

Caseins and Casein Hydrolysates. 2. Antioxidative Properties and Relevance to Lipoxygenase Inhibition

Sandrine G. Rival,* Carmen G. Boeriu, and Harry J. Wichers

Agrotechnological Research Institute (ATO), P.O. Box 17, 6700 AA Wageningen, The Netherlands

The antioxidant activity of caseins and casein-derived peptides was evaluated by using three free radical producing reactions—the lipoxygenase- and AAPH-catalyzed oxidation of linoleic acid and the hemoglobin-catalyzed oxidation of linoleic acid hydroperoxide. Caseins and casein-derived peptides were able to inhibit enzymatic and nonenzymatic lipid peroxidation, suggesting they were preferred targets for the free radical intermediates. The antioxidative feature was not lost with the dephosphorylation or the proteolysis of the proteins. The fractionation of the tryptic β -casein digest yielded peptides with antioxidant activity. A structure–function relationship between the amino acid sequence and the antioxidant capacity and effectiveness is proposed. In addition, indirect evidence suggested that the trapping of free radicals by the proteins/peptides was accompanied by the oxidation of proteins/peptides, according to a sequence-specific mechanism.

Keywords: *Lipoxygenase-catalyzed reaction; free radical-generating systems; antioxidant peptides; radical scavenger; peptide oxidation*

INTRODUCTION

Soybean lipoxygenase 1 (Lox; EC 1.13.11.12) catalyzes the peroxidation of unsaturated fatty acids (linoleic acid), in the presence of molecular oxygen, into the corresponding hydroperoxides (Siedow, 1991). Despite the simplicity of this reaction and a long history of study, the Lox mechanism is still not understood in detail, notably the role of the enzyme iron, essential for catalysis. Lox contains 1 mol of non-heme iron cofactor per mole of enzyme (Chan, 1973; Pistorius and Axelrod, 1974), which is usually in the ferrous form (inactive) when isolated. The two main mechanisms proposed for the Lox reaction depend on the ability of the metal ion to participate in redox chemistry and to coordinate substrates, intermediates, or reaction products. In both mechanisms, Lox must be in the oxidized or ferric form for the Lox-catalyzed reaction to proceed, requiring an activation step during which Fe^{2+} is oxidized to Fe^{3+} by the product. In the mechanism proposed by de Groot et al. (1975), called the radical mechanism, the role of the iron is to oxidize the substrate to a radical species that adds oxygen directly. In the mechanism proposed by Corey and Nagata (1987), the role of the ferric ion is to form a σ -organoiron complex with the substrate, and dioxygen inserts into the Fe–C bond. Even though both mechanisms are supported by experimental proof, the radical mechanism is favored on the grounds of strong EPR evidence [for reviews, see Brash (1999), Feussner and Wasternack (1998), and Nelson and Seitz (1994)]. It is generally accepted that hydrogen abstraction from linoleic acid at the C-11 methylene group, accompanied with the reduction of Lox to the Fe^{2+} form, gives rise to a delocalized radical (Figure 1). The pentadienyl radical is then trapped by dioxygen to yield a peroxy radical of linoleic acid as a catalytic intermediate. The final step of the reaction is the reduction of the radical to the

hydroperoxide product and the subsequent reoxidation of Lox to the Fe^{3+} form, with the release of the product from the enzyme.

Caseins and casein-derived peptides were previously shown to inhibit lipoxygenase-catalyzed oxidation of linoleate (Part 1, Rival et al., 2000). Further research was needed to elucidate the mechanism of action of these compounds. The lipoxygenase-catalyzed mechanism (literature data) was then examined to set up specific experiments to provide new information, especially with regard to presumed antioxidative properties of caseins and casein-derived peptides.

Considering the radical mechanism (Figure 1), caseins and casein-derived peptides could act on Lox-catalyzed reaction by scavenging free radical intermediate(s) and/or the product of the reaction and/or by blocking the redox cycle of the iron. The effects of the test compounds were therefore assayed with two free radical generating systems (chemically induced oxidation of linoleic acid and hemoglobin-catalyzed oxidation of linoleic acid hydroperoxide). In addition, their effects on a stable free radical species were analyzed. The chelating activity of the test compounds was assayed on Fe^{2+} because a coordination of the iron in the active site would also result in inhibition.

MATERIALS AND METHODS

Materials. The various types of bovine casein, except β -casein, BSA (hydroperoxide- and fatty acid-free), soybean lipoxygenase 1 (Lox), linoleic acid (LA, ~99%), 13(S), (9Z,11E)-hydroperoxyoctadecadienoic acid (Hpode), ferrozine or (3,12-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, DPPH radical or 1,1-diphenyl-2-picrylhydrazyl, and *n*-propyl gallate, were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Bovine β -casein [90% β -casein based on weight, 95% β -casein based on nitrogen (w/w)], was purchased from Eurial (Rennes, France) and contained mainly the genetic variants A¹ and A². All of the proteins were of analytical grade. Casein-derived peptides (casein and β -casein proteolytic digests, FPLC-fractionated β -casein tryptic digest peaks 1–3,

* Corresponding author (fax 31.317.475347; e-mail sgrival@excite.fr).

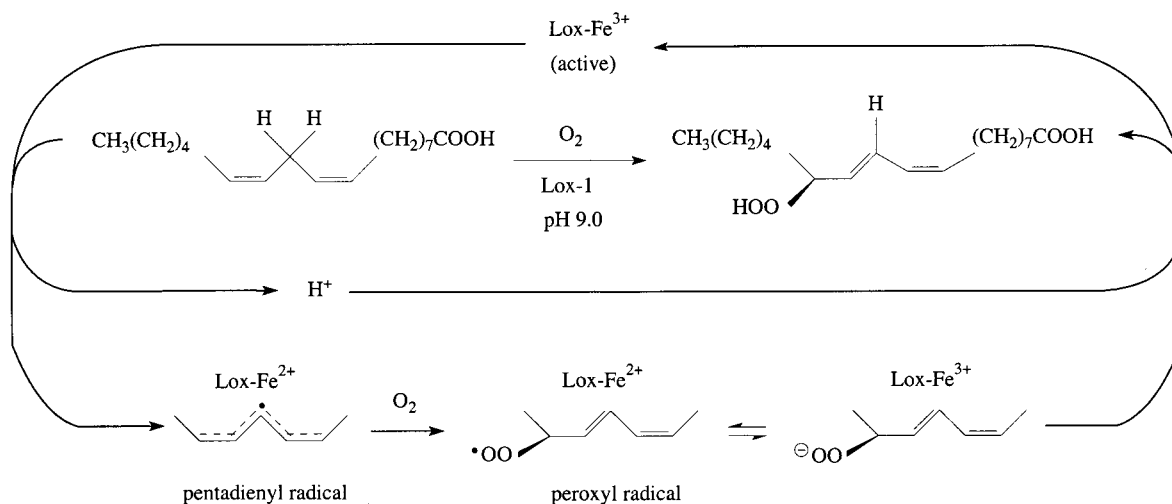


Figure 1. Radical mechanism for lipoxygenase-catalyzed oxidation of linoleic acid under aerobic conditions [modified from de Groot et al. (1975)].

Table 1. Sequence of the Synthesized Peptides

synthesized peptide ^a	amino acid sequence
spps 3	VKEAMAPK (β -casein residues 98–105)
spps 4	AVPYPQR (β -casein residues 177–183)
spps 5	KVLPVPQK (β -casein residues 169–176)
spps 6	VLVPVQK (β -casein residues 170–176)
control peptides	VFQFLGKTIHHVGNFVHGFSHFV AAQVGIGFAKPFKLI KIIHHVGNFVHGFSHFV DILDKVENAIHNAAQV

^a The spps sequence was authenticated by cochromatography with the HPLC-collected peptides (hplc 3–6), previously sequenced by ESI-MSⁿ (Part 1). Identity and purity of control peptides were not checked.

and β -casein tryptic peptides hplc/spps 3–6) were obtained and characterized as described in Part 1. β -Casein tryptic peptides (spps), and some control peptides (see Table 1), were prepared by the fluorenylmethoxycarbonyl (Fmoc) strategy, using a multiple peptide synthesizer (model AMS 422, ABIMED, Langenfeld, Germany), as described in Part 1. Side-chain-protected amino acid derivatives, in the L configuration, resins for peptide assembly, and coupling reagents were purchased from Novabiochem (Läufelfingen, Switzerland). All other reagents were from Biosolve (Valkenswaard, The Netherlands). The oxidation initiator, AAPH or 2,2'-azobis(2-amidinopropane) dihydrochloride, was purchased from Sigma-Aldrich. Lox- and AAPH-catalyzed reactions were monitored as the rate of oxygen consumption using a biological oxygen monitor (YSI model 5300, Yellow Springs Instruments, Yellow Springs, OH). The leucomethylene blue derivative (LMB) or 10-benzoyl-3,7-bis(dimethylamino)-10*H*-phenothiazine was purchased from Brunschwig Chemie (Amsterdam, The Netherlands). The LMB colorimetric reagent was prepared as described by Auerbach et al. (1992). Because the authors warned about the importance of the purity of Triton X-100 to achieve long-term stability of the LMB colorimetric reagent and low background absorbance, Triton X-100 reduced, peroxide- and carbonyl-free (Sigma X-100R-PC) was used. Hpode, used in the LMB methodology, was daily prepared by enzymatic peroxidation of linoleic acid with soybean lipoxygenase 1 [procedure described by Schilstra et al. (1992)]. The purification step was not performed: the yield of the reaction was ~95%, on the basis of Hpode concentration, determined spectrophotometrically at 234 nm (absorption coefficient 25000 M⁻¹ cm⁻¹; Galey et al., 1988). Enzymatic Hpode was authenticated with commercial Hpode by analytical RP-HPLC (equipment described in Part 1), using a linear gradient of acetonitrile in acidic water (0.1% trifluoroacetic acid) and 234 nm as detection wavelength.

Measurement for Antioxidant Activity of Caseins and Casein-Derived Peptides against Enzymatically Induced Linoleic Acid Oxidation: (LA/Lox) Oxidation System.

The procedure described in Part 1 was used to perform complementary experiments with the synthesized β -casein tryptic peptides (spps) and several control peptides (sequences summarized in Table 1). Diluted linoleic acid stock solution (6.4 mM) was added (150 μ L) in air-saturated 0.1 M sodium borate buffer, pH 9.0 (in absence or in the presence of 450 μ M peptides), for a total volume of 3.0 mL. The reaction was started by the injection of the enzyme (2 nM, final concentration), and the initial rate was determined from the linear phase. The relative antioxidant activity (RAA), mean value of three experiments, was calculated by dividing the initial rate in the control by that in the test sample. An RAA value (x) above 1 indicates an antioxidative effect, the rate being x times slower than that of the control. The data previously obtained with caseins and casein-derived peptides (Part 1) were also expressed in RAA values.

Measurement for Antioxidant Activity of Caseins and Casein-Derived Peptides against Chemically Induced Oxidation of Linoleic Acid: (LA/AAPH) Oxidation System.

The antioxidative activity of caseins and casein-derived peptides in the AAPH-induced oxidation system was measured as reported by Chen et al. (1998). Linoleic acid in 0.1 M sodium phosphate buffer, pH 7.0, containing Triton X-100 (1%, w/v) was emulsified by sonication for 5 min. The linoleic acid emulsion (3.2 g/L, final concentration) was mixed with the test compounds (2.4 g/L, except spps assayed at 70, 100, and 300 μ M, final concentration) dissolved in the same buffer and stirred at 37 °C. The dissolved oxygen content in the emulsion was monitored with the oxygen electrode. When the baseline was stable, the water-soluble initiator AAPH (1.6 g/L, final concentration) was injected into the emulsion to accelerate the oxidation of linoleic acid. In any case, the oxygen uptake was linearly related to the time of the experiment (~15 min), allowing the calculation of the reaction rate. The results (mean value of three replicates) are expressed as RAA.

Measurement for Antioxidant Activity of Caseins and Casein-Derived Peptides against Hemoglobin-Catalyzed Oxidation of Linoleic Acid Hydroperoxide (Methylene Blue-Based Methodology): (Hpode/Hb) Oxidation System.

The assays were performed according to Ohishi's methodology (Ohishi et al., 1985; Yagi et al., 1986), as adapted by Auerbach et al. (1992). The experiments were performed in a 96-well microtiter plate. The test compounds (150 μ g), solubilized with 0.1 M sodium borate buffer, pH 9.0, were mixed with increasing concentrations of Hpode (from 1 to 12 or 14 nmol) in a total volume of 50 μ L, and the LMB color reagent was added (100 μ L). After 15 min at room temperature, the absorbance at 660 nm was measured using a microtiter plate

reader. The hydrogen peroxide concentration was determined by linear regression using a calibration curve [$A_{660} = f(C_{H_2O_2})$], built from control experiments performed with Hpode and the buffer, pH 9.0. The results (mean value of three experiments) were then expressed as RAA. RAA was calculated by dividing the slope in the control by that in the test sample. Accordingly, an RAA value (x) above 1 indicates an antioxidative effect, the reaction in the presence of the test compound being inhibited x times.

Measurement for Antiradical Activity of Caseins and Casein-Derived Peptides against DPPH. The test compounds were solubilized with 0.1 M sodium phosphate buffer, pH 7.0, containing Triton X-100 (1%, w/v), except the spps and control peptides, which were solubilized with methanol. To check that the buffer did not interfere with the assay, a known scavenger (*n*-propyl gallate) was solubilized with the buffer or with methanol and the results were compared. The solutions of test compounds (1.2 g/L, except the spps and control peptides, 100 μ M) were mixed with 100 μ M of DPPH radical in methanol. The mixture was shaken vigorously and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. The radical scavenging activity (RSA) was calculated as the absorbance ratio in the control (DPPH in buffer) to that in the test sample and is the mean value of three replicates. An RSA value above 1 indicates a scavenging activity.

Measurement for Fe²⁺ Chelating Activity of Caseins and Casein-Derived Peptides. The chelating activity of caseins and casein-derived peptides was measured as reported by Decker and Welch (1990) and Yen and Wu (1999). The test compounds (2.4 g/L except the spps, 500 μ M, final concentration, aqueous solutions) were mixed with FeCl₂ (0.001–0.050 g/L, final concentration, aqueous solutions). The mixture was then reacted with ferrozine (200 μ M, final concentration, aqueous solution) for 10 min. The absorbance of the mixture (formation of the ferrous iron–ferrozine complex) was measured spectrophotometrically at 562 nm. The standard curve (control) was performed in the same way using FeCl₂ and water. The chelating effect of the test compounds on Fe²⁺ was calculated as follows: chelating effect (%) = [1 – (absorbance of sample at 562 nm/absorbance of control at 562 nm)] \times 100.

RESULTS AND DISCUSSION

Antioxidative Activity of Caseins and Casein-Derived Peptides in the (LA/Lox) Oxidation System. We have previously shown that caseins and some casein-derived peptides were acting on lipoxygenase kinetics by decreasing the oxygen consumption rate (Part 1). We assumed that only specific amino acid sequences within the protein molecules were responsible for this effect, and some bioactive sequences were identified upon proteolysis. Depending on the protease specificities and the hydrolyzed molecules, the proteolysis liberated bioactive peptides and also several inactive peptides (subtilisin digest of casein, clostripain digest of β -casein, and the FPLC-fractionated peak 3 from the β -casein tryptic digest) (Table 2). The most potent Lox inhibitor was the unfractionated tryptic digest of β -casein (RAA = 1.38). Its fractionation into pools of peptides (FPLC peaks 2 and 3) or into individual peptides (hplc 3, 4, 6, and 6) did not enhance but, most importantly, did not abolish the Lox inhibitory property. The peptides (hplc) were identified and synthesized as reported previously (Part 1) to allow more extensive studies. The corresponding synthesized peptides (spps 3–6) were examined with regard to their Lox inhibitory properties (Table 2). They surprisingly did not exhibit an activity comparable to that of the HPLC-isolated peptides: even at 450 μ M, they hardly inhibited the enzymatic reaction. A synergistic mechanism of the HPLC-fractionated peptides with phosphate groups, or a different confor-

Table 2. Antioxidative Properties of Caseins and Casein-Derived Peptides^a

test compound	relative antioxidative activity			RSA DPPH radical ^e
	(LA/Lox) ^b	(LA/AAPH) ^c	(Hpode/Hb) ^d	
undigested proteins				
Cas	1.30	1.56	1.83	1.31
Deph Cas	1.25	1.76	1.97	1.07
β -Cas	1.21	1.16	1.51	1.10
Deph β -Cas	1.09	1.49	1.59	0.94
Deph α -Cas	1.19	1.58	2.14	1.22
κ -Cas	1.36	1.37	1.88	1.17
BSA	1.31	1.47	1.33	1.17
digested proteins				
Trp D Cas	1.31	1.69	1.84	0.82
Clost D Cas	1.03	1.28	1.17	1.09
Subt D Cas	1.22	1.62	1.53	0.86
Trp D β -Cas	1.38	1.33	1.18	0.98
Subt D β -Cas	1.03	1.44	1.25	1.04
FPLC-fractionated peptides				
FPLC peak 1	1.18	1.07	1.15	0.83
FPLC peak 2	1.11	1.55	1.25	0.82
FPLC peak 3	1.05	1.18	1.15	0.82
HPLC-fractionated peptides				
hplc 3	1.19	ND	ND	ND
hplc 4	1.10	ND	ND	ND
hplc 5	1.26	ND	ND	ND
hplc 6	1.11	ND	ND	ND
synthetic peptides				
spps 3	1.05	1.12	1.67	0.95
spps 4	1.02	1.35	4.22	1.00
spps 5	1.03	1.13	1.00	0.99
spps 6	1.06	1.22	1.06	1.05

^a The results are expressed as relative antioxidant activity (RAA) and radical scavenging activity (RSA) as reported by Chen et al. (1998) to compare the data obtained with the different lipid oxidation catalysts used in this study and the present data to the authors' data. For conditions used, see Materials and Methods.

^b The data are from Part 1, except spps (this study). The peptide concentrations (spps) were 450 μ M for (b) and 100 μ M (spps 3), 300 μ M (spps 4), 70 μ M (spps 5), and 100 μ M (spps 6) for (c). The test compound concentrations were 3 g/L for (d) and 1.2 g/L, except spps, 100 μ M for (e). Also assayed: propyl gallate (10 μ M, RAA = 2.16), and control peptides (100 μ M, RAA = 1.00) in system c; DOPA and propyl gallate (5 and 10 g/L, RAA = ∞ , i.e., 100% of quenching), propyl gallate (10 and 100 μ M, RAA = 1.05 and 38.41, respectively), L-Arg (3 g/L, RAA = 1.29), L-Val and L-Ile (3 g/L, RAA = 1.00) in system d; propyl gallate (500 μ M, RSA = 3.58), control peptides (100 μ M, RSA = 1.00), Deph β -Cas (2.4 g/L, RSA = 1.34), Deph α -Cas (2.4 g/L, RSA = 2.20), and κ -Cas (2.4 g/L, RAA = 1.95) in system e. Standard deviations in systems b (spps), c, d, and e were <5%.

mation of the synthesized peptides, could account for the change in reactivity. The HPLC-isolated peptides were MS-detected as singly and doubly charged ions, but also as phosphate adducts (Part 1). The presence of phosphate groups, which are evidenced as Lox inhibitors (Glickman and Klinman, 1995) and also as strong iron chelators, can enhance the effect of the peptides on Lox activity. Furthermore, the phosphorylated β -casein fragment (33–48) contaminated hplc 5, acting perhaps as a synergistic agent. Alternatively, a slight difference in the conformations of the synthesized and purified proteolytic peptides, arising from the presence of phosphate adducts and the consequent molecular electrostatic potential changes, could lead to a decrease in reactivity. Regarding the possible biologically active conformation, even a small peptide can indeed exist in multiple conformation stages. However, certain conformations are preferred depending on the amino acid residues in the peptide chain and the molecular environment (Schlimme and Meisel, 1995).

The results from the sole lipoxygenase assay did not provide many clues about the mechanism of action of

caseins and casein-derived peptides. Lipoxygenase-catalyzed reaction is based on consecutive reactions producing carbon-centered and then oxygen-centered radicals (Figure 1), and caseins and casein-derived peptides can interfere at each stage of the chain. Therefore, simple nonenzymatic reactions, susceptible to represent an individual step, were examined.

Antioxidative Activity of Caseins and Casein-Derived Peptides in the (LA/AAPH) Oxidation System. Caseins and casein-derived peptides were able to significantly inhibit the AAPH-initiated lipid peroxidation (RAA from 1.07 to 1.76; Table 2). The most potent compounds were less active than the commercial antioxidant propyl gallate. However, their effect was 106–120% higher than that of BSA, described as a free radical scavenger (Kanner et al., 1987). The dephosphorylated casein exhibited the highest antioxidant activity. The activity of casein or β -casein was enhanced upon proteolysis. The unfractionated digests (except the clostripain digest of casein) had higher antioxidant activity than the undigested corresponding proteins. FPLC fractionation of the tryptic β -casein digest yielded three antioxidant fractions, but only one (peak 2) exhibited a higher effect than the digest. The synthesized peptides, mimicking the HPLC fractions, also showed inhibiting effects. However, only spps 4 was as active as the unfractionated digest (Table 2). Accordingly, the antioxidative feature was not lost with dephosphorylation or digestion of the proteins or with fractionation of the tryptic β -casein digest (as observed in the case of the enzymatically induced oxidation). In addition, the synthesized peptides (spps) were much more potent in preventing the chemically induced, rather than the enzymatically induced, oxidation of linoleate (RAA from 1.12 to 1.35, at the concentrations yielding the maximal activities; Table 2).

The dephosphorylated casein and β -casein molecules were better antioxidants than their respective native molecules. The removal of phosphate groups enhanced their hydrophobicity, presumably optimizing their interaction with the fatty acid intermediates. The HPLC-isolated peptides, as well as the synthesized ones, are composed of hydrophobic amino acids such as Val and Leu. The most effective peptides (spps 4 and 6) contain two hydrophobic residues at the N-terminal side, and the addition of a Lys (spps 5) at the N-terminal position of spps 6 dramatically reduced its activity (Table 2). Nevertheless, the factor of hydrophobicity, important for the accessibility to hydrophobic targets (Murase et al., 1993), does not fully explain the observed effects. A scale for hydrophobicity, built with the retention times obtained by RP-HPLC (Part 1), allows the classification of the peptides by decreasing hydrophobicity, spps 6 > spps 5 > spps 4 > spps 3, and to demonstrate that hydrophobicity was unrelated to activity, spps 4 > spps 6 > spps 5 \geq spps 3 (decreasing RAA). Therefore, a residue–activity relationship can be highlighted among the β -casein tryptic peptides (Table 1). Spps 4 (the most efficient) contains a Tyr residue, which is a potent hydrogen donor. β -Casein contains a high amount of proline residues (~16%), and the active peptides contain at least one proline (Table 1). The effects obtained with the β -casein peptides, although deprived of His, were in the same order of magnitude as His-containing peptides showing antioxidant properties, which were designed on the sequence of LLPHH from soybean β -conglycinin (Chen et al., 1998; PHH, 50 μ M, had the

highest RAA, 1.30). The undigested proteins and some digests yielded higher effects (RAA up to 1.76). However, our control peptides (His-containing or not) were not antioxidant, suggesting that the antioxidative property can indeed be characteristic of the amino acid sequence and/or the physicochemical property of the peptides. This is in agreement with previous observations that antioxidant peptides isolated or designed from soybean β -conglycinin contained both hydrophobic amino acids at the N-terminal side (Chen et al., 1995) and His and Pro residues (Chen et al., 1996, 1998).

AAPH catalyzes lipid oxidation via a free radical mechanism. According to Porter et al. (1980, 1981), the hydroperoxidation of linoleic acid initiated by an azo-compound is a two step reaction. An intermediate pentadienyl radical is formed first (as for the Lox-catalyzed oxidation) to which oxygen adds reversibly, resulting in cis-trans and trans-trans peroxy radicals (as opposed to Lox stereospecific product). The test compounds inhibited the reaction by decreasing the oxygen consumption rate, suggesting they were targeting an intermediate that had not yet undergone the oxygen attack. These and other data lead us to propose that caseins and casein-derived peptides are preferred targets for the free radical intermediates (presumably the carbon-centered ones) in comparison with the peroxidizable linoleic acid.

Antioxidative Activity of Caseins and Casein-Derived Peptides in the Redox (Hpode/Hb) Oxidation System. The activity of caseins and casein-derived peptides as antioxidants should be evaluated on the basis of both lipid and protein oxidation, because the two previous lipid oxidation catalysts (Lox and AAPH) produced free radicals. In the (Hpode/Hb) system used, the lipid hydroperoxide can react with hemoglobin to form a hemoglobin-derived peroxy radical, for example, an oxygen-centered radical. This intermediate has then the possibility to react either with LMB or with other oxidizable species present in the reaction medium. In the latter case, LMB is not oxidized into the colorful methylene blue derivative, and no color generation is observed. Kelman et al. (1994) demonstrated that glutathione, the tripeptide γ -Glu-Cys-Gly, was oxidized by the myoglobin-derived peroxy radical formed from the reaction of myoglobin with hydrogen peroxide, analogous to the reaction of hemoglobin with Hpode. The antioxidant capacity as well as the sensitivity of the compounds for being oxidized can then be indirectly evaluated by the (Hpode/Hb) system.

Caseins and casein-derived peptides (except spps 5) were able to quench the color generation. The magnitude of the quenching was dependent on the considered molecule. The quenching was proportional to the hydroperoxide concentration, in the range assayed: a linear relationship ($R > 0.97$; data not shown) was obtained when the absorbance values were plotted against increasing Hpode concentrations. An example of the curve patterns is given in Figure 2. The same feature was observed, as expected, with known antioxidants (propyl gallate, L-3,4-dihydroxyphenylalanine), and also with BSA, already described as an antioxidant, and Arg, an amino acid prone to oxidation. The amino acid residues Ile and Val were ineffective.

Caseins and casein-derived peptides, except the clostripain casein digest, the subtilisin β -casein digest, FPLC peaks 1 and 3, and spps 5 and 6, were better antioxidants than BSA (Table 2). The synthesized

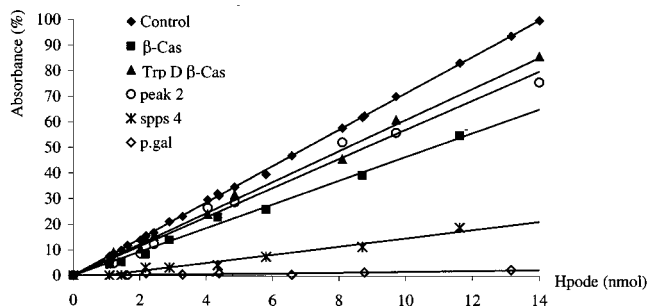


Figure 2. Effect of some molecules on color generation in the (Hpode/Hb) oxidation system (LMB-based methodology). For conditions used, see Materials and Methods. The standard deviation was <5%. The propyl gallate concentration was 100 μ M.

peptide spps 4 exhibited the highest activity (RAA = 4.22). The most potent antioxidant among the undigested proteins was the dephosphorylated α -casein. In this oxidation system, too, the activity of the test compounds was not solely related to phosphate groups. Digests and fractionated digests retained the property to inhibit the redox catalyst, in addition to the other oxidation catalysts, Lox and AAPH. Furthermore, the reactivity order of the unfractionated digests was exactly the same as that shown in the (LA/AAPH) oxidation system: casein tryptic digest \gg casein subtilisin digest \gg β -casein subtilisin digest $>$ β -casein tryptic digest \geq casein clostripain digest (decreasing RAA). The unfractionated digests of β -casein were more active than the undigested β -casein, but the digests of whole casein were less effective except the tryptic digest, which exhibited the same activity as whole casein.

Qualitatively, the nonenzymatic oxidation systems pointed at the same property: caseins and casein-derived peptides showed antioxidant activity and, thus, susceptibility to react with free radical intermediates, both carbon- and oxygen-centered. Certain types of caseins, digests, fractions, and especially spps 4 (Figure 2) were preferred targets for oxygen-centered radicals, considering the high ability of these compounds to quench the color formation in the (Hpode/Hb) oxidation system. This effect is most likely related to the molecule's primary structure, certain amino acids among a sequence being more sensitive to oxidation than others. Considering notably the structure-activity of the synthesized peptides (spps), Met, Tyr, and Arg can be highlighted as key residues. Moreover, compared to other proteins including BSA, caseins and therefore casein-derived peptides contain an unusually high amount of proline. The proline residues in collagen and other prolyl polypeptides were reported as target amino acids for oxidative damage in metal-catalyzed free radical generating systems, as well as His, Phe, Lys, and Arg residues, to a lesser extent (Uchida et al., 1992). Furthermore, spps 4 (AVPYPQR) was identified by Yang et al. (1997) as a specific site of oxidation in bovine β -casein by hypochlorite, and the tyrosyl residue was oxidized into the corresponding quinone methide. In addition, spps 3 probably owes its activity to the Met residue. This assumption is supported by the work of Yang et al. (1997), who showed that Met residues in β -casein were also preferred targets for oxidation. A selective process of oxidation, strongly related to the particular amino acid sequence of the proteins/peptides, can then be proposed to explain the discrepancies in the reactivity of caseins and casein-derived peptides.

Antiradical Activity of the Caseins and Casein-Derived Peptides against the Nitrogen-Centered Radical DPPH. The previous results demonstrated that caseins and casein-derived peptides were antioxidants. The antioxidative mechanism of antioxidants can result from metal chelation, free radical scavenging (hydrogen donating capability or free radical quenching), or cooperative effects of these properties. The ability of the test compounds to donate hydrogen was checked by using the stable free radical DPPH. In the presence of hydrogen donors, DPPH is reduced and a stable free radical is formed from the scavenger.

Significant radical trapping activities were obtained for only five proteins including BSA (Table 2), a known free radical scavenger (Kanner et al., 1987). None of the proteins was as effective as the commercial antioxidant propyl gallate (RSA = 3.58 at 500 μ M). Tyrosine and histidine residues were most likely responsible for the antiradical effect of the caseins. The dephosphorylated molecules of casein and β -casein (inactive at 1.2 g/L; Table 2) demonstrated scavenging properties at a higher concentration (2.4 g/L). Unfractionated and fractionated digests as well as synthesized peptides (β -casein peptides and control peptides) were not hydrogen donors at the concentration assayed. Therefore, the antioxidant effect of caseins and casein-derived peptides is not fully based on the capability to donate hydrogen. However, the reactivity of the nitrogen-centered DPPH radical and the free radicals (carbon- and/or oxygen-centered) generated by the three other lipid oxidation catalysts may strongly differ. The solubility of the radical can also play an important role. Dean et al. (1991) demonstrated that hydrophobic radicals were poorly able to attack proteins in solution (BSA) compared to aqueous radicals. The assumption that casein and casein-derived peptides can be attacked by certain hydrophilic radicals (AAPH-derived carbon-centered radicals or Hb-derived oxygen-centered radicals), and to a lesser extent by hydrophobic radicals (Lox-produced carbon-centered radicals), might be appropriate.

Chelating Activity of the Caseins and Casein-Derived Peptides on Fe^{2+} . The chelating activity of the compounds on Fe^{2+} was also determined because iron chelators could inhibit Lox- and metal-catalyzed lipid oxidation. Metals are well-known initiators of unwanted oxidative processes in food products.

Undigested caseins, digested caseins, and FPLC-fractionated digests of β -casein coordinated the free iron (Figure 3). Except for β -casein (Figure 3A) and FPLC peak 3 (Figure 3B), their chelating property diminished with increasing concentrations of iron, suggesting a saturation mechanism. β -Casein chelating properties were obviously due to its phosphorylated groups because the dephosphorylated molecule was not a chelator over the range studied (Figure 3A). Manson and Cannon (1978) and Emery (1992) have already described this characteristic of casein. The synthesized peptides (spps) were very weak chelators even at high concentration (500 μ M, data not shown). For an iron concentration of 0.025 g/L, the chelating effect was \sim 15% for spps 4 and 5 and \sim 7% for spps 3 and 6. However, metal-chelating properties are reported for amino acids and small peptides (Yee and Shipe, 1981; Yamashoji et al., 1977), but no dissociation constant values are available. The measured chelating activity of our synthesized peptides as determined by the formation of the iron-ferrozine

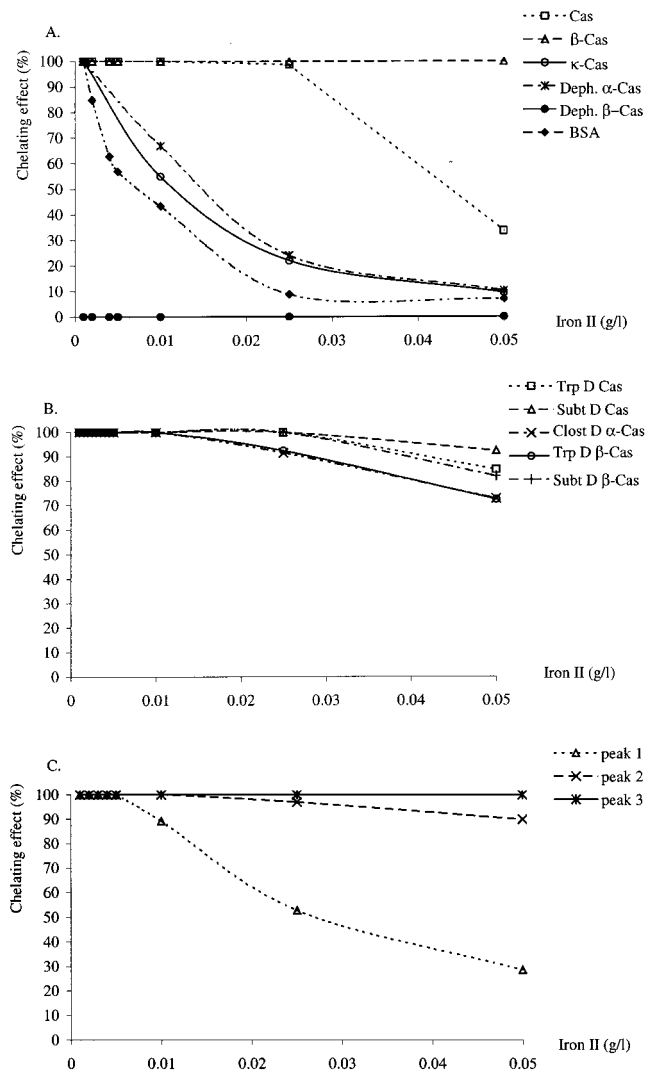


Figure 3. Chelating properties of caseins and casein-derived peptides on Fe²⁺: (A) undigested caseins and BSA; (B) unfractionated digests; (C) FPLC-fractionated peptides (tryptic digest β -casein). For conditions used, see Materials and Methods.

complex is perhaps underestimated because ferrozine could extract some iron in weaker complexes. As for the antiradical activities on DPPH, the iron-chelating activities of the test compounds were not proportional to their antioxidant or Lox-inhibitory activities. Therefore, the Lox inhibitory properties of the caseins and casein-derived peptides cannot be (totally) explained by the coordination of the iron in the Lox active site. The coordination of the iron in the active site of Lox would indeed interrupt the redox cycle between Fe²⁺ and Fe³⁺ (Figure 1), forming dead-end complexes (Summers et al., 1987) and inhibiting the reaction. However, it is not clear at present how the compounds could gain access to the iron atom and interact with it because the Lox metal is not free but partly coordinated by residues in the active site. The metal ion is generally believed to be in an octahedral environment, including three histidine and one asparagine side chain, the terminal carboxylate, and a water molecule (Minor et al., 1996). In conclusion, the Lox inhibitory and antioxidative properties of caseins and casein-derived peptides are probably not linked to a chelation property, the test compounds being able to inhibit non-metal-catalyzed fatty acid oxidation.

Hypotheses about the Action Mechanism of Caseins and Casein-Derived Peptides on Lox-Catalyzed Reaction. The caseins and casein-derived peptides decreased the oxygen consumption in both Lox- and AAPH-catalyzed oxidation, suggesting they were targets of carbon-centered radical(s) formed before oxygen entered the reaction. They were also sensitive to oxygen-centered radicals such as those generated in the (Hpode/Hb) oxidation system. The three lipid oxidation catalysts (including Lox) are susceptible to generate oxyl radicals. The Lox reaction is supposed to proceed with sequential addition of substrates, the fatty acid binding first and C–H bond cleavage occurring before oxygen enters the reaction (Prigge et al., 1997). By EPR experiments, Nelson et al. (1994) have detected both a linoleyl- and a peroxy-based radical, supporting the idea that C–H bond cleavages produce an organic radical that then reacts with molecular oxygen. In addition, the radical-producing mechanism for the Lox-catalyzed dioxygenation of polyunsaturated fatty acids is supported by many investigators (Chamulitrat and Mason, 1989; Nelson et al., 1994; Nelson and Cowling, 1990). The data obtained in the present study with the nonenzymatic lipid oxidation catalysts strongly suggest the ability of caseins and casein-derived peptides to scavenge free radicals, and this ability could account for their effect on the Lox reaction, giving indirect support to the radical mechanism. However, the relative antioxidative activities of the test compounds on the different lipid oxidation catalysts (Lox, AAPH, and Hb) were not strictly correlated, and no mathematical relationship could be proven. Nevertheless, the highest RAA values were obtained with the same compounds in the (LA/AAPH) and (Hpode/Hb) oxidation systems. Moreover, the Lox-catalyzed reactions were performed under initial rate conditions, assuming that <10% of Hpode (and then free radical intermediates) were formed. Accordingly, the lower effectiveness of the test compounds in the Lox system compared to the two other systems is perhaps due to a concentration factor. On the other hand, the Lox fatty acid radicals are normally bound to the enzyme, whereas the other free radical generating systems used in the present study produced unbound radicals. In addition, the radicals formed in the different oxidation systems may differ in hydrophobicity, stereochemistry, or reactivity, modifying the interaction with caseins and casein-derived peptides. Spss 4 (β -casein 177–183) was a strong antioxidant [RAA = 4.2 in the (Hpode/Hb) system, RAA = 1.35 in the (LA/AAPH) system] but almost inactive on Lox kinetics (RAA = 1.02). Other compounds such as vitamin E and probucol are reported to be antioxidants in the (Hpode/Hb) system without being significant Lox inhibitors (Auerbach et al., 1992). Nevertheless, most of the caseins and casein-derived peptides were both antioxidants and inhibitors and were presumably oxidized by free radical intermediate(s) formed during the course of the Lox-catalyzed reaction. Although structurally unrelated, antioxidants such as nordihydroguaiaretic acid (NDGA) or quercetin are also Lox inhibitors and are transformed into oxidation metabolites in the Lox reaction (Van der Zee et al., 1989, for NDGA; Takahama, 1985, for quercetin). A structurally related compound, carnosin, the dipeptide β -Ala-His, was shown to exhibit both antioxidant and lipoxygenase inhibitory properties (Decker and Faraji, 1990). Its effectiveness was not correlated in the different oxidation systems

used. Its capacity to scavenge hydroxyl radicals, demonstrated in the autoxidation process of linoleic acid (Chan and Decker, 1994), could account for its effect on Lox reaction.

Therefore, we propose that caseins and casein-derived peptides interact with free radical intermediate(s) in the Lox reaction, resulting in the observed inhibition and in the oxidation of specific amino acid residues within the sequence. The sequence-specific reactivity of the test compounds, as previously described, is supported by the literature (Tannenbaum et al., 1969; Byczkowski et al., 1991; Uchida and Kawakishi, 1992; Murase et al., 1993; Chen et al., 1996; Hatate, 1998). Furthermore, the interaction between lipid radicals and proteins can lead to the formation of protein radicals (Davies et al., 1995) and further to fragmentation products (Hunt et al., 1988; Uchida and Kawakishi, 1986, 1988). The iron in the Lox active site could become the target of such protein-derived radicals, although it is not immediately apparent how caseins could navigate the narrow channel in the published structure of Lox (Minor et al., 1996). In the radical and σ -organoiron mechanisms, the iron oscillates from two oxidation states, between Fe^{3+} (active enzyme) and Fe^{2+} (inactive enzyme) during the catalytic cycle. Both mechanisms are based on the "product activation model" (Schilstra et al., 1994) in which Hpode is required to activate the enzyme by oxidizing the ferrous ion to the ferric ion. Provided that the assumption that the compounds are degraded into free radical metabolites is correct, caseins and casein-derived peptides may inhibit Lox according to the one-electron-reducing mechanism proposed by Nelson et al. (1991). On the other hand, the sole subtraction of Hpode from the reaction medium, as a result of the scavenging of intermediate(s) by the inhibitors, would prevent the active ferric Lox from being regenerated in the product activation model.

Conclusions. Casein and casein-derived peptides inhibited enzymatic and nonenzymatic lipid peroxidation, most likely by being a preferred target over fatty acid free radical intermediate(s). Indirect evidence led us to suggest that they can be oxidized during the process, according to a site- or sequence-specific mechanism. The use of proteins and peptides such as caseins and casein-derived peptides as antioxidants should therefore be evaluated on the basis of both lipid and protein oxidation. One can suppose that proteins and peptides can, together with the fatty acid, be the target of oxidative degradations and either initiate further unwanted radical-mediated side reactions or terminate chain reactions depending on the stability of the resulting protein/peptide radical.

ABBREVIATIONS USED

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; BSA, bovine serum albumin; Clost, clostripain; D, digest; Deph, dephosphorylated; DOPA, L-3,4-dihydroxyphenylalanine; DPPH radical, 1,1-diphenyl-2-picrylhydrazyl; EPR, electron paramagnetic resonance; ferrozine, (3,12-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; Fmoc, fluorenylmethoxycarbonyl; Hb, hemoglobin; hplc, RP-HPLC-fractionated peptides; Hpode, linoleic acid hydroperoxide [13(S),9Z,11E]-hydroperoxyoctadecadienoic acid; LA, linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid); LMB, leucomethylene blue derivative [10-benzoyl-3,7-bis(dimethylamino)-10H-phenothiazine]; Lox, lipoxygenase (EC 1.13.11.12); ND, not

determined; NDGA, nordihydroguaiaretic acid; RAA, relative antioxidant activity; RSA, relative scavenging activity; spps, hplc-mimicking peptides, synthesized by solid-phase peptide synthesis; Subt, subtilisin; Trp, trypsin.

ACKNOWLEDGMENT

We thank E. J. M. van Kan for performing the peptide synthesis and T. A. Richey for revising the English of the document.

LITERATURE CITED

- Auerbach, B. J.; Kiely, J. S.; Cornicelli, J. A. A spectrophotometric microtiter-based assay for the detection of hydroperoxy derivatives of linoleic acid. *Anal. Biochem.* **1992**, *201*, 375–380.
- Brash, A. R. Lipoxygenases: Occurrence, functions, catalysis, and acquisition of substrate. *J. Biol. Chem.* **1999**, *274*, 23679–23682.
- Byczkowski, J. Z.; Ramgoolie, P. J.; Kulkarni, A. P. Proline hydroxylation by soybean lipoxygenase. *Biochem. Int.* **1991**, *25*, 639–646.
- Chamulitrat, W.; Mason, R. P. Lipid peroxy radical intermediates in the peroxidation of polyunsaturated fatty acids by lipoxygenase. Direct electron spin resonance investigations. *J. Biol. Chem.* **1989**, *264*, 20968–20973.
- Chan, H. W.-S. Soy-bean lipoxygenase: an iron-containing dioxygenase. *Biochim. Biophys. Acta* **1973**, *327*, 32–35.
- Chan, K. M.; Decker, E. A. Endogenous skeletal muscle antioxidants. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 403–426.
- Chen, H.-M.; Muramoto, K.; Yamauchi, F. Structural analysis of antioxidative peptides from soybean β -Conglycinin. *J. Agric. Food Chem.* **1995**, *43*, 574–578.
- Chen, H.-M.; Muramoto, K.; Yamauchi, F.; Nokihara, K. J. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food Chem.* **1996**, *44*, 2619–2623.
- Chen, H.-M.; Muramoto, K.; Yamauchi, F.; Fujimoto, K.; Nokihara, K. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J. Agric. Food Chem.* **1998**, *46*, 49–53.
- Corey, E. J.; Nagata, R. Evidence in favor of an organoiron-mediated pathway for lipoxygenation of fatty acids by soybean lipoxygenase. *J. Am. Chem. Soc.* **1987**, *109*, 8107–8108.
- Davies, M. J.; Fu, S.; Dean R. T. Protein hydroperoxides can give rise to reactive free radicals. *Biochem. J.* **1995**, *305*, 643–649.
- Dean, R. T.; Hunt, J. V.; Grant, A. J.; Yamamoto, Y.; Niki, E. Free radical damage to proteins: The influence of relative delocalization of radical generation, antioxidants, and target proteins. *Free Radical Biol. Med.* **1991**, *11*, 161–168.
- Decker, E. A.; Faraji, H. Inhibition of lipid oxidation by carnosine. *J. Am. Oil Chem. Soc.* **1990**, *67*, 650–652.
- Decker, E. A.; Welch, B. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.* **1990**, *38*, 674–677.
- de Groot, J. J. M. C.; Veldink, G. A.; Vliegthart, J. F. G.; Boldingh, J.; Wever, R.; Van Gelder, B. F. Demonstration by EPR spectroscopy of the functional role of iron in soybean 1-lipoxygenase. *Biochim. Biophys. Acta* **1975**, *377*, 71–79.
- Emery, T. Iron oxidation by casein. *Biochem. Biophys. Res. Commun.* **1992**, *182*, 1047–1052.
- Feussner, E.; Wasternack, C. Lipoxygenase catalyzed oxidation of lipids. *Fett/Lipid* **1998**, *100*, 146–152.
- Galey, J.-B.; Bombard, S.; Chopard, C.; Girerd, J.-J.; Lederer, F.; Thang, D.-C.; Nam, N.-H.; Mansuy, D.; Chottard, J.-C. Hexanal phenylhydrazine is a mechanism-based inactivator of soybean lipoxygenase 1. *Biochemistry* **1988**, *27*, 1058–1066.

- Glickman, M. H.; Klinman, J. P. Nature of rate-limiting steps in the soybean lipoxygenase-1 reaction. *Biochemistry* **1995**, *34*, 14077–14092.
- Hataate, H. Isolation of an active peptide fragment from human serum albumin and its synergism with α -tocopherol. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1135–1139.
- Hunt, J. V.; Simpson, J. A.; Dean, R. T. Hydroperoxide-mediated fragmentation of proteins. *Biochem. J.* **1988**, *250*, 87–93.
- Kanner, J.; German, J. B.; Kinsella, J. E. Initiation of lipid peroxidation in biological systems. *CRC Crit. Rev. Food Sci. Nutr.* **1987**, *25*, 317–364.
- Kelman, D. J.; DeGray, J. A.; Mason, R. P. Reaction of myoglobin with hydrogen peroxide forms a peroxy radical which oxidizes substrate. *J. Biol. Chem.* **1994**, *269*, 7458–7463.
- Manson, W.; Cannon, J. The reaction of α_{S1} - and β -casein with ferrous ion in the presence of oxygen. *J. Dairy Res.* **1978**, *45*, 59–67.
- Minor, W.; Steczko, J.; Stec, B.; Otwinowski, Z.; Bolin, J. T.; Walter, R.; Axelrod, B. Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. *Biochemistry* **1996**, *35*, 10687–10701.
- Murase, H.; Nagao, A.; Terao, J. Antioxidant and emulsifying activity of *N*-(long-chain-acyl)histidine and *N*-(long-chain-acyl)carnosine. *J. Agric. Food Chem.* **1993**, *41*, 1601–1604.
- Nelson, M. J.; Cowling, R. A. Observation of a peroxy radical in samples of “purple” lipoxygenase. *J. Am. Chem. Soc.* **1990**, *112*, 2820–2821.
- Nelson, M. J.; Seitz, S. P. The structure and function of lipoxygenase. *Curr. Opin. Struct. Biol.* **1994**, *4*, 878–884.
- Nelson, M. J.; Batt, D. G.; Thompson, J. S.; Wright, S. W. Reduction of the active-site iron by potent inhibitors of lipoxygenases. *J. Biol. Chem.* **1991**, *266*, 8225–8229.
- Nelson, M. J.; Cowling, R. A.; Seitz, S. P. Structural characterization of alkyl and peroxy radicals in solutions of purple lipoxygenase. *Biochemistry* **1994**, *33*, 4966–4973.
- Ohishi, N.; Ohkawa, H.; Miike, A.; Tatano, T.; Yagi, K. A new assay method for lipid peroxides using a methylene blue derivative. *Biochem. Int.* **1985**, *10*, 205–211.
- Pistorius, E. K.; Axelrod, B. Iron, an essential component of lipoxygenase. *J. Biol. Chem.* **1974**, *249*, 3183–3186.
- Porter, N. A.; Weber, B. A.; Weenen, H.; Khan, J. A. Autoxidation of polyunsaturated lipids. Factors controlling the stereochemistry of product hydroperoxides. *J. Am. Chem. Soc.* **1980**, *102*, 5597–5601.
- Porter, N. A.; Lehman, L. S.; Weber, B. A.; Smith, K. J. Unified mechanism for polyunsaturated fatty acid autoxidation. Competition of peroxy radical hydrogen atom abstraction, β -scission, and cyclization. *J. Am. Chem. Soc.* **1981**, *103*, 6447–6455.
- Prigge, S. T.; Boyington, J. C.; Faig, M.; Doctor, K. S.; Gaffney, B. J.; Amzel, L. M. Structure and mechanism of lipoxygenases. *Biochimie* **1997**, *79*, 629–636.
- Rival, S. G.; Fornaroli, S.; Boeriu, C. G.; Wichers, H. J. Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *J. Agric. Food Chem.* **2000**, *48*, 287–294.
- Schilstra, M. J.; Veldink, G. A.; Verhagen, J.; Vliegthart, J. F. G. Effect of lipid hydroperoxide on lipoxygenase kinetics. *Biochemistry* **1992**, *31*, 7692–7699.
- Schilstra, M. J.; Veldink, G. A.; Vliegthart, J. F. G. The dioxygenation rate in lipoxygenase catalysis is determined by the amount of iron(III) lipoxygenase in solution. *Biochemistry* **1994**, *33*, 3974–3979.
- Schlimme, E.; Meisel, H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **1995**, *39*, 1–20.
- Siedow, J. N. Plant lipoxygenase: structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145–188.
- Summers, J. B.; Gunn, B. P.; Mazdiyasn, H.; Goetz, A. M.; Young, P. R.; Bouska, J. B.; Dyer, R. D.; Brooks, D. W.; Carter, G. W. In vivo characterization of hydroxamic acid inhibitors of 5-lipoxygenase. *J. Med. Chem.* **1987**, *30*, 2121–2126.
- Takahama, U. Inhibition of soybean lipoxygenase-dependent oxidation by quercetin: mechanism of antioxidative function. *Phytochemistry* **1985**, *24*, 1443–1446.
- Tannenbaum, S. R.; Barth, H.; LeRoux, J. P. Loss of methionine in casein during storage with autoxidizing methyl linoleate. *J. Agric. Food Chem.* **1969**, *17*, 1353.
- Uchida, K.; Kawakishi, S. Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system. *Biochem. Biophys. Res. Commun.* **1986**, *138*, 659–665.
- Uchida, K.; Kawakishi, S. Selective oxidation of tryptophan and histidine residues in protein through the copper-catalyzed autoxidation of L-ascorbic acid. *Agric. Biol. Chem.* **1988**, *52*, 529–535.
- Uchida, K.; Kawakishi, S. Sequence-dependent reactivity of histidine-containing peptides with copper(II)/ascorbate. *J. Agric. Food Chem.* **1992**, *40*, 13–16.
- Uchida, K.; Kato, Y.; Kawakishi, S. Metal-catalyzed oxidative degradation of collagen. *J. Agric. Food Chem.* **1992**, *40*, 9–12.
- Van der Zee, J.; Eling, T. E.; Mason, R. P. Formation of free radical metabolites in the reaction between soybean lipoxygenase and its inhibitors. An ESR study. *Biochemistry* **1989**, *28*, 8363–8367.
- Yagi, K.; Kiuchi, K.; Saito, Y.; Miike, A.; Kayahara, N.; Tatano, T.; Ohishi, N. Use of a new methylene blue derivative for determination of lipid peroxides in foods. *Biochem. Int.* **1986**, *12*, 367–371.
- Yamashoji, S.; Yoshida, H.; Kajimoto, G. Studies on the metal-protein complex. II. Effect of ionic properties of polypeptides on metal-catalyzed fatty acid oxidation. *J. Jpn. Oil Chem. Soc.* **1977**, *26*, 433–435.
- Yang, C.-Y.; Gu, Z.-W.; Yang, H.-X.; Yang, M.; Wiseman, W. S.; Rogers, L. K.; Welty, S. E.; Viswanatham, K.; Rohde, M. F.; Smith, C. V. Oxidation of bovine β -casein by hypochlorite. *Free Radical Biol. Med.* **1997**, *22*, 1235–1240.
- Yee, J. J.; Shipe, W. F. Using enzymatic proteolysis to reduce copper-protein catalysis of lipid oxidation. *J. Food Sci.* **1981**, *46*, 966–967.
- Yen, G.-C.; Wu, J.-Y. Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. *Food Chem.* **1999**, *65*, 373–379.

Received for review March 24, 2000. Revised manuscript received September 19, 2000. Accepted September 21, 2000. This research was supported by a grant from the European Union, Agriculture and Fisheries Program (Contract FAIR-98-5005).

JF0003911