manner (**Figure 6**). With 2.0% casein calcium peptides, the inhibition rate was 69.7%. The activity of casein calcium peptides was elucidated effectively showing antioxidant activity in the meat model system.

The antioxidant activity of casein calcium peptides was assayed using several different test systems. Recent investigations show differences between the test systems in determining

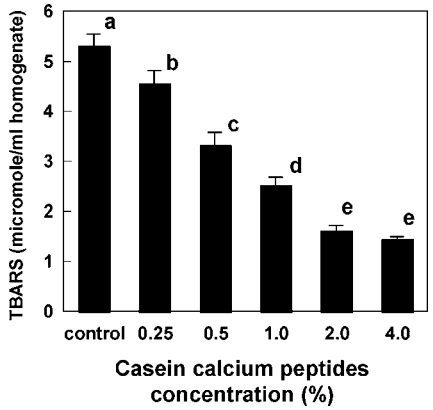

Figure 6. Effect of casein calcium peptides on the formation of TBARS in ground beef homogenates. Data represent the mean ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

antioxidant activity (*44*, *45*). Using at least two methods is recommended. In this study, we used several methods showing different sensitivity and using different systems. First, we evaluated the antioxidant activity of casein calcium peptides by the β-carotene bleaching assay, because β-carotene shows strong biological activity and is physiologically an important compound (*35*). Casein calcium peptides showed strong effects against the discoloration of β-carotene, that is, casein calcium peptides delayed the degradation of β-carotene by acting as antioxidants. Next, scavenging activity against free radicals such as superoxide radicals, DPPH radicals, and hydroxyl radicals was investigated. The model systems of scavenging these free radicals are simple methods to evaluate the antioxidant activity of antioxidants. With 0.5% of casein calcium peptides, the inhibition rates of superoxide radicals, DPPH radicals, and hydroxyl radicals were 72.6, 48.3, and 78.4%, respectively. Casein calcium peptides showed antioxidant activity in all of the different in vitro assay systems. These results suggest that casein calcium peptides are a good source of natural antioxidant.

When incorporated into ground beef homogenates, casein calcium peptides (2.0%) effectively inhibit about 70% of lipid oxidation in homogenates. Casein calcium peptides may be as useful in meat processing as other naturally occurring antioxidants, helping to prevent the formation of an off-flavor in meat products thereby increasing shelf life. Further works on the characterization of antioxidant compounds in casein calcium peptides are in progress to establish the connection between antioxidant activity and chemical composition.

LITERATURE CITED

(1) Lin, C. C.; Liang, J. H. Effect of antioxidants on the oxidative stability of chicken breast meat in a dispersion system. *J. Food Sci.* **2002**, *67*, 37−41.

(2) Wang, B.; Pace, R. D.; Dessai, A. P.; Bovel-Benjamin, A.; Philips, B. Modified extraction method for determining 2-thiobarbituric acid values in meat with increased specificity and simplicity. *J. Food Sci.* **2002**, *67*, 2833−2836.

(3) Kikuzaki, H.; Nakatani, N. Antioxidant effects of some ginger constituents. *J. Food Sci.* **1993**, *58*, 1407−1410.

(4) Kim, J. S.; Godber, J. S. Oxidative stability and vitamin E levels increased in restructured beef roasts with added rice bran oil. *J. Food Qual*. **2001**, *24*, 17−26.

(5) Shin, F. F.; Daigle, K. W. Antioxidant properties of milled-rice coproducts and their effects on lipid oxidation in ground beef. *J. Food Sci*. **2003**, *68*, 2672−2675.

(6) Buxing, S.; Fukuhara, M. Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoid on the activation of mutagens and drug-metabolizing enzymes in mice. *Toxicology* **1997**, *122*, 61−72.
(7) Hirose, M.; Takesada, Y.; Tanaka, H.; Tamano, S.; Kato, T.; Shirai, T. Carcinogenicity of antioxidants BHA, caffeic acid, sesamol, 4-methoxyphenol and catechol at low doses, either alone or in combination and modulation of their effects in a rat medium-term multi-organ carcinogenesis model. *Carcinogenesis* **1998**, *19*, 207−212.
(8) Bishov, S. J.; Henick, A. S. Antioxidant effect of protein hydrolysates in freeze-dried model system. *J. Food Sci*. **1975**, *40*, 345−348.
(9) Nagai, T.; Inoue, R.; Inoue, H.; Suzuki, N. Preparation and antioxidant properties of water extract of propolis. *Food Chem*. **2003**, *80*, 29−33.
(10) Shahidi, F.; Wanasundara, P. K. J. P. D. Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr*. **1992**, *32*, 67−103.
(11) Lu, C. L.; Baker, R. C. Characteristic of egg yolk phosvitin as an antioxidant for inhibiting metal-catalyzed phospholipids oxidations. *Poult. Sci*. **1986**, *65*, 2065−2070.
(12) Cervato, G.; Cazzola, R.; Cestaro, B. Studies on the antioxidant activity of milk caseins. *Int. J. Food Sci. Nutr*. **1999**, *50*, 291−296.
(13) Amarowicz, R.; Shahidi, F. Antioxidant activity of peptide fraction of capelin protein hydrolysates. *Food Chem*. **1997**, *58*, 355−359.
(14) Pena-Ramos, E. A.; Xiong, Y. L. Antioxidant activity of soy protein hydrolysates in a liposomal system. *J. Food Sci*. **2002**, *67*, 2952−2956.
(15) Lemieux, L.; Amiot, J. High-performance liquid chromatography of casein hydrolysates phosphorylated and dephosphorylated. I. Peptide mapping. *J. Chromatogr*. **1990**, *519*, 299−321.
(16) Silk, D. B.; Marrs, T. C.; Addison, J. M.; Burston, D.; Clark, M. L.; Mattews, D. M. Absorption of amino acids from an amino acid mixture simulating casein and a tryptic hydrolysates of casein in man. *Clin. Sci. Mol. Med*. **1973**, *45*, 715−719.
(17) Imondi, A. R.; Stradley, R. P. Utilization of enzymatically hydrolyzed soybean protein and crystalline amino acid diets by rats with exocrine pancreatic insufficiency. *J. Nutr*. **1974**, *104*, 793−801.
(18) Taylor, M. J.; Richardson, T. Antioxidant activity of skim milk: Effect of sonication. *J. Dairy Sci*. **1980**, *63*, 1938−1942.
(19) Cervato, G.; Cazzola, R.; Cestaro, B. Studies on the antioxidant activity of milk caseins. *Int. J. Food Sci. Nutr*. **1999**, *50*, 291−296.
(20) Diaz, M.; Dunn, C. M.; McClement, D. J.; Decker, E. A. Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *J. Agric. Food Chem*. **2003**, *51*, 2365−2370.
(21) Shimooka, I.; Kayashita, J.; Nakajoh, M.; Mori, S.; Itokawa, Y. Measurement of intestinal absorption of calcium-casein peptide in rats by portal cannulation. *J. Jpn. Soc. Nutr. Food Sci*. **1992**, *45*, 339−345.
(22) Gutierrez, M. A.; Mitsuya, T.; Hatta, H.; Koketsu, M.; Kobayashi, R.; Juneja, L. R.; Kim, M. Comparison of egg-yolk protein hydrolysate and soybean protein hydrolysate in terms of nitrogen utilization. *Br. J. Nutr*. **1998**, *80*, 477−484.
(23) Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Miller, A. A novel method of measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci*. **1993**, *84*, 407−412.
(24) Wanasundara, U.; Amarowicz, R.; Shahidi, F. Isolation and identification of an antioxidative component in canola meal. *J. Agric. Food Chem*. **1994**, *42*, 1285−1290.
(25) Kauer, C.; Kapper, H. C. Anti-oxidant activity and total phenolic content of some Asian vegetables. *Int. J. Food Sci. Technol*. **2002**, *37*, 153−161.
(26) Parejo, I.; Viladomat, F.; Bastida, J.; Rosas-Romero, A.; Flerlage, N.; Burillo, J.; Codina, C. Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants. *J. Agric. Food Chem*. **2002**, *50*, 6882−6890.
(27) Cos, P.; Ying, L. Y.; Calomme, M.; Hu, J. H.; Cimanga, K.; Van Poel, B.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. Structure−activity relationships and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod*. **1998**, *61*, 71−76.
(28) Chung, S. K.; Osawa, T.; Kawakishi, S. Hydroxyl radical-scavenging effects of species and scavengers from brown mustard (*Brassica nigra*). *Biosci., Biotechnol., Biochem*. **1997**, *61*, 118−123.
(29) Nagai, T.; Inoue, R. Preparation and the functional properties of water extract and alkaline extract of royal jelly. *Food Chem*. **2004**, *84*, 181−186.
(30) Gutteridge, J. M. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochem. J*. **1984**, *224*, 761−767.
(31) Gutteridge, J. M. Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. *Biochem. J*. **1987**, *243*, 709−714.
(32) Lee, B. J.; Hendricks, D. G. Antioxidant effects of L-carnosine on liposomes and beef homogenates. *J. Food Sci.* **1997**, *62*, 931−934.
(33) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol*. **1978**, *52*, 302−304.
(34) McDonald, R. E.; Hultin, H. O. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J. Food Sci*. **1987**, *52*, 15−27.
(35) Sarkar, A.; Bishayee, A.; Chatterjee, M. Beta-carotene prevents lipid peroxidation and red blood cell membrane protein damage in experimental hepato carcinogenesis. *Cancer Biochem. Biophys.* **1995**, *15*, 111−125.
(36) Kumazawa, S.; Taniguchi, M.; Suzuki, Y.; Shimura, M.; Kwon, M. S.; Nakayama, T. Antioxidant activity of polyphenols in carob pods. *J. Agric. Food Chem*. **2002**, *50*, 373−377.
(37) Fridovich, I. The biology of oxygen radicals. *Science* **1978**, *201*, 875−880.
(38) Nice, D. J.; Robinson, D. S. Inhibition of lipid autoxidation by bovine superoxide dismutase. *Food Chem*. **1992**, *45*, 99−103.
(39) Hatano, T.; Takagi, M.; Ito, H.; Yoshida, T. Phenolic constituents of liquorice. VII. A new calcone with a potent radical scavenging activity and accompanying phenols. *Chem. Pharm. Bull*. **1997**, *45*, 1485−1492.
(40) Shimoji, Y.; Tamura, Y.; Nakamura, Y.; Nanda, K.; Nishidai, S.; Nishikawa, Y.; Ishihara, N.; Uenakai, K.; Ohigashi, H. Isolation and identification of DPPH radical scavenging compounds in kurosu (Japanese unpolished rice vinegar). *J. Agric. Food Chem*. **2002**, *50*, 6501−6503.
(41) Benvenuti, S.; Pellati, F.; Melegari, M.; Bertelli, D. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. *J. Food Sci*. **2004**, *69*, FCT164−FCT169.
(42) Frankel, E. N. Recent advances in lipid oxidation. *J. Sci. Food Agric*. **1991**, *54*, 495−511.
(43) Yen, G. C.; Chang, Y. C.; Chen, J. P. Antioxidant activity of mycelia from *Aspergillus candidus*. *J. Food Sci*. **2002**, *67*, 567−572.
(44) Schlesier, K.; Harwat, M.; Böhm, V.; Bitsch, R. Assessment of antioxidant activity by using different in vitro methods. *Free Radical Res*. **2002**, *36*, 177−187.
(45) Gahler, S.; Otto, K.; Böhm, V. Alterations of vitamin C, total polyphenols, and antioxidant capacity as affected by processing tomatoes to different products. *J. Agric. Food Chem*. **2003**, *51*, 7962−7968.

Received for review July 23, 2004. Revised manuscript received October 24, 2004. Accepted November 3, 2004.

JF0487699