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Milk Casein: Inhibitor of Lipoxygenase-Catalyzed Lipid Peroxidation

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Various milk products inhibited lipoxygenase-catalyzed lipid peroxidation in a model system consisting of linoleic acid, one of the two purified soybean isozymes, and the inhibitor to be tested. The inhibition by milk was not dependent on its fat content, pasteurization, dialyzation, or heating. The inhibitory effect was associated with the isoelectrically precipitated casein fraction. This effect was verified with a commercial casein product. However, on the basis of kinetic grounds neither did the absorption of substrate to casein nor did calcium cause the inhibition. Luminometric measurements showed that casein inhibition was accompanied by a loss of chemiluminescence emission. This suggests that casein either has radical trapping properties or changes the mode of attack of the enzyme so that such radicals are not formed. The inhibition was not limited to the purified enzymes but a 50% reduction of oxygenation in various plant homogenates was achieved by 0.06–3.2% (w/w) casein supplementation.

The range of known or potential lipoxygenase inhibitors is limited. This presents a significant problem for the storage and processing of lipid-rich plant materials. Especially in cases where heat treatment is not wanted, cracking or bruising of intact tissue promotes lipoxygenase action and results in peroxidation of unsaturated fatty acids accompanied by flavor alterations and pigment destruction.

Poisons such as cyanide, azide, or *p*-(chloromercuri)-benzoate do not inhibit the enzymes (Holman, 1947). Polyunsaturated fats with trans double bonds do inhibit the enzymes (Mitsuda et al., 1967), but because their effect is competitive with the *cis,cis*-pentadiene fatty acid substrates, they are of less practical value in food processing. Although numerous reports on the inhibitory effect of thiols and divalent cations, especially Ca^{2+}, have emerged, there exists no consistent picture of their application to lipoxygenases. Currently, antioxidants are widely used as lipoxygenase inhibitors. They also inhibit the autoxidation of lipoxygenase substrates. However, a limited number of safe antioxidants remains when dealing with plant materials intended for human consumption. The development of a general lipoxygenase inhibitor with the same role as a flavor-improving additive seems an ambitious plan. A realistic approach toward this goal is the search for naturally occurring inhibitors from living tissues and biological fluids because biological systems have defense mechanisms against the damaging effects of activated forms of oxygen and free radical intermediates generated during lipoxygenase catalysis (Galliard and Chan, 1980). Consequently, the search for compounds presumably having either oxygen scavenger or general radical trapping capabilities is tempting.

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The motivation to study cow's milk as an inhibitor of lipid peroxidation arose during attempts to analyze various biological fluids for the content of unsaturated fatty acids by modifying the luminometric lipoxygenase assay of Lilius and Laakso (1982). In these experiments milk samples were found to inhibit the emission of chemiluminescence, suggesting that they contained a factor able to interfere with the free radical mechanism of enzymic lipid peroxidation. Therefore, in this work casein and other milk proteins were studied for their possible role as "biological inhibitors". This report gives evidence that milk casein has a direct effect on the mechanism of lipoxygenase catalysis. Milk casein stabilized suspensions of various plant seeds including soybean, green peas, wheat, rye, oats, barley, and turnip rape against their lipoxygenase-catalyzed peroxidation of unsaturated lipids.

EXPERIMENTAL SECTION

Materials. Linoleic acid (grade III) was from Fluka AG, Switzerland. Casein was the "vitamin-free" product of Sigma Chemical Co. Powdered skim milk (1% fat) was a commercial product of Kuivamaito Oy, Finland. Pasteurized and homogenized cow's milk with varying fat content (0, 2.9, and 3.5%) were commercial dairy products (Kulmala Oy, Finland). Untreated cow's milk was a mixture of five different collection tanks from various Finnish dairy farms. Dialyzed milk was prepared from 50 mL of pasteurized low-fat milk (2.9% fat) by dialysis at 8 °C for 48 h against 3 L of deionized water followed by two changes of 3 L of 50 mM sodium phosphate buffer, pH 6.5. Isoelectrically precipitated casein was obtained by adjusting the pH of a 50-mL portion of pasteurized low-fat milk (2.9% fat) to 4.6 with 0.5 N HCl and by incubating this mixture at 25 °C for 1 h. The precipitate was collected by centrifugation at 5000*g* and 25 °C for 15 min and washed with 100 mL of 50 mM sodium phosphate buffer, pH 6.5. This washed precipitate was dialyzed

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against 3 L of the washing buffer at 25 °C and diluted to 50 mL with the same buffer prior to its use. Other reagents used in enzyme purification and reaction mixtures were of reagent grade and used without purification.

Representative of legumes, cereal grains, and oilseeds to be tested for were purchased as follows: soybean (*Glycine max*), green pea (*Pisum sativum* subsp. *sativum*), wheat (*Tritivum aestivum*), rye (*Secale cereale*), oats (*Avena sativa*), barley (*Hordeum vulgare*), and turnip rape (*Brassica rapa* subsp. *rapa*). Immediately prior to use about 10 g of legumes or seeds (dry weight) was milled at room temperature in a Janke & Kunkel disk homogenizer for 30 s each, and 100 mg of meal passing a 60-mesh screen was used in assays without defatting.

Purification of Soybean Lipoxygenase Isozymes. Soybean lipoxygenase-1 (L-1) and lipoxygenase-2 (L-2) were purified essentially according to the method of Axelrod et al. (1981) with only the following modifications: 150 mg of lyophilized acetone powder from Sigma was used without defatting as starting material in the purification of L-1 whereas L-2 was extracted from freshly made soybean meal defatted immediately after milling to minimize the loss of activity due to the presence of fatty acid substrates in soybean oil. The purified L-1 had a specific activity of 180 units/mg with linoleic acid as the substrate at pH 9.0 and L-2 49 units/mg with arachidonic acid as the substrate at pH 6.5.

The isozymes migrated as separate bands in $NaDodSO_4$ disc electrophoresis at pH 9.5 (Christopher et al., 1970). L-2 failed to produce conjugated carbonyl compounds absorbing at 280 nm from linoleic acid under both aerobic and anaerobic conditions whereas L-1 could do so under anaerobic conditions, suggesting that no contamination from each other existed in these preparations (Axelrod et al., 1981).

Enzyme Assay. Lipoxygenase activities were determined at 25 °C as the rate of oxygen consumption in a magnetically stirred reaction mixture (Christopher et al., 1970) consisting of 0.4 mM linoleic acid substrate in 1.0 mL of either 0.2 M sodium borate buffer, pH 9.0 (L-1), or 0.2 M sodium phosphate buffer, pH 6.5 (L-2). Approximately 20 milliunits of L-1 (0.1 μg) and L-2 (0.5 μg) or 100 mg of each meal sample was used to initiate the reactions. When inhibitors were added, their volume was compensated for by the buffer to obtain a 1.0-mL reaction mixture. The linoleic acid substrate was pipetted from a 10 mM stock solution in Tween 20 (Surrey, 1964). The equipment was a Hansatech D.W. oxygen electrode unit equipped with Goertz RE 511 recorder. The enzyme unit was defined as the amount of micromoles of oxygen consumed per minute at 25 °C.

The enzyme reaction was also followed luminometrically. In this procedure the generation of free radical intermediates by a lipoxygenase is coupled to the oxidation of luminol (5-amino-2,3-dihydrophtalazine-1,4-dione from Sigma), and the resulting chemiluminescence is recorded as the rate of the reaction. The conditions of the assay and the equipment used were the same as described previously (Lilius and Laakso, 1982).

RESULTS AND DISCUSSION

Because vegetable oils are generally extracted from sources rich in enzymes that oxidize unsaturated fats, we commenced a study to determine the effect of milk supplementation on the rate of oxygenation of essential unsaturated fatty acids. Milk proteins were added in amounts equivalent to the enzyme level in a model reaction consisting of linoleic acid and one of the two isozymes of soybean lipoxygenases purified to homogeneity. The enzymes, both catalyzing the oxygenation of *cis,cis*-1,4-pentadiene fatty acids as their primary reaction, were chosen on the basis of their distinctly different pH optimum and response to anaerobic reaction conditions as well as the availability of the most advanced methodology to establish their identity during purification (Axelrod et al., 1981).

Table I. Inhibition of Soybean Lipoxygenase Isozymes by Milk and Its Constituents

supplement[a]	% enzyme act.[b] L-1	L-2
none	100	100
pasteurized milk (0% homogenized fat)	45	46
pasteurized milk (2.9% homogenized fat)	41	50
pasteurized milk (3.5% homogenized fat)	41	45
boiled milk (2.9% homogenized fat)	42	
untreated milk	40	46
isoelectrically precipitated casein (27 mg/mL)	48	46
dialyzed milk	42	46
$CaCl_2$ (1.2 mg/mL)	45	118

[a] 30 μL of each supplement was added to a 1-mL reaction mixture containing 20 milliunits of lipoxygenase-1 and 10 μL to reaction mixtures containing 20 milliunits of lipoxygenase-2. [b] Each value represents the average of duplicates.

With both isozymes the reaction rate, followed as the rate of oxygen consumption, was found to decrease with addition of increasing concentrations of pasteurized low-fat milk. The inhibition was instantaneous and no time-dependent inhibition of either isozymes was detected. Therefore, in further assays fixed concentrations of L-1 (0.1 μg/1-mL assay) and L-2 (0.5 μg/1-mL assay) were chosen, and an apparent I_{50} value of 25 μL of milk for the former and 5 μL for the latter was obtained. According to these I_{50} values, different types of milk products were tested and compared for their inhibitory capabilities (Table I).

The fat content of pasteurized milk did not play a role in the inhibition, nor did dialysis of low-fat milk (2.9%) for 48 h abolish the inhibitory effect. Therefore, free low molecular weight compounds are not responsible for the inhibition. Experiments with untreated raw milk show that the inhibitory characteristics were not developed during the pasteurization process.

The first evidence that the whole casein fraction might be responsible for the inhibition was obtained from the data with isoelectrically precipitated casein. Isoelectric precipitation was performed at pH 4.6 (40 °C) followed by dissolution of the casein fraction to correspond to the milk's original casein content. With this preparation the degree of inhibition corresponded to that of pasteurized milk used as a reference. Casein from a commercial source at a concentration of 27 mg/mL in 0.2 M buffer either at pH 9.0 (L-1) or at pH 6.5 (L-2) reproduced this result.

The possibility that the observed inhibition by casein was an artifact due to absorption of the fatty acid substrate to casein was studied by using albumin, the fat carrier protein from bovine blood serum, as a reference. The degree of inhibition by 0.7 mg of either casein or albumin was found to be of the same order of magnitude (56 and 64%, respectively) when the proteins were added to 1.0-mL reaction mixtures containing 0.4 μmol of linoleate and 24 milliunits of lipoxygenase-1. However, a distinctly different behavior in enzyme activity resulted when the concentration of substrate was gradually increased. Figure 1 demonstrates the point where albumin becomes saturated with linoleic acid and any further additions of substrate thereafter are reflected as increments in the oxy-

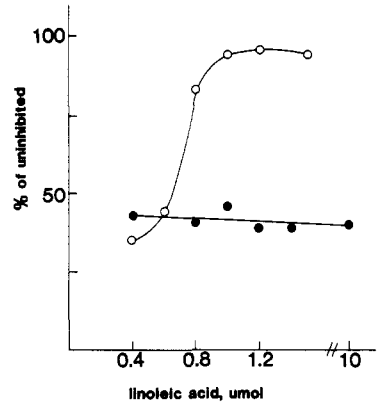

Figure 1. Inhibition of lipoxygenase activity by casein and albumin in the presence of different concentrations of linoleic acid. Reaction mixtures in 1.0 mL of 0.2 M sodium borate buffer, pH 9.0, consisted of 20 milliunits of lipoxygenase-1 and either 0.7 mg of albumin (○) or 0.7 mg of casein (●). The initial velocities of oxygen consumption were recorded at linoleic acid concentrations from 0.4 to 1.5 μmol/mL and compared to that obtained with 0.4 μmol/mL linoleic acid without an inhibitor present (100%).

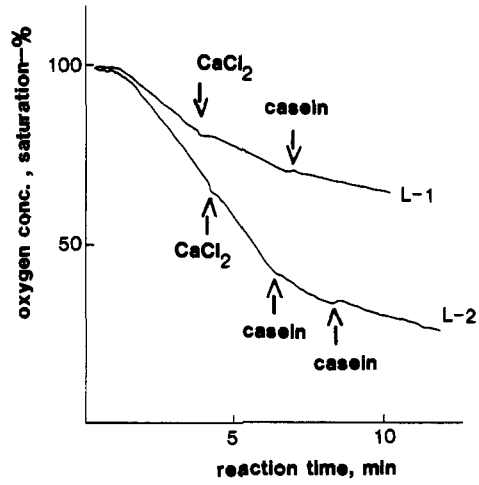

Figure 2. Effect of $CaCl_2$ and casein on reaction velocity curves of lipoxygenase reaction. The reaction mixtures consisted of 0.4 mM linoleic acid and either 17 milliunits of lipoxygenase-1 (L-1) or 24 milliunits of lipoxygenase-2 (L-2) in 1.0 mL of 0.2 M buffer at the appropriate pH. The arrows indicate the points of addition of $CaCl_2$ (90 μg) and casein (0.8 mg) from their stock solutions in 0.2 M sodium phosphate buffer, pH 6.5.

genation rates until the saturation concentration of substrate was reached.

In contrast, casein inhibition remained unchanged with addition of increasing concentrations of linoleate. Attempts to overcome the inhibition with up to 10 μmol/mL linoleate (all data not shown in Figure 1) failed, suggesting that saturation concentrations of unbound substrate were present. Further support for this came from the observation that 0.7 mg of the albumin, when added to casein-inhibited reactions, did not reduce the reaction rates at linoleate concentrations above 0.8 μmol/mL.

Because ionically associated calcium and colloidal calcium phosphate are lost to the whey as their ionized forms during isolation of casein by isoelectric precipitation, these are unlikely inhibitors in the casein preparations used. In addition, $CaCl_2$ solution prepared to approximate the content of total calcium in milk inhibited L-1 isozyme, giving about the same I_{50} value determined for pasteurized milk (Table I). A slight activation occurred in the case of L-2 enzyme, which previously was found to be even more sensitive to inhibition by milk (I_{50} = 5 μL).

As shown in Figure 2, casein inhibited both enzymes also in the presence of Ca^{2+}. In these experiments the enzymes were quantitated to give a linear reaction velocity curve until the concentration of dissolved molecular oxygen was reduced to 20% of its initial level (about 13 and 10 min for L-1 and L-2, respectively). However, the addition of $CaCl_2$ to L-1 reactions at 80% oxygen saturation instantaneously changed the slope from the initial 17 to 10 nmol/min. Supplementation of this Ca^{2+}-inhibited reaction with casein at 70% oxygen saturation further reduced the reaction rate to 4 nmol/min, suggesting that their effects are additive for L-1. This view is supported by the facts that the inhibition could be reproduced by adding both Ca^{2+} and casein prior to initiation of the reaction whereas the activities varied between 9 and 11 nmol/min when only one of the inhibitors was added. Consequently, if casein and calcium were equally inhibitory for L-1, a 2-fold volume of $CaCl_2$ solution should be used to achieve the same degree of inhibition as milk. However, this was not the case.

Attempts to measure lipoxygenase-1 reaction by chemiluminescence according to the method of Lilius and Laakso (1982) were unsuccessful at casein concentrations above 0.1 mg per a 1-mL reaction mixture although the catalyst concentration was raised up to 5 units of L-1. Because free radicals normally dissociated from the ES complex are believed to be responsible for the light emission during lipoxygenase attack on linoleic acid, casein may play a role as a radical scavenger in lipoxygenase reactions. However, this might not be the primary effect of casein because several compounds are able to interfere with the chemiluminescent reaction without a significant loss of the oxygenating activity (Lilius and Laakso, 1982).

Instead, the inhibition by casein may be related to the observation that immobilization of lipoxygenases to polymer supports abolished chemiluminescence while reducing only partially the main process (Laakso, 1981). The hydrophobic nature of lipoxygenases (Allen et al., 1977) might also cause their spontaneous coupling to casein. According to this view the "immobilized" species represent either inactive or catalytically changed chemiluminescence-silent enzyme. In spite of the fact that lipoxygenases from various sources differ, e.g., with respect to optimum reaction conditions and product specificity (Axelrod, 1974; Gardner and Weisleder, 1970), their mechanism of attack on unsaturated fatty acids seems analogous (Spaapen et al., 1977). Consequently, if casein is able to interfere with this mechanism, the inhibition observed in the case of soybean isozymes should apply to lipoxygenases from other sources.

This was tested by using representatives of leguminous seeds, cereal grains, and oilseeds as lipoxygenase sources. The oxygraph was found useful in direct monitoring of oxygenation activity in freshly prepared meal samples both with and without exogenously added fatty acid substrates as well as in the presence of casein. The seeds were milled at room temperature and used without defatting only in reaction mixtures buffered at pH 6.5. The values in Table II do not necessarily represent absolute enzyme contents and are for comparative purposes only. Although markedly different responses to exogenously added linoleic acid were demonstrated among the samples tested, in each case the inhibition of oxygenation was dependent on the concentration of casein added. The degree of inhibition showed a correlation with the original oxygenation rate without the inhibitor present, and therefore, I_{50} values were calculated for each enzyme source. The required casein/meal ratio is obviously a critical point when the use of casein

Table II. Effect of Casein on Oxygenation Rates in Suspensions of Some Legumes and Cereal Grains

source of enzyme[a]	rate of oxygen consumption, nmol/min		casein/meal at I_{50}, % (w/w)	
		linoleate supplemented (0.4 μmol)		linoleate supplemented (0.4 μmol)
green pea	58	93	2.0	3.2
wheat	19	494	0.06	1.6
barley	28	28	0.2	1.6
oats	35	36	1.8	2.7
rye	15	108	0.2	1.2
turnip rape	9	33	*b*	2.5
soybean	77	200	0.4	1.2

[a] For each assay 100 mg of meal without defatting was used in a 3.0-mL reaction mixture. [b] Reaction rates at I_{50} were below the detection limit of the assay.

as a lipoxygenase inhibitor is considered. Table II enables the comparison of different plant meals with respect to casein content required at I_{50}. The use of skim milk in quantities corresponding to the content of casein reproduced the inhibition within experimental error (approximately 15%).

As a conclusion from these observations it is suggested that milk casein offers an alternative to the use of antioxidants and heat treatments in the stabilization of essential unsaturated fatty acids in plant materials.

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Comparative Degradation of the Pyrethroids Tralomethrin, Tralocythrin, Deltamethrin, and Cypermethrin on Cotton and Bean Foliage

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Residues on cotton and bean foliage up to 20 days after treatment with nonstabilized formulations of tralomethrin and tralocythrin consist of the parent pyrethroids and various ester photoproducts, i.e., significant amounts of deltamethrin and (1R,αS)-*cis*-cypermethrin from debromination and *trans*-deltamethrin and -cypermethrin from 1*R*-cis → 1*RS*-cis,trans isomerization and minor levels of 1′-bromodeltamethrin and 1′-bromocypermethrin from dehydrobromination. Small amounts of the α*R* enantiomers of deltamethrin and (1*R*)-*cis*-cypermethrin are also detected. The acid moiety of tralomethrin undergoes rapid debromination on cotton foliage. Additional products include polar conjugates and compounds not readily recovered on extraction with chloroform–acetonitrile. With the exception of the initial debromination and dehydrobromination reactions, the degradation processes and ultimate residues of tralomethrin and tralocythrin appear to be essentially the same as those of deltamethrin and (1*R*,α*S*)-*cis*-cypermethrin.

Tralomethrin and tralocythrin with 3-tetrahaloethyl substituents (Roussel-Uclaf, 1978a) are readily debrominated to deltamethrin and (1*R*,α*S*)-*cis*-cypermethrin with 3-dihalovinyl substituents in insects (Ruzo et al., 1981) and rats (Cole et al., 1982) and upon photolysis (Ruzo and Casida, 1981) (Figure 1). Deltamethrin and cypermethrin are degraded on or in plants primarily by photoisomerization, ester cleavage, and conjugation reactions (Roberts, 1981; Ruzo and Casida, 1979; Wright et al., 1980). The present study compares the degradation of tralomethrin, tralocythrin, deltamethrin, and (1*R*,α*S*)-*cis*-cypermethrin on cotton and bean leaves with emphasis on modification occurring in the acid moiety.

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MATERIALS AND METHODS

Chemicals. Figure 1 gives the structures, names, and abbreviations for the compounds under investigation. It also indicates the labeling positions for the [^{14}C]pyrethroids examined (40–60 mCi/mmol; supplied by Roussel-Uclaf, Paris, France; Cole et al., 1982). Cypermethrin as used here refers to the 1*R*,α*S*-cis isomer unless indicated otherwise.

Treatment of Plants. Cotton leaves (0.3–0.5 g) on greenhouse-grown plants (20–22 cm high) were individually treated with each [^{14}C]pyrethroid (~0.3 μg/leaf) by using cold ether (30 μL) to apply the samples in the shade and

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