Caseins and Casein Hydrolysates. 1. Lipoxygenase Inhibitory Properties

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Whole casein from bovine origin, the different casein subtypes α, *β*, and *κ*, and the related dephosphorylated proteins were assayed as modulators of soybean lipoxygenase 1 activity and were found to inhibit it. To define the lipoxygenase inhibitory domain, whole casein and *β*-casein were digested by proteases (trypsin, clostripain, and subtilisin). The *β*-casein tryptic digest and the tryptic and subtilisin digests of whole casein retained their inhibitory properties. The tryptic *β*-casein digest was the most potent inhibitor of lipoxygenase activity and was further fractionated by FPLC or HPLC. The collected peptides inhibited the lipoxygenase-catalyzed reaction to different extents. The active fractions were analyzed by ESI-MS, and the sequences of several lipoxygenase inhibitory peptides, corresponding mainly to the C-terminal moiety of *β*-casein, were identified.

Keywords: *Soybean lipoxygenase 1; Lox-1 inhibitory β-casein peptides; FPLC; RP-HPLC; ESI-MS*

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) is a key enzyme in polyunsaturated fatty acid metabolism (Samuelsson et al., 1987) and is found in both the plant and animal kingdoms. Lipoxygenase (Lox) is a nonheme iron-containing enzyme (Chan, 1973; Pistorius and Axelrod, 1974) that carries out the stereo- and regiospecific conversion of 1,4-cis,cis double bonds in unsaturated fatty acids to chiral (cis, trans) conjugated dienoic monohydroperoxides (Brash, 1999). The most extensively studied form of the enzyme, which was used in this study, is the isoenzyme 1 from soybeans. This enzyme shows a strong preference for oxygenation of the substrate (linoleate) at the *ω*−6 position when the reaction is carried out at pH 9 (Hamberg and Samuelsson, 1967) and produces 13-hydroperoxy-9,11-octadecadienoic acid (Hpode). In plants, Lox-derived hydroperoxy fatty acids are further metabolized by several enzymes of the Lox pathway (Blée, 1996; Hamberg, 1995). In foods, lipid oxidation is a major cause of chemical spoilage, and hydroperoxides are known to be potentially reactive to cause deterioration of proteins or amino acids (Gardner, 1979). Moreover, lipid hydroperoxides can decompose to form alkoxyl and peroxyl radicals, which participate in chain propagation reactions, or a great variety of aldehydes following *β*-cleavage of the lipid alkoxyl radicals (Gardner, 1987; Grosch, 1987).

Considerable research effort has therefore been devoted to optimizing methods to prevent lipid oxidation and to inactivate catalysts such as Lox. Blanching of food products is the main common process, but it may also adversely affect the flavor of food products. In model systems, lipoxygenase activity is inhibited by various antioxidants (O'Connor and O'Brien, 1991), by substrate analogues (Corey et al., 1986; Galey et al., 1988; Clapp et al., 1995), and by product analogues (Funk and Alteneder, 1983). However, a limited number of safe inhibitors remain when one is dealing with plant materials intended for human consumption (Schuler, 1990). While screening biological fluids for their activity toward Lox, Laakso and Lilius (1982) found that milk and milk products were able to inhibit Lox. Further experiments showed that casein, a mixture of proteins of relatively low molecular weight (20−24 kDa), was the compound responsible for this inhibition.

Casein consists of four principal primary proteins, α_{S1}-, α_{S2}-, *β*-, and *κ*-casein, in the ratio 38:11:38:13 in bovine milk. The four main proteins not only differ in amino acid sequence and length but also vary in degree of phosphorylation and glycosylation, disulfide-linked polymerization, and genetic polymorphism (Fiat and Jollès, 1989). Phosphate groups are esterified as serine monoesters. *κ*-Casein is the only glycosylated casein, probably arranged as tri- or tetrasaccharides linked to threonine or serine. α_{S1}- and *β*-casein do not contain any cysteinyl residues, making sulfhydryl−disulfide cross-linking impossible (Swaisgood, 1993). The caseins are strongly hydrophobic in the order $\beta > \kappa > \alpha_{S1} > \alpha_{S2}$. Hydrophobic and hydrophilic residues are not uniformly distributed throughout the sequences, making the structure highly amphipathic. Phosphoseryl residues tend to cluster, which has a major influence on the metal-binding properties.

The aim of this study was first to evaluate the effects of various types of bovine casein on the activity of lipoxygenase in vitro. The second step focused on the identification of the domain within the protein responsible for the inhibitory activity. To define this lipoxygenase inhibitory domain or sequence, whole casein and *β*-casein were enzymatically hydrolyzed using trypsin, subtilisin, and clostripain. Some of the obtained digests retained their lipoxygenase inhibitory properties, the *β*-casein tryptic digest being the most potent. The latter hydrolysate was submitted to further separation by FPLC or RP-HPLC. The collected peptides were assayed as potential Lox inhibitors, and the active ones were identified by ESI-MS.

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MATERIALS AND METHODS

Materials. The various types of bovine casein, except β-casein, and bovine serum albumin (BSA; hydroperoxide- and fatty acid-free) 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 A^1 and A^2. All of the proteins were of analytical grade. Linoleic acid (~99%) and the enzymes trypsin (EC 3.4.21.4), subtilisin (EC 3.4.21.62), clostripain (EC 3.4.22.8), and soybean lipoxygenase 1 (EC 1.3.11.12) were purchased from Sigma-Aldrich. Side-chain-protected amino acid derivatives, in the L configuration, resins for peptide assembly, and coupling reagents were obtained from Novabiochem (Läufelfingen, Switzerland). All other reagents were of analytical grade from Biosolve (Valkenswaard, The Netherlands).

Hydrolysis of Sodium Caseinate and β-Casein by Proteases. The proteolyses were performed at room temperature with magnetic stirring. The proteins (25 g/L) were dissolved in 50 mM Tris-HCl buffer, pH 7.8, 10 mM $CaCl_2$ for the tryptic digestions or in 50 mM Tris-HCl buffer for the subtilisin (pH 7.8) and the clostripain (pH 7.4) digestions. The reactions were started by the addition of the enzyme (2 mg/L). The extent of the reactions was monitored by electrophoresis (SDS−PAGE) on the PhastSystem (Pharmacia-LKB, Uppsala, Sweden). The reactions were stopped by thermal inactivation when the undigested substrate was not detected further (24−28 h of hydrolysis, data not shown). Sodium caseinate was not completely digested at the chosen concentration of clostripain; the reaction medium was therefore ultrafiltrated to remove the undigested substrate (membrane PM 30 from Amicon, Inc., Beverly, MA). The digestion media were then freeze-dried.

Fractionation of the Peptides from the Tryptic β-Casein Digest. *Fractionation Using Ion Exchange Chromatography.* The FPLC system was from Pharmacia-LKB: Pharmacia-LKB controller LCC-500 Plus, high-precision pump P-500, monitor UV-M 280 nm, recorder REC-481, and FRAC-100 collector. The fractionation was performed at 4 °C. The freeze-dried sample (tryptic digest of β-casein) was dissolved in 20 mM NH_4HCO_3, pH 7.9 (buffer A), and filtered through a 22 μm syringe filter. The solution was applied to a Resource Q 1 mL anion exchange column (Pharmacia-LKB) and then eluted with a gradient at a flow rate of 1 mL/min. The injection volume was 500 μL. The gradient was as follows: 0% B in 2 column volumes (CV); 0−10% B in 2 CV; 10−11% B in 4 CV; 11−50% B in 2 CV; 50−100% B in 3 CV. Buffer B was 20 mM NH_4HCO_3, pH 7.9, 1 M NaCl. The eluent was monitored at 280 nm, and peaks were collected. Several runs were performed to collect sufficient material and were subsequently freeze-dried. The freeze-dried samples were assayed as Lox inhibitors, and the active samples were analyzed by ESI-MS.

Fractionation Using RP-HPLC. The HPLC system was from Pharmacia-LKB: Pharmacia-LKB VWM 2141 UV lamp, LKB gradient pump 2249, gradient mixer and controller. The fractionation was performed at room temperature. The RP-HPLC sample was prepared as follows. The freeze-dried sample (tryptic digest of β-casein) was diluted with Milli-Q water (12.5 g/L final concentration) and submitted to a 50% ultrafiltration using a YM1 membrane from Amicon (molecular weight cutoff of 1000 Da). The retentate was rediluted with Milli-Q water and submitted to a second 50% ultrafiltration. The retentate (twice concentrated) was centrifuged for 20 min at 10000 rpm to remove insoluble materials. The supernatant was precipitated with 0.1% trifluoroacetic acid (TFA). The acidic mixture was finally centrifuged for 20 min at 10000 rpm. The supernatant, containing TFA-soluble peptides, was then injected into the column. The conditions were as follows: HS HYPER PREP 100 BDS C-18 column (8 μm, 250 × 22 mm) and C-18 guard column from Alltech Associates Inc. (Breda, The Netherlands), flow rate = 5 mL/min, injection volume = 2 mL, detection at 214 nm. The peptides were eluted from the column using an isocratic elution followed by a gradient of acetonitrile (ACN) in acidic water: 0−40 min, 0% B; 40−100 min, 0−100% B. Eluent A was 85:15:0.1 (H_2O/ACN/TFA, v/v/v) and eluent B was 5:95:0.1 (H_2O/ACN/TFA, v/v/v). Several runs were performed to collect adequate material and were then dried by evaporation at reduced pressure (rotary evaporator, water bath set at 40 °C). The samples resulting from the HPLC separation were assayed as Lox inhibitors, and the active fractions were sequenced by ESI-MS^n. An analytical RP-HPLC column, HYPERSIL BDS C-18 (5 μm, 250 × 4.6 mm), from Alltech, was also used to authenticate, by cochromatography, the sequenced and the synthesized peptides. The purified and synthesized peptides, both dissolved in 100 μL of acidic water (0.1% TFA, eluent A), were eluted using a linear gradient of ACN in acidic water (5:95:0.1, H_2O/ACN/TFA, v/v/v; eluent B) with a flow rate of 1 mL/min. The gradient was as follows: 0−1 min, 0% B; 1−31 min, 0−100% B. The peptides were detected at 214 nm.

ESI-MS Analysis of the Fractionated Peptides from the Tryptic β-Casein Digest. Electrospray mass spectrometry (ESI-MS) analyses were performed on an LCQ mass spectrometer equipped with electrospray interface (Thermoquest, San Jose, CA). The samples (dried collected fractions) were dissolved in 200 μL of MeOH/H_2O/TFA (50:50:0.05, v/v/v) and injected into the ion source at a flow rate of 3 μL/min. Full-scan mass spectra were recorded at mass-to-charge ratios (m/z) from 50 to 1999. The first MS (MS^1) generated and selected the parent ion (singly charged ion or $[M + H]^+$) of the peptide, which was subsequently fragmented (MS^n). The sequence-specific product ions were then mass analyzed in the second mass spectrometer (MS^2), and the collision-induced decomposition (CID) spectrum of the precursor was recorded. MS^n analyses were performed to sequence the peptide(s) contained in the HPLC samples, but only full-scan MS (MS^1) analyses were realized for the FPLC samples.

Solid-Phase Peptide Synthesis. A multiple-peptide synthesizer was used (AMS 422 multiple peptide synthesizer, ABIMED, Langenfeld, Germany). Peptides were prepared by the fluorenylmethoxycarbonyl (Fmoc) strategy with the phosphonium reagent PyBop [benzotriazole-1-yloxytris(pyrrolidinophosphonium) hexafluorophosphate] for carboxyl activation and preloaded Wang resins: Fmoc-Lys(Boc)-Wang resin, Fmoc-Arg(Pmc)-Wang resin for peptide-assembly. The following N-protected amino acid derivatives were used: Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)OH, Fmoc-Tyr(tBu)OH, Fmoc-Arg(Pbf)-OH (see Abbreviations Used). The calculations were made on the basis of an amount of 25 μmol of peptide, the excess in amino acids being 4. The cleavage was performed in 90% TFA/7% triisopropylsilane/3% H_2O.

Measurement for Inhibition Activity of Caseins and Casein-Derived Peptides against Lox-Catalyzed Linoleate Oxidation (Initial Rate Kinetics). Lipoxygenase activity was determined at 30 °C as the rate of oxygen consumption in a magnetically stirred reaction mixture, using a biological oxygen monitor (Clark type electrode) YSI model 5300 (Yellow Springs Instrument, Yellow Springs, OH). A stock 32 mM linoleic acid (LA) solution was prepared with Tween 20 (1%) according to the method of Grossman and Zakut (1979) and kept frozen until use. In the Lox assays, the final percentage of Tween 20 (0.05%) and the ionic strength were kept constant, independent of the substrate concentration used. The standard reaction medium was 150 μL of LA diluted stock solution in air-saturated 0.1 M sodium borate buffer, pH 9.0 (or in solutions containing the test compounds), for a total volume of 3.0 mL. The test compounds were dissolved in the same buffer (1.7 g/L in the case of the undigested and digested caseins). In the case of the HPLC-collected samples, the dried samples were resolubilized with the minimum volume of 0.1 M ammonium hydroxide to adjust the pH at 9.0. The volume of the fractions was then reduced to ~1 mL by a stream of nitrogen. The HPLC samples (100 μL) or 0.1 M ammonium hydroxide (100 μL, control) were then added to the standard medium. The reaction was started by injection of enzyme solution (6−12 nM). The reaction rate was determined from the linear phase (initial rate conditions). The results (mean

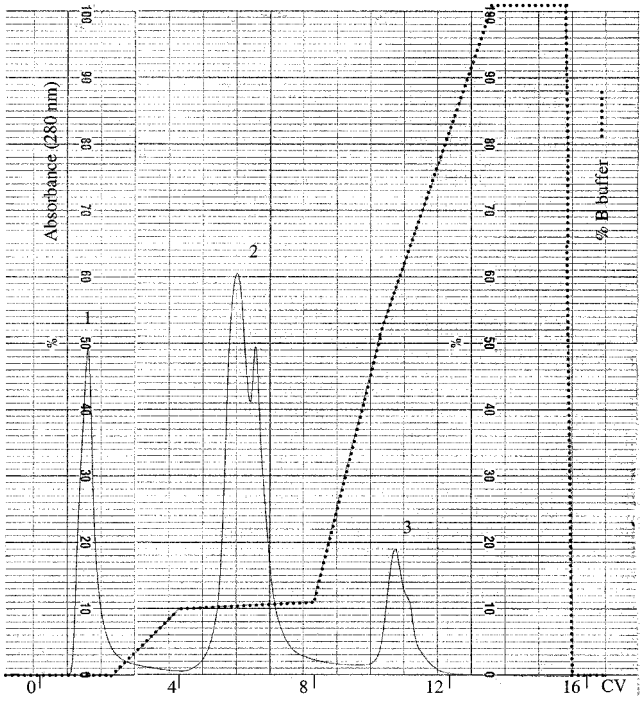

Figure 1. FPLC chromatogram (Mono Q column) of the tryptic digest of β-casein. CV: column volume (mL). For conditions used, see Materials and Methods.

value of three experiments) are expressed as residual activities (initial rate in the test sample/initial rate in the control) × 100.

RESULTS AND DISCUSSION

Proteolysis of Sodium Caseinate and β-Casein. The proteases used have different specificities. Trypsin is arginine- and lysine-specific, clostripain is strictly arginine-specific, and subtilisin has a broad specificity toward neutral and acidic amino acids (Keil, 1992). The β-casein molecule contains 4 arginine and 11 lysine residues, α-casein is characterized by a high number of acidic residues, and κ-casein is an amphipatic molecule (Fiat and Jollès, 1989). As a consequence, fragments of different sizes and properties were obtained. Moreover, peptides arising from clostripain digestion include, as a part of their amino acid sequence, the sequence of tryptic casein peptides. According to the patterns of SDS−PAGE (data not shown), clostripain digests contained the larger fragments (apparent molecular weight around 20000 Da), as expected due to the narrow specificity of the enzyme. Subtilisin digests comprised many small fragments (bands near 1700 and 2500 Da). The trypsin digests included peptides of intermediate molecular weights (bands around 1700 and 2500 Da and between 6200 to 8150 Da).

Fractionation of the Peptides from the Tryptic β-Casein Digest. *Fractionation According to pI*: *Anion Exchange Chromatography*. Because most of the expected tryptic peptides of β-casein have a p*I* <8 (Nau et al., 1993, 1995; Leadbeater and Ward, 1987), anion exchange chromatography with a buffer of pH 7.9 was chosen. The tryptic digest of β-casein was separated into three peaks (the second one with a shoulder), eluting at NaCl concentrations of 0, 10, and ∼40% (Figure 1). The elution profile was highly reproducible. The first peak (peak 1), which eluted before application of the salt gradient, contained peptides of p*I* >8. Two groups of peptides (peaks 2 and 3) were clearly individualized with the course of the gradient. Peak 2 included peptides with a p*I* of less than or around 8, whereas peak 3 was formed of peptides with an acidic p*I*. The second and third peak collections were desalted using ultrafiltration (membrane of molecular weight cutoff 1000 Da, retentate kept) before being freeze-dried. FPLC peaks 1−3 were assayed as Lox inhibitors. FPLC peaks 1 and 2, with inhibiting activity, were submitted to MS analysis.

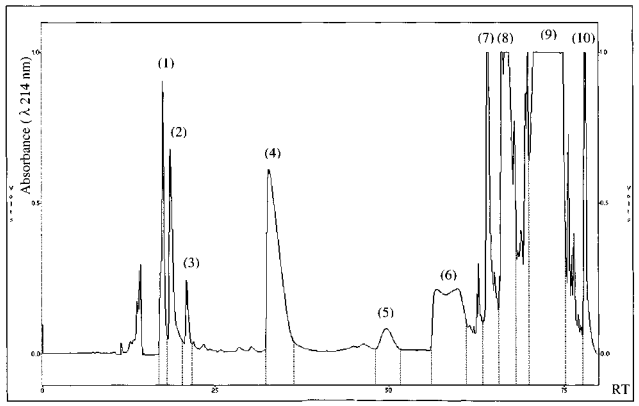

Figure 2. RP-HPLC chromatogram (preparative column) of the tryptic digest of β-casein. The retention times (RT, in minutes) of the collected fractions were (1) 17.7, (2) 18.6, (3) 20.9, (4) 32.7, (5) 49.7, (6) 58.0, (7) 64.3, (8) 66.7, (9) 72.8, and (10) 78. For conditions used, see Materials and Methods.

Fractionation According to Hydrophobicity: *RP-HPLC*. β-Casein is rated among the most hydrophobic proteins, and its digestion can release even more hydrophobic fragments (Léonil et al., 1988). RP-HPLC with gradient elution was therefore chosen to fractionate the tryptic digest of β-casein. Several runs, exhibiting high separation reproducibility (retention time, area, and peak resolution), were performed, and ∼200 mg (in total) of β-casein tryptic digest was loaded onto the preparative column. Ten fractions (hereafter called hplc 1 to hplc 10) were collected, with retention times (RT) ranging from 17.7 to 78.0 min (Figure 2). Their activity against the Lox-catalyzed reaction was determined. Fractions hplc 3−6, which showed Lox-inhibiting effects, were submitted to MS^n analysis.

Identification of β-Casein Peptides in the Collected Fractions According to Their Molecular Weight: ESI-MS. The collected fractions (FPLC, HPLC) were at first submitted to MS in the single mode, and full mass spectra were recorded. Fragmentation of peptides in the ion source is for all practical purposes nonexistent with electrospray ionization. All ions observed in the mass spectrum (MS^1) can therefore be attributed to molecular ions or adducts and their different charged states (Covey et al., 1991). The peptides were indeed detected as single ions $[M + H]^+$ (masses <1000 Da), as doubly charged ions $[M + 2H]^{2+}$, and as adducts $[M + Na]^+$, $[M...H_3PO_4 + H]^+$ (data not shown). Tryptic peptides of masses <∼1000 Da usually show abundant singly charged as well as doubly charged ions on the basis of the characteristic hydrolysis products of trypsin (Covey et al., 1988). Thus, individual components of a mixture may be distinguished and their molecular weight determined, although their actual identification may require additional information. The proposed sequences (Table 1) are based on the molecular weight of the most probable peptides resulting from the tryptic digestion of β-casein (variants A^1 and A^2) and,

Table 1. Results of ESI-MS Analyses of the Active Fractions, Obtained after Fractionation of the β-Casein Tryptic Digest by FPLC or RP-HPLC

fraction	$[M + H]^+$ obsd	relative abundance (%)	$[M + H]^+$ calcd	β-casein residues	theor p*I*[j]
peak 1	802.5	100	802.5	134−140[a]	ND
	764.5	80	764.4	108−113[b]	ND
	824.6	65	824.4	120−125[c]	ND
	780.5	55	780.5	170−176	10.10
	830.5	44	830.5	177−183	9.80
	786.5	35	788.4	106−111[d]	ND
	874.5	20	873.5	98−105[e]	9.89
	742.5	18	742.5	203−209[f]	6.05
peak 2	786.5	100	788.3	106−111[g]	ND
	887.4	29	ND	ND	ND
	1101.7	21	1101.4	164−171[h]	ND
hplc 3	873.5	100	873.5	98−105	9.89
hplc 4	830.5	100	830.5	177−183	9.80
hplc 5	908.6	100	908.5	169−176	ND
	1031.9[i]	27	2061.8	33−48	4.00
hplc 6	780.5	100	780.5	170−176	10.10

[a] Result from chymotrypsin action. Should not belong to the mentioned peak considering the p*I*. [b] One oxidized Met in the sequence. [c] Two PO_4 in the sequence. Result from chymotrypsin action. [d] $[M + H]^+$ differ by 2 units from the calculated ones. Can also fit with β-casein residues (1−6), resulting from chymotrypsin action, with 2 Glu residues instead of Gln (calculated $[M + H]^+ = 786.5$). [e] $[M + H]^+$ differ by 1 unit from the calculated ones. [f] Should not belong to the mentioned peak considering the p*I*. [g] $[M + H]^+$ differ by 2 units from the calculated ones. Should not belong to the mentioned peak considering the p*I*. [h] Three PO_4 in the sequence. Result from chymotrypsin action. [i] $[M + 2H]^{2+}$. [j] The p*I* values are from Nau et al. (1993, 1995).

in the case of the HPLC peptides, on their MS sequencing (Figure 3).

FPLC peak 1 contained at least eight peptides (including the HPLC peptides 3, 4, and 6), and FPLC peak 2 at least three peptides (Table 1). Two peptides in peak 1 and two peptides in peak 2 could not be identified as tryptic peptides. Three of them could result from chymotrypsin activity, which often contaminates trypsin preparations. However, in the amino acid sequence of β-casein, seven peptides with, for instance, an $[M + H]^+$ of 802 ± 1 (major peptide from peak 1, identified as β-casein residue 134−140) can be found: fragments 133−139, 134−140, 140−145, 174−180, 175−181, 176−182, and 186−192. Nevertheless, none of the latter fragments fit with trypsin or chymotrypsin specificity. The hydrolysis of β-casein (variant A^1 or A^2) by trypsin or chymotrypsin was simulated (program MS-Digest 3.1.1., ProteinProspector 3.2.1, The Regents of the University of California, on Internet, 1995, 1999), and the 886 or 785 molecular weight fragments (FPLC peak 2) were absent from the list. In the case of the 785 molecular weight fragment, the fragment with the closest mass corresponds to the residue 106−111 (molecular weight = 787), resulting from combined tryptic and chymotryptic activities. On the other hand, if the assumption that two of the three Glu residues in β-casein variants are modified into Gln is valid, the 785 molecular weight fragment can fit with chymotryptic β-casein residue 1−6 (molecular weight 785).

HPLC fractions 3, 4, and 6 contained single peptides, but fraction 5 had one major contaminant (relative abundance of 27%). The doubly charged ion $[M + 2H]^{2+}$ of this peptide had a mass-to-charge ratio (m/z) of 1031.9, which yields a molecular weight of 2061.8, and leads to its identification as the tryptic β-casein(33−48) peptide (molecular weight 2062).

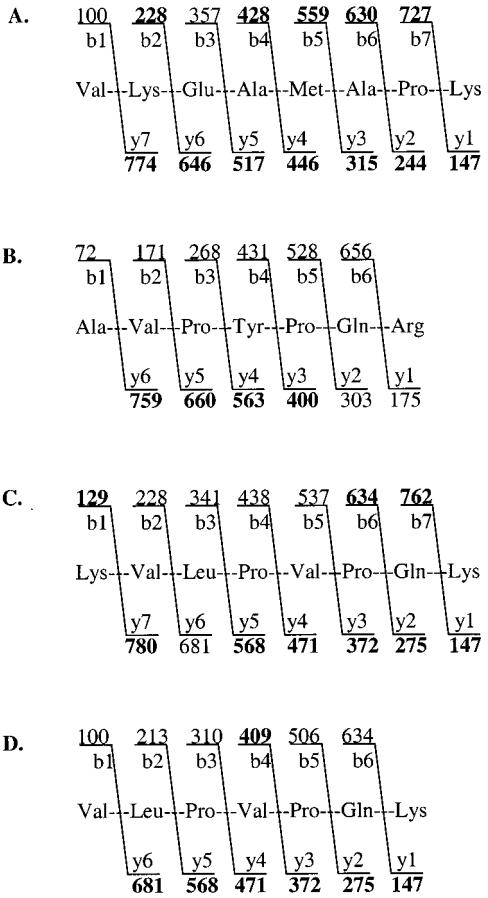

Figure 3. Daughter ions from the ESI-MS^n of the active tryptic β-casein fragments, obtained after fractionation by RP-HPLC of the tryptic digest of β-casein: (A) hplc 3, $[M + H]^+ = 873.5$, also observed were internal fragments EAMA (403) and EAM (332); (B) hplc 4, $[M + H]^+ = 830.5$, also observed were immonium ion of Pro (70), internal fragments PQ (226), PYPQ (486), and YP (261); (C) hplc 5, $[M + H]^+ = 908.5$, also observed were internal fragments PQ (226), VL (213), and PV (197); (D) hplc 6, $[M + H]^+ = 780.5$, also observed were immonium ion of Pro (70), internal fragment PQ (226). The expected nominal masses are shown on the sequence with those in bold observed in the mass spectra. The fragment attribution was checked by MS simulation on the Internet: MS-Product 3.1.1, Protein-Prospector 3.2.1 (The Regents of the University of California, 1995, 1999).

Sequencing of the HPLC-Collected Peptides (hplc 3−6): ESI-MS^n. HPLC-collected fractions were submitted to MS^n, and CID spectra of the major peptides (singly charged ions) in the fractions were recorded. The spectra were then analyzed using the fragmentation rules (de Hoffmann et al., 1996) and the simulation program MS-Product 1.4.1, Protein-Prospector 3.2.1, (The Regents of the University of California, on the Internet, 1995, 1999). The spectra provided sufficient fragmentation information to allow 100% of the sequence to be determined. Among the different fragments expected in MS^n (x, y, a, b, ...), mainly y and a few b fragments were observed, but internal fragments and the immonium ion of proline [nomenclature of Roepstorff and Fohlman (1984) and Biemann (1988)] were also observed. The mass difference between consecutive ions within a series allowed us to determine the identity of the consecutive amino acids and thus to deduce the peptide sequence (Figure 3). The assigned structures of the four peptides, as deduced from the different spectra, are summarized in Table 2, and the sequences are supported by the known

Table 2. Results of ESI-MS^n Analyses of the Active RP-HPLC Fractions from the β-Casein Tryptic Digest

HPLC fraction	sequence	β-casein residues	expected?	origin
3	VKEAMAPK	98−105	yes	trypsin
4	AVPYPQR	177−183	yes	trypsin
5	KVLPVPQK	169−176	no	unknown
6	VLPVPQK	170−176	yes	trypsin

sequence of β-casein. Isobar amino acids (Lys/Gln) or isomers (Ile/Leu) were discriminated on the basis of the known sequence of β-casein (Fiat and Jollès, 1989). Furthermore, the structure was confirmed by synthesis of the peptides. The elution times by analytical RP-HPLC of the synthetic peptides were identical to those of the isolated peptides (data not shown).

The presence of basic amino acids at the C-terminal amino acid side of the peptides explains the high occurrence of y MS fragments. Furthermore, the formation of internal fragments was a favored process because the peptides contained proline(s) (Heerma et al., 1991). Residues 98−105, 177−183, and 170−176 resulted from the typical action of trypsin that specifically cleaves bonds involving a lysine or an arginine in the P_1 position [nomenclature of Schechter and Berger (1967)]. The same three peptides were also detected among the peptides of FPLC peak 1, which was not surprising considering their p*I* values are much higher than 8 (Table 1).

Sixteen end-product peptides are theoretically expected in a β-casein tryptic digest (Leadbeater and Ward, 1987), including the fragments 170−176 (hplc 6) and 177−183 (hplc 4). Léonil et al. (1988) evidenced that β-casein disappearance was strongly associated with the early appearance of the stable fragments 100−105, 170−176, and 177−183. The fragment 100−105 results from the breakdown of the unstable intermediate 98−105 (hplc 3). In our proteolysis conditions, the 99−100 bond was not or only partially cleaved, and the fragment 98−105 was still present after 24 h of proteolysis. In contrast to the conditions used by Léonil et al. (1988), we used an initial β-casein concentration above its critical micellar concentration (35 mg/L; Hossain and Fenton, 1998), and we observed the temporary precipitation of hydrophobic intermediates. The 25−26 arginyl bond and the 28−29 lysyl bond are among the first cleavage sites, and very hydrophobic intermediates are formed, some of them being more hydrophobic than the whole β-casein (Léonil et al., 1988). The precipitation depends on the ratio of enzyme to substrate and the initial content in β-casein (above 1 g/L). It seems then that hydrolysis kinetics can be influenced by the aggregation state of the β-casein molecule. The β-casein fragment 169−176 was unexpected because its formation requires an unexplained cleavage at the Ser−Lys bond, but the assigned structure represents the only sequence of amino acids, present in the β-casein molecule, capable of yielding the observed fragmentation ions (Figure 3C). Formation of this fragment can be explained by atypical cleavage due to chymotrypsin activity present in the trypsin preparation (Visser et al., 1995), to unusual and unexplained enzyme activity, or even to unknown origin (Jones et al., 1991).

Inhibition Activity of Caseins and Casein-Derived Peptides against Lox-Catalyzed Linoleate Oxidation (Initial Rate Kinetics). The effect of caseins and casein-derived peptides on Lox-1 activity is summarized in Table 3. Among the different types of casein assayed, the glycosylated protein, κ-casein, and the whole casein, a mixture of components, were the most efficient. The dephosphorylation of the proteins did not significantly modify their inhibitory properties. To identify the active sequence within the molecules, caseins were hydrolyzed. Sodium caseinate and β-casein were chosen as molecule models because they were commercially available in large quantities and at reasonable costs. Three digests of five retained the inhibition properties of the undigested proteins, but to different extents (Table 3). The tryptic digest of whole casein was as potent as the undigested protein, decreasing the initial Lox rates by ~25%. Neither the subtilisin/clostripain digests of whole casein nor the subtilisin digest of β-casein was a significant inhibitor. On the other hand, the tryptic digest of β-casein exhibited a higher inhibition activity (27%) than the undigested β-casein (17%). Thus, it was assumed that bioactive peptides were hidden in an inactive state within the amino acid sequence of β-casein and that digestion liberated active peptide(s) from the inactive ones.

Table 3. Effect of Caseins and Casein-Derived Peptides on Lox-1 Activity at pH 9.0 (30 °C) As Determined by Oxygen Uptake (Means ± SD)

	test compd[a]	residual activity (%)
undigested proteins[b]	Cas	76.8 ± 3.3
	Deph Cas	80.0 ± 2.8
	β-Cas	82.5 ± 9.0
	Deph β-Cas	91.6 ± 4.6
	Deph α-Cas	84.3 ± 9.0
	κ-Cas	73.4 ± 5.7
	BSA	76.2 ± 1.0
digested proteins[c]	Trp D Cas	76.2 ± 5.2
	Clost D Cas	96.7 ± 4.4
	Subt D Cas	81.8 ± 8.7
	Trp D β-Cas	72.5 ± 1.3
	Subt D β-Cas	96.7 ± 4.3
FPLC-fractionated peptides (tryptic digest β-casein)[d]	peak 1	84.7 ± 2.0
	peak 2	90.1 ± 5.0
	peak 3	95.2 ± 1.2
HPLC-fractionated peptides (tryptic digest β-casein)[e]	hplc 3	84.0 ± 5.0
	hplc 4	91.2 ± 5.9
	hplc 5	79.5 ± 3.0
	hplc 6	90.0 ± 2.0

[a] Abbreviations: Cas, casein; Deph, dephosphorylated; Trp, trypsin; Clost, clostripain; Subt, subtilisin; D, digest. [b] The assay was performed with 320 μM linoleic acid, 1.7 g/L test compounds (prepared as outlined under Materials and Methods), and 12 nM Lox. [c] The assay was performed with 320 μM linoleic acid, 1.7 g/L test compounds (prepared as outlined under Materials and Methods), and 8 nM Lox. [d] The assay was performed with 320 μM linoleic acid, 1.7 g/L test compounds (prepared as outlined under Materials and Methods), and 12 nM Lox. [e] The assay was performed with 320 μM linoleic acid, 100 μL of hplc peptides (prepared as outlined under Materials and Methods), and 6 nM Lox.

The aim of the next steps was to determine the active peptide(s) and, therefore, the tryptic digest of β-casein was submitted to fractionation (FPLC or RP-HPLC methods). The activity was not enhanced by the fractionation of the peptides according to their p*I*. Compared to the digest, the FPLC fractions had approximately the same (peak 1) or lower (peaks 2 and 3) inhibition effects (Table 3). The activity of the undigested β-casein was found in FPLC peak 1 with a common ability to decrease ~15% of the Lox initial rate. The tryptic digest of β-casein was then fractionated according to the hydrophobicity of its constitutive peptides. The effect of the 10 HPLC fractions on Lox activity was analyzed. Four

peptides with retention times of 20.9 min (hplc 3), 32.7 min (hplc 4), 49.7 min (hplc 5), and 58 min (hplc 6), as eluted from the preparative column (i.e., of increasing hydrophobicity), were found to decrease the Lox activity (Table 3). The highest inhibition value (20%) was obtained with hplc 5, identified as β-casein(169−176) with a trace amount of β-casein(33−48), a phosphorylated peptide. Its effect was higher than that of FPLC peak 1 or the undigested protein (17%) but lower than that of the unfractionated digest (27%). Hplc 3, sequenced as β-casein(98−105), inhibited the reaction to the same extent as FPLC peak 1 or the undigested protein (16%). Hplc 4 or β-casein(177−183) and hplc 6 or β-casein(170−176) decreased ~10% of the reaction rate. Hence, they were less active than the undigested or the digested protein and FPLC peak 1. The isolated peptides retained the inhibition properties of native and digested β-casein. Hplc 3, hplc 4, and hplc 6 were inhibitors as components of a mixture (identified among the eight peptides contained in FPLC peak 1) as well as individual peptides. To our knowledge, no biological activity is reported for these peptides, except for the fragment 177−183. The β-casein(177−183) peptide is known as a casokinin, with antihypertensive properties (Schlimme et al., 1989; Schlimme and Meisel, 1995). Other bioactive peptides such as exorphins (casomorphin), phosphopeptides, casokinins, and immunopeptides are derived from casein (Meisel et al., 1989; Schlimme et al., 1989). A "strategic zone" containing immunostimulating and opioid peptides was located in bovine and human β-casein (Fiat and Jollès, 1989). Furthermore, bitter peptides (Minagawa et al., 1989), emulsifying peptides (Caessens et al., 1997), tyrosinase-inhibiting peptides (Tomita et al., 1991), and calcium-enhancing absorption peptides (Fiat and Jollès, 1989) have also been described.

The specificity for the effect of caseins and casein-derived peptides on Lox kinetics was evaluated with another protein, BSA, a well-known fatty acid carrier. The Lox-inhibiting activity of BSA was comparable to that of undigested casein (Table 3). However, in contrast to casein, BSA was shown to form complexes with linoleate, resulting in substrate-limited Lox kinetics (Laakso and Lilius, 1982). To check such a coating of the substrate by the proteins of interest, β-casein, its tryptic digest, and FPLC fractions were assayed against different linoleate concentrations (80−320 μM; data not shown). The inhibition effect was not enhanced at low substrate concentrations, at which a saturation of linoleate by casein molecules would be optimal, suggesting a chemical rather than a physical mechanism of action for caseins and casein-related peptides. This is in agreement with the results of Laakso and Lilius (1982), who proposed that casein inhibits Lox reaction either by trapping free radicals or by interacting with the enzyme. In addition, the coating mechanism, which can occur with large molecules such as caseins, is excluded with shorter molecules, as it was observed for casein digests and β-casein hepta- and octapeptides. As a consequence, the effect of caseins and casein-derived peptides on Lox activity can result from a true inhibition, an iron complexation, and/or an antioxidation mechanism.

To investigate if caseins and casein-derived peptides interact with the enzyme, kinetic studies were performed with β-casein, tryptic β-casein digest, and FPLC peaks. The data obtained did not fit with classical inhibition models (i.e., competitive, not competitive, uncompetitive; data not shown). A possible explanation resides in the heterogeneity of the test compounds. Also, caseins and casein-derived peptides bear little resemblance to substrate or products of the Lox reaction. Consequently, formation of a complex [enzyme−inhibitor], as observed with substrate analogues and half-product analogues (Zhu and Funk, 1996), seems unlikely. Furthermore, it is not immediately apparent how these compounds could gain access to the vicinity of the active site (steric hindrance). The structure of Lox-1 resolved to 1.4 Å shows that the enzyme has two domains (Minor et al., 1996). The smaller, domain I, comprises the 146 residues of the amino terminus. Domain II consists of the remaining 693 residues and contains the active site. The iron is at the center of the major domain. Two sides of the iron center face two internal cavities that are probably the conduits through which the fatty acid and the molecular oxygen gain access to the metal (Prigge et al., 1997). There is no consensus on how substrates gain access to the iron center or any definite information on substrate binding. In addition, it is not clear at present whether the diverse substances gain access to the iron atom through the substrate channel or by some other path.

Similarly, caseins and casein-derived peptides, having few or no sulfhydryl groups, cannot inhibit Lox via the same mechanism as cysteine, 2-mercaptoethanol, and glutathione. The inhibition of Lox by these compounds was attributed to hydrogen peroxide, formed in the oxidation of thiol groups in the presence of trace amounts of metals (O'Connor and O'Brien, 1991).

On the other hand, compounds able to coordinate or reduce the iron at the Lox active site (Kemal et al., 1987; Mansuy et al., 1988; Clapp et al., 1985; Nelson et al., 1991; Desmarais et al., 1994), as well as compounds that interfere with intermediate(s) of the enzyme-catalyzed reaction (Decker and Faraji, 1990), are inhibitors of Lox. Proteins, including casein (Laakso, 1984; Taylor and Richardson, 1980; Tannenbaum et al., 1969) and peptides (Chen et al., 1996, 1998; Murase et al., 1993), were described as antioxidants, and the antioxidative mechanism was investigated, except in the case of casein.

Conclusion. In this study, it was shown that caseins and casein-derived peptides had lipoxygenase inhibitory properties. Eventual iron-chelating or general antioxidant properties of caseins and casein-derived peptides should be investigated in order to understand the chemical basis for their measured Lox inhibitory activity.

ABBREVIATIONS USED

ACN, acetonitrile; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; Cas, casein; Clost, clostripain; D, digest; Deph, dephosphorylated; ESI-MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; FPLC, fast pressure liquid chromatography; hplc, RP-HPLC-fractionated peptides; LA, linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid); Lox, lipoxygenase (EC 1.13.11.12); MeOH, methanol; ND, not determined; OtBu: *tert*-butoxy; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBop, benzotriazole-1-yloxytris(pyrrolidinophosphonium) hexafluorophosphate; RP-HPLC, reverse-phase high-performance liquid chromatography; RT, retention time; SDS−PAGE, polyacrylamide gel electrophoresis performed with sodium

dodecyl sulfate; Subt, subtilisin; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Trp, trypsin.

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