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Received for review March 8, 1988. Revised manuscript received July 11, 1988. Accepted October 11, 1988.

Changes Induced in β-Lactoglobulin B following Interactions with Linoleic Acid 13-Hydroperoxide

Francisco J. Hidalgo and John E. Kinsella*

β-Lactoglobulin B (βLG B) undergoes a number of deteriorative changes when exposed to linoleic acid 13-hydroperoxide. In the first stage, the lipid hydroperoxide caused destruction of tryptophan and disulfide cross-linking of βLG B. Concurrently though more slowly, the hydroperoxide itself or some secondary product (SP) reacted with free amino groups of βLG B to generate fluorescent compounds and promoted polymerization. The presence of other degradation compounds, such as short-chain aldehydes, reacted with exposed amino groups of βLG B to form additional but different fluorescent and nonfluorescent compounds. However, the development of the principal fluorescence was closely related to the formation of stable dimers of βLG B. The blockage of the amino groups in βLG B by reductive alkylation prevented the development of fluorescence and the formation of stable dimers, suggesting that the presence of free amino groups is necessary for these types of reactions.

Lipid oxidation is one of the major causes of food spoilage and is undesirable not only from an acceptibility and economic point of view but also because oxidative reactions can decrease the nutritional quality of food and generate oxidation products that are potentially toxic (Matsuo, 1962; Morton, 1977; Nawar, 1985; Richardson, 1984). Among the negative effects produced by peroxidizing lipids in foods, their chemical interactions with proteins have received considerable attention (Desai and Tappel, 1963;, Gardner, 1983; Karel et al., 1975; Pokorny et al., 1988). This is an important deteriorative mechanism in the processing and storage of foods causing loss in flavor, color, functional properties, and nutritive value and, also, causing changes in biological tissues and is a basic pathological process in vivo (Funes et al., 1982; Kanner and Karel, 1976; Kanner et al., 1987; Tappel, 1973).

Exposure of proteins to peroxidizing lipids or their secondary breakdown products can produce changes in proteins, including loss of enzyme activity, polymerization, insolubilization, scission, and formation of lipid–protein complexes, some of which are fluorescent (Chio and Tappel, 1969; Funes et al., 1982; Gardner, 1979; Pokorny and Janicek, 1975). These changes occur through two basic mechanisms, namely via protein–amino condensation reactions involving lipid peroxidation breakdown products and via reactions of proteins with lipid oxidation products (lipid free radicals, hydroperoxides, and volatile secondary products), resulting in the formation of protein-centered free radicals (Karel, 1977).

Most studies of interaction between peroxidizing lipids and proteins have been carried out between a model lipid, mostly linoleic acid or its hydroperoxides, and model proteins, usually lysozyme, egg albumin, or bovine serum albumin (BSA), and almost all have studied the changes produced in the protein.

The objective of this research was to determine the kinetics and products of the interaction between linoleic acid 13-hydroperoxide (13-LOOH) and β-lactoglobulin B (βLG B).

βLG B is a well-characterized protein that has not been used in these types of studies. However, it should be useful because its composition and secondary and tertiary

Institute of Food Science, Cornell University, Ithaca, New York 14853.

0021-8561/89/1437-0860$01.50/0

structures are known (Papiz et al., 1986), it has a free thiol group, and it has a high binding affinity for apolar compounds (O'Neill and Kinsella, 1987). The system composed of 13-LOOH and βLG B may be considered as a model for lipid–protein interactions and may be useful in elucidating the mechanisms of reactions involving lipoproteins, whose oxidation in vivo has lately attracted much attention (Esterbauer et al., 1987).

MATERIALS AND METHODS

Protein. β-Lactoglobulin (βLG) was isolated from bovine milk as a mixture of A and B (Armstrong et al., 1967) and was fractionated to obtain pure βLG B (Piez et al., 1961). Native slab polyacrylamide electrophoresis (12% acrylamide) confirmed the purity of the samples (O'Neill and Kinsella, 1987).

Reductive alkylated βLG B (RβLG B) was used as reference protein in these studies. This was prepared by modification of a procedure of Means and Feeney (1968), to obtain protection of 95% of amino groups. A solution (100 mg of βLG in 20 mL of 0.1 M sodium borate buffer, pH 9) at 0 °C was treated with 10 mg of $NaBH_4$, and then five sequential additions of 10 μL of HCHO were made at intervals of 6 min. After 30 min, 10 mg of $NaBH_4$ was added and the procedure was repeated with 20 μL of HCHO in each addition. The whole procedure was repeated once again with 10 mg of $NaBH_4$ and five additions of 20 μL of HCHO. Then the sample was dialyzed and freeze-dried. The percentage of free amino groups was determinated according to the method of Habeeb (1966) using picrylsulfonic acid and 4% sodium bicarbonate, pH 8.5. The RβLG B contained one SH group per monomer (Brinegar and Kinsella, 1981).

Lipid. The 13-LOOH was prepared from pure linoleic acid by mixing the linoleic acid (184 mg) with Tween 20 (180 mg) and homogenizing the mixture in 10 mL of water without oxygen. Then, ≈1.5 mL of 0.5 N NaOH was added until a transparent solution was obtained which was diluted to 50 mL with water. The solution (13.1 mM sodium linoleate) was divided into 1.5-mL aliquots and frozen until use.

Soybean lipoxygenase (Type I, Lot 127F-0327; Sigma, St. Louis, MO; specific activity 146 000 units/mg of protein) was used for preparation of linoleic acid hydroperoxide. A solution of lipoxygenase was prepared in 0.1 M sodium borate, pH 9.0, and its activity determinated according to Axelrod et al. (1981).

Linoleic Acid Oxidation. Sodium borate buffer (25 mL of 0.1 M), pH 9.0, was oxygenated for 15 min at 0 °C by bubbling in a slow stream of oxygen. After this time, 24 units of lipoxygenase and 10.5 mL of sodium linoleate (13.1 mM) were added. The lipoxygenation was carried out for 6 min at 0 °C in the presence of oxygen, the pH was then adjusted to pH 3, and the lipids were extracted three times with ether. The organic layers were pooled and dried over magnesium sulfate, and the solvent was evaporated. The 13-LOOH was purified by TLC with ether–hexane–acetic acid (40:60:1) as eluent (Zamora et al., 1986). The yield was 40%.

The same procedure was employed when labeled linoleic acid ([1-^{14}C]linoleic acid) was used to prepare [1-^{14}C]LOOH. In this case, 0.05 μCi of [1-^{14}C]linoleic acid was added per 10 mg of unlabeled linoleic acid.

Lipid–Protein Interactions. The interaction between βLG B and 13-LOOH was studied in incubations carried out on 5 mg of lipid and 10 mg of protein in 2 mL of standard buffer with 0.015% Triton X-100 at 37 °C. Unless noted otherwise, the standard buffer was 116 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM sodium phosphate, pH 7.4. The 13-LOOH initially formed an emulsion in the buffer, but this quickly disappeared upon mixing with βLG B. Sample aliquots were removed from the reaction mixture at different intervals of time during the incubation and analyzed by fluorescence and electrophoresis, and the content of free amino groups in the protein–lipid complex was determinated. At the end of the 24-h incubation period, after which no further increase in the fluorescence intensity was observed, the aldehydic lipid peroxidation products were extracted and analyzed. Then the ether-soluble reactants and products remaining in the incubation mixture were extracted with diethyl ether (4 × 2 mL) and the extracted lipids discarded. The protein remaining after ether extraction was precipitated by addition of 8 mL of ethyl alcohol and pelleted by centrifugation (6000 rpm). The precipitated protein was washed with ethyl ether–ethyl alcohol (1:1) (4 × 2 mL) to extract the remaining lipids. The protein was then analyzed by Sephadex column chromatography in order to fractionate protein polymers.

Separate reaction assays between βLG B and aldehydes were also carried out on pure aliphatic aldehydes propanal, butanal, pentanal, and hexanal (Aldrich Chemical Co., Milwaukee, WI) under analogous conditions used for the interaction between βLG B and 13-LOOH.

Fluorescence spectra were determined to monitor changes in the fluorescence of the original protein and the formation of new fluorescent compounds during the reaction. Spectra were recorded on a Perkin-Elmer Model 650-40 fluorescence spectrophotometer of 20-μL samples diluted with 2.5 mL of standard buffer. A slit width of 5 nm was used, and the instrument was standardized with quinine sulfate (0.1 μg/mL in 0.1 N H_2SO_4) to give a fluorescence intensity of 20 at 450 nm, when excitation was done at 350 nm.

The formation of protein polymers during the reaction was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of samples of the reaction mixture. The method of Laemmli (1970) was used with running gels containing 12% acrylamide. Samples from the incubation mixture (10 μL) were diluted with the same quantity of sample buffer, pH 6.8, containing 0.3% glycine, 0.12% EDTA, 4% SDS, 0.1% bromophenol blue, 27% sucrose, and, in some cases, 10% β-mercaptoethanol and heated 5 min or more at 80–90 °C. Gels were run at 20 mA for 2 h at 20 °C, fixed for 1 h in 7.5% acetic acid and 20% methanol, stained for 12 h in 0.1% Coomassie Brilliant Blue R, 30% methanol, and 8% acetic acid, and destained in 7.5% acetic acid and 20% methanol. The gels were scanned by an E-C densitometer (EC Co., St. Petersburg, FL) interfaced with a Hewlett-Packard computing integrator.

The decrease in free amino groups of the protein during the incubation was quantified by the method of Habeeb (1966). Samples of the reaction mixture (50 μL) were diluted to 1 mL with standard buffer before the reactive was added (picrylsulfonic acid). Unreacted βLG B was used as standard.

The formation of aldehydic lipid peroxidation products in the reaction between 13-LOOH and βLG B was determined by modification of a procedure of Esterbauer et al. (1982). Samples (100 μL) of the reaction mixture were collected, diluted with 1 mL of standard buffer, and treated with 1 mL of 2 mM dinitrophenylhydrazine (DNPH) in 1 N HCl. The mixture was held 2 h in the darkness and then extracted with $CHCl_3$ (3 × 2 mL). The organic layers were collected and evaporated to dryness and the residues purified by TLC (R_f 0.5–0.75, benzene as eluent) to isolate the aldehyde band. The aldehyde dinitrophenylhydrazones in this band were eluted with methanol (3 × 2 mL), which was evaporated to dryness. The residue was dissolved in 500 μL of methanol, and the aldehydes were separated by HPLC on a Whatman PartiSphere 5-μm C_{18} column using methanol–water (80:20) as eluent. A flow rate of 1 mL/min was used in all the experiments.

The final extraction of the incubation mixture after 24 h of reaction was made to remove lipid products and dissociate lipid–protein complexes. The presence of these lipoprotein complexes complicated the subsequent fractionation of the different protein products formed in these reactions. The removal of non chemically bound lipids by extractions was monitored by measuring radiactivity in those incubations containing [1-^{14}C]linoleic acid 13-hydroperoxide ([1-^{14}C]LOOH) and βLG B. Samples of the incubation mixture and the protein (when this was precipitated) were taken, and the radioactivity was counted on a scintillation spectrometer (Packard Model Minaxi Tri-Carb, 4000 Series).

Protein polymers formed during the incubation between 13-LOOH and βLG B were fractionated by Sephadex column chromatography. Sephadex G-100 and G-150 columns were equilibrated with standard buffer or a buffer containing 0.5 M Tris-HCl, pH 8.8, and 0.1% SDS. In the first case, the sample was dissolved in the same buffer, and in the second one, the sample buffer also contained 10% β-mercaptoethanol to break disulfide bonds. Three-milliliter fractions were collected and tested for protein by absorption at 280 nm and for fluorescence using

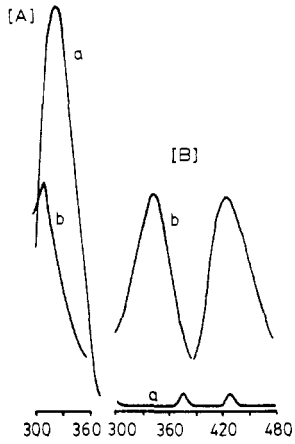

Figure 1. (A) Fluorescence emission spectra of βLG B at the beginning of incubation with 13-LOOH (a) and after 1 h (b). Excitation was done at 285 nm. (B) Fluorescence spectra of fluorescent substances formed from 13-LOOH and βLG B. Spectra were taken at the beginning of the reaction (a) and after 20 h (b). All the spectra were taken under the same conditions.

fluorescence excitation at 360 nm and emission at 430 nm.

Some samples of extracted and precipitated protein were submitted to sulfitolysis to break S–S bonds by the method of Cole (1967).

RESULTS

The reaction between 13-LOOH and βLG B proceeded rapidly with binding of 13-LOOH to βLG B, development of fluorescence, loss of free amino groups, and polymerization of protein. When the 13-LOOH emulsion was mixed with βLG B, the solution became clear and, concurrently, the fluorescence emission spectrum of the protein at 327 nm (excitation at 280 nm) decreased substantially (Figure 1A). The remaining peak was mainly contributed by the buffer and remained unchanged during the reaction.

A marked formation of fluorescent substances, other than that of βLG B was observed with time (Figure 1B). These fluorescent substances showed the same fluorescence spectra (with excitation and emission maxima at 350–360 and 420–430 nm, respectively) as those formed, for example, in the reaction between phosphatidylcholine hydroperoxide and 1-aminopentane (Iio and Yoden, 1988) or the fluorescent lipofuscin pigments (associated with aging) found in animal tissues (Tappel, 1973). The development of this fluorescence was linear for the first 20 h until a maximum was attained, and, subsequently, there was a slight decrease (Figure 2). This fluorescence was not observed when the reaction was carried out with Rβ LG B (data not shown), showing that presence of free amino groups was critical for the development of the fluorescent materials.

The free amino groups of βLG B decreased continuously without attaining a minimum (Figure 2). This behavior did not parallel the development of the fluorescence (Figure 2) and suggests that amino groups were involved not only in this fluorescence but also in other reactions.

Aldehydes are important secondary products of lipid autoxidation (Pokorny et al., 1987) and can react with amino groups forming Schiff bases which may be further dimerized by aldolization reactions with abstraction of an amine (Patrick, 1952) or trimerized to pyridinium salts (Suyama and Adachi, 1979). Analysis of lipid reaction products between 13-LOOH and βLG B revealed the presence of hexanal, propanal, butanal, and pentanal (Figure 3). When pure aldehydes were reacted with βLG B, they produced a substantial decrease of free amino groups (Figure 4A), and fluorescent substances were concurrently formed (Figure 4B).

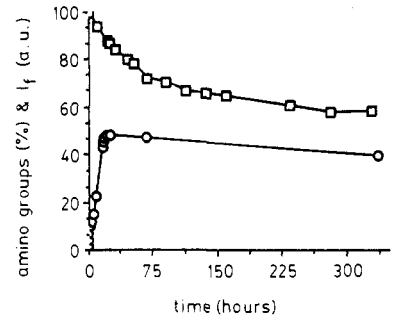

Figure 2. Changes in fluorescence (○) and percentage of free amino groups (□) of the complex formed during the reaction of 13-LOOH with βLG B.

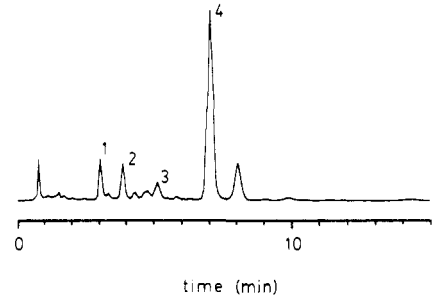

Figure 3. Separation by HPLC of the dinitrophenylhydrazone derivatives of carbonyl compounds produced in the reaction between 13-LOOH and βLG B. Peaks: 1, propanal; 2, butanal; 3, pentanal; 4, hexanal. Other peaks are unknown.

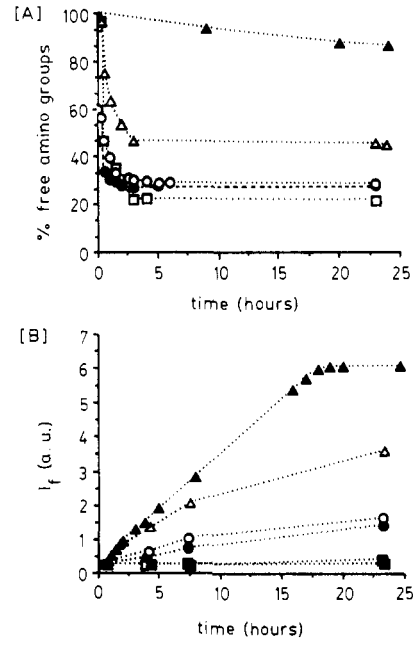

Figure 4. Changes in percentage of free amino groups (A) and fluorescence intensity (B) of complexes formed in the reactions between 13-LOOH and βLG B (▲), hexanal and βLG B (△), pentanal and βLG B (○), butanal and βLG B (●), and propanal and βLG B (□).

The proteins from reaction between 13-LOOH and βLG B showed different SDS-PAGE patterns when the sample was treated with β-mercaptoethanol and when the reducing agent was absent. When the sample buffer contained β-mercaptoethanol, SDS-PAGE showed the presence of three bands, two of molecular weights corresponding to a monomer and one corresponding to a dimer of βLG (Figure 5, line c). When β-mercaptoethanol was absent from the sample buffer, one band of molecular weight corresponding

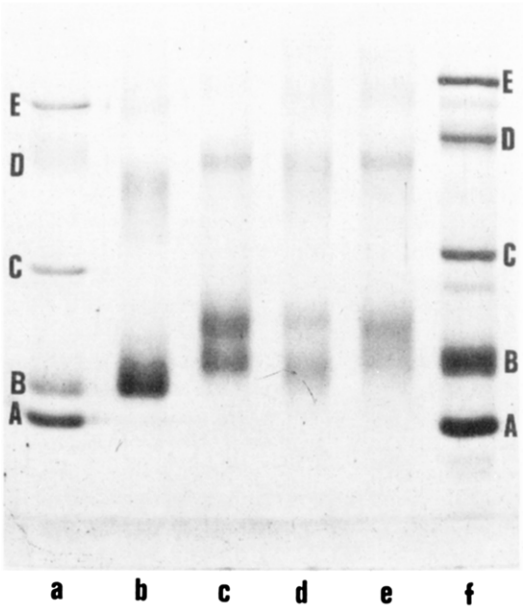

Figure 5. SDS-PAGE of protein products obtained in the incubation of 13-LOOH and βLG B after 24 h (b, c) and after the sample was submitted for sulfitolysis (d, e). (a) and (f) correspond to molecular weight standards: lysozyme, 14 300 (A); β-lactoglobulin, 18 400 (B); α-chymotrypsinogen, 24 000 (C); egg albumin, 45 000 (D); BSA, 66 000 (E). Lines a, b, and d correspond to samples where β-mercaptoethanol was omitted from the sample buffer, and lines c, e, and f were obtained when the reductive agent was present.

to a monomer of βLG B and another broad band corresponding to a dimer were observed (Figure 5, line b). This suggested that two different types of dimers of βLG B were present, one of which could be easily broken by β-mercaptoethanol. To confirm this, protein extracted from an aliquot of the reaction mixture was submitted to sulfitolysis to break disulfide bonds. SDS-PAGE of the protein obtained after this treatment did not show any difference with or without treatment with β-mercaptoethanol (Figure 5, lines d and e). This confirmed the presence of two types of dimers, one being a S–S dimer and the other a C–C dimer or C–N dimer (C–C dimer) that was not broken by reduction with β-mercaptoethanol. The presence of a small band corresponding to a trimer of βLG B was also observed and was not affected by the presence of β-mercaptoethanol in the sample buffer. The substitution of βLG B with RβLG B in the reaction mixture prevented the formation of stable polymers.

The relative rates of formation of the disulfide- and non-disulfide-linked polimers were studied by SDS-PAGE. Samples of the reaction mixture between 13-LOOH and βLG B were collected at different intervals of time, diluted with sample buffer containing β-mercaptoethanol, and analyzed by SDS-PAGE. The protein bands on the gels were quantified densitometrically, and the kinetics of their formation are shown in Figure 6. The results showed rapid formation of the S–S dimer and a slower rate of formation of the C–C dimer.

Separation of proteins from the reaction mixture on Sephadex G-150 after 24 h of reaction revealed several peaks corresponding to different products (Figure 7). These data suggested the presence of several lipoprotein complexes in addition to the polymerized βLG B. To eliminate the presence of these lipid protein complexes that impeded the fractionation of the different polymers of βLG B, the lipid–protein complexes were exhaustively extracted with diethyl ether and the protein was precip-

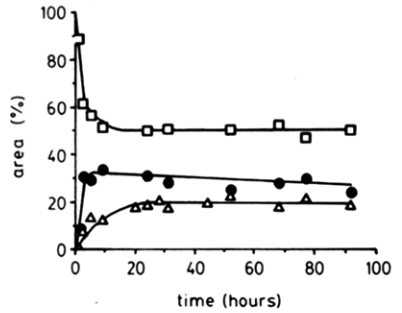

Figure 6. Kinetics of formation of S–S (●) and C–C (△) dimers and disappearance of the initial monomer (□) as determined by densitometry of SDS-PAGE of samples of incubation between 13-LOOH and βLG B. A small quantity of trimer was also present.

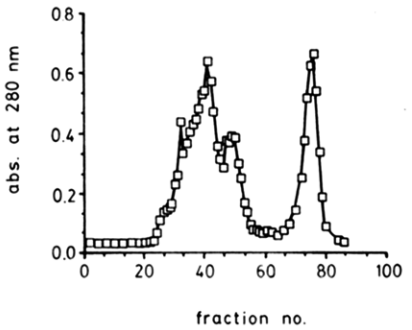

Figure 7. Separation of proteins from incubation mixture between 13-LOOH and βLG B after 24 h on Sephadex G-150. The column was equilibrated with standard buffer, and proteins were eluted with the same buffer and monitored by UV at 280 nm.

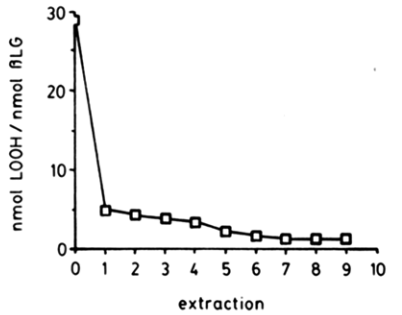

Figure 8. Radioactivity remaining in the protein phase of reaction between [1-^{14}C]LOOH and βLG B during sequential extraction with ether as described in the text. Point 0 corresponds to the reaction mixture before extraction, 1–4 correspond to the reaction mixture after extraction of lipids with ether, 5 corresponds to the protein after precipitation, and 6–9 correspond to the protein after washing with ether–ethanol (1:1).

itated according to the previously described procedure. The extent of removal of lipids was monitored by measuring radioactivity in the ether and polar phases. The radioactivity remaining in the system was measured, and the results showed that the exhaustively washed and precipitated βLG B had a radioactivity that corresponded approximately to one molecule of fatty acid per 18 000 molecular weight of the initial βLG B (Figure 8).

Proteins from the interaction between 13-LOOH and βLG B were also fractionated on a Sephadex G-100 column equilibrated with SDS/Tris-HCl, pH 8.8. The sample buffer contained 10% β-mercaptoethanol. Four peaks were obtained in this separation, the first two corresponding to the C–C dimer and monomers (some from the S–S dimer), respectively (Figure 9). Peak a (C–C dimer) showed most of the fluorescence, suggesting that fluorescence and non-disulfide polymerization were related. The amount

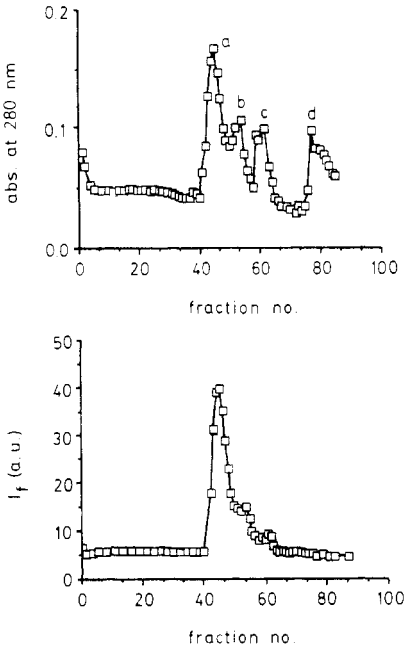

Figure 9. Separation of proteins from reaction mixture between 13-LOOH and βLG B on Sephadex G-100 after extraction of lipids as described in the text. The column was equilibrated with 0.5 M Tris-HCl, pH 8.8, and 0.1% SDS, and the sample was introduced in the same buffer containing 10% β-mercaptoethanol. Proteins were eluted with SDS/Tris buffer and monitored by UV at 280 nm and by fluorescence with excitation and emission maxima at 360 and 430 nm, respectively.

of protein in peak b, though it showed a lower absorbance at 280 nm, was greater than peak a, in agreement with Figure 6 where the monomer plus S–S dimer represented ≈75% of total protein peak area.

DISCUSSION

When the 13-LOOH emulsion was mixed with βLG B, the solution became clear, suggesting that the lipids bound to βLG B (O'Neill and Kinsella, 1987; Smith et al., 1983; Spector and Fletcher, 1969). This lipid–protein complex (C) facilitated further interaction and oxidation.

The first observed effect was the disappearance of the fluorescence emission spectrum of the protein (Figure 1A). Fluorescence emission spectrum in proteins is due to the presence of tryptophan and tyrosine residues (Freifelder, 1982). The disappearance of this band from the fluoresecence spectrum suggests a destruction of tryptophan residues. Tyrosine residues may be quenched (Freifelder, 1982). Tryptophan residues are among the most sensitive to degradation by peroxidizing lipids (Gardner, 1979; Kanazawa et al., 1975), and its transformation into β-oxyindolalanine and *N*-formylkynurenine by peroxidzing methyl linoleate has been studied in detail (Yong et al., 1980). The position of tryptophan residues on the surface of βLG B (Papiz et al., 1986) may have facilitated this reaction.

The disappearance of the intrinsic fluorescence of the protein was accompanied by the formation of new fluorescent substances within 20 h (Figures 1B and 2). The formation of these new fluorescent substances reflects interaction of the oxidized lipid with amino groups in the protein because when the incubation was made between 13-LOOH and RβLG B, no fluorescent compound was observed. The kinetics of development of fluorescence and loss of free amino groups in the protein were not parallel (Figure 2). Thus, the formation of fluorescence seems to be related to an initial reaction that took place during the first 20 h after which decreases only in free amino groups were observed, reflecting reactions with other products, e.g. aldehydes from degradation of 13-LOOH.

These aldehydes are major secondary products of lipid autoxidation (Pokorny et al., 1987), and they have been shown to interact with free amino groups in model peptides (Zamora et al., 1987, 1989). Thus, analysis of HPLC showed the presence of short-chain aldehydes (Figure 3). When pure samples of these were reacted with βLG B under the same conditions as 13-LOOH, the free amino groups in the protein decreased (Figure 4A) and fluorescent compounds were developed (Figure 4B). This suggests that some of the reactions observed may be due to the reaction of these aldehydes from 13-LOOH with free amino groups, which formed complexes that contributed to the fluorescence of the mixture. The slight decrease in the number of free amino groups in the reaction between 13-LOOH and βLG B during the first 24 h (Figure 4A) suggests that the contributions by the reactions involving the short-chain aldehydes to the fluorescence is unimportant, and the major fluorescent chromophore was formed between amino groups of the protein and the linoleic hydroperoxide itself or some secondary products other than the short-chain aldehydes.

SDS-PAGE of the reaction mixture between 13-LOOH and βLG B after 24 h showed the presence of one monomer of βLG B and two dimers, one being a S–S dimer and the other a non-disulfide-linked polymer (C–C dimer) (Figure 5). It is known that the oxidation of cysteine in peptides under exposure to oxidizing agents (including linoleic acid hydroperoxide) produces a number of dimers of these peptides (Finley et al., 1981). This reaction is fast, and when it was carried out between 13-LOOH and glutathione (GSH), only 12% of the initial SH was retained after 8 h of oxidation (Finley et al., 1981). It is reasonable to suggest that a similar reaction can occur with βLG B in the presence of 13-LOOH because of the relative proximity between the SH group of the tryptophan (Papiz et al., 1986) and the ease of access of the acyl hydroperoxide to the tryptophan residues, which were quickly destroyed (Figure 1A).

The kinetics of formation of S–S and C–C dimers (Figure 6) showed a quick formation of the S–S dimer, analogous to the rate for the reaction of 13-LOOH with GSH (Finley et al., 1981). The formation of the non-disulfide-linked dimer was slower and did not reach maximum until 24 h. The similarity between the kinetics of development of fluorescence (Figure 2) and formation of C–C dimer (Figure 6) may indicate a relationship between both processes. This was confirmed by separation of the delipidated and reduced protein products on Sephadex G-100 (Figure 9). The main fluorescent peak was the C–C dimer.

The apparent decrease in the proportion of S–S dimer with time (Figure 6) may indicate that the S–S dimer was transformed into C–C dimer without necessarily destroying the disulfide bond.

Finally, the fact that, after extraction of non chemically bound lipids, the protein retained radioactivity when the incubation was conducted with [1-^{14}C]LOOH implies the presence of bound lipid molecules in the final complex. Oxidized lipids have been found in protein–lipid complexes formed without chain breaking (Gardner, 1979; Iio and Yoden, 1988).

In summary, on the basis of the above results, we propose a reaction scheme for the interaction between the 13-LOOH and the βLG B that might be general for this type of reactions (Figure 10). When the lipid is mixed

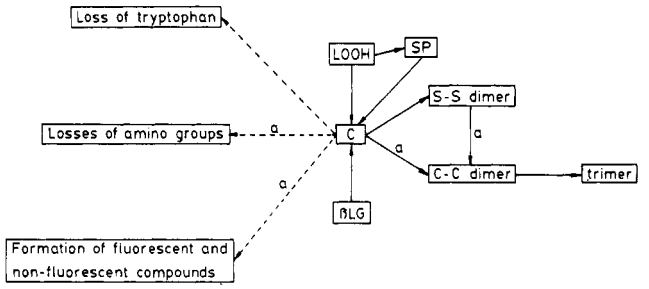

Figure 10. General scheme showing the possible reactions that occurred in the incubation between 13-LOOH and βLG B. The initial interaction between 13-LOOH and βLG B involved the binding of the lipid to the protein. This complex (C) facilitated the oxidizing action of the hydroperoxide that caused loss of tryptophan and formation of a disulfide-linked dimer (S–S dimer). The formation of a fluorescent non-disulfide-linker dimer (C–C dimer), which evolved to higher polymers, was also observed. Secondary products (SP) of decomposition of the hydroperoxide complicated the process by reacting with free amino groups of the protein and forming both fluorescent and nonfluorescent compounds. The blockage of protein amino groups prevents all the reactions marked a.

with the protein the formation of a lipid–protein complex (C) is observed. This facilitates the chemical interaction that begins with the hydroperoxide, producing oxidative changes in tryptophan and cysteine residues. At the same time, but more slowly, the hydroperoxide itself or some secondary product reacts with protein amino groups to generate fluorescent non-disulfide dimers, the structure of which is unknown at present. The reaction is complicated for other secondary products, such as short-chain aldehydes, that react with free amino groups and form both fluorescent and nonfluorescent compounds. The blockage of protein amino groups prevents all the reactions marked a.

Registry No. 13-LOOH, 18320-18-8; Trp, 73-22-3; Cys, 52-90-4; propanal, 123-38-6; butanal, 123-72-8; pentanal, 110-62-3; hexanal, 66-25-1.

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Received for review August 4, 1988. Accepted December 27, 1988. Supported in part by the National Dairy Board and Wisconsin Milk Marketing Board.

Bleaching of β-Carotene by Trout Gill Lipoxygenase in the Presence of Polyunsaturated Fatty Acid Substrates

Richard A. Stone and John E. Kinsella*

The 12-lipoxygenase (LO) from trout gill effectively bleaches β-carotene in conjunction with the peroxidation of different polyunsaturated fatty acid (PUFA) substrates. The maximum velocity of bleaching differed significantly between LO from gill and the 15-LO from soybean for six PUFA substrates, compared to linoleic acid. The lag times before reaching maximum velocity of bleaching were shorter for trout gill LO than were those catalyzed by soybean LO for six of the eight PUFA substrates tested. This may reflect the presence of oxidative cofactors in the trout gill LO preparation.

Plant lipoxygenases (LO) have been extensively studied with regard to fatty acid peroxidation (Yoon and Klein, 1979; Klein et al., 1984). The enzyme has been implicated as a major contributor to off-flavors in legumes (Kalbrener et al., 1974) and in causing chemical changes in fruits and vegetables (Tressl et al., 1981). LO also plays a role in bread making by increasing mixing tolerance, serving as a bleaching agent, and improving dough rheology (Faubion and Hoseney, 1981). Recently, researchers have prepared LO from fish and other animal sources, in sufficient purity, for studying lipid oxidation rates (German and Kinsella, 1985; Yokoyama et al., 1986). The LO isolated from trout converts polyunsaturated fatty acids (PUFA) from fish muscle into PUFA hydroxyperoxides that can generate flavors and off-flavors (German and Kinsella, 1985; Hsieh and Kinsella, 1989). This LO action may cause discoloration of certain fish (Tsukuda, 1970), conceivably by bleaching of carotenoids following free-radical quenching (Krinsky and Deneke, 1982; Halevy and Sklan, 1987; Kanner et al., 1987). In order to assess this possibility, we compared the relative capacities of the LO isolated from trout gill and soybean to catalyze β-carotene bleaching in the presence of various PUFA substrates of both $n-6$ (ω6) and $n-3$ (ω3) families.

MATERIALS AND METHODS

The polyunsaturated fatty acids (PUFA; 99+% purity) linoleic (18:2, $n-6$), eicosadienoic (20:2, $n-6$), homo-γ-linolenic (20:3, $n-6$), eicosatrienoic (20:3, $n-3$), arachidonic (20:4, $n-6$), docosatrienoic (22:3, $n-3$), docosatetraenoic (22:4, $n-6$), and docosahexaenoic acid (22:6, $n-3$) were obtained from Nu-Chek Prep (Elysian, MN). These unsaturated fatty acids were dissolved in ethanol, diluted to a concentration of 25 mM, and stored at −70 °C under a nitrogen atmosphere.

Soybean 15-lipoxygenase (Type II, E.C. 1.13.13) (Sigma Chemical Co., St. Louis, MO) (10 mg) was dissolved in 100 mL of 0.05 M phosphate buffer (pH 7.8) and used as such. Trout gill containing LO (5 mg of protein), prepared by the method of German et al. (1986), was dissolved in 100 mL of 0.05 M phosphate buffer (pH 7.8) and used as such. Both LO preparations readily caused peroxidation of PUFA as assessed by oxygen consumption and autoradiography (Hsieh et al., 1988).

β-Carotene bleaching was assessed with pure β-carotene dispersed via the method of Aziz et al. (1971). Thus, 25 mg of β-carotene was dissolved in 25 mL of chloroform with 1 mL of Tween 80 detergent (ICI America's Inc., Wilmington, DE). Of this solution, 1 mL was evaporated to dryness on an aspirator and reconstituted in 10 mL of distilled water to yield a clear micellar solution (19 mM) of β-carotene.

The reaction mixture of trout gill LO contained 75 μg of trout gill protein dissolved in 1.5 mL of 0.05 M phosphate buffer (pH 7.8), 0.3 mL of emulsion containing β-carotene (30 mM), and different PUFA substrates (180 μM) for a total reaction volume of 1.8 mL.

The reaction mixture using soybean LO contained 150 μg of LO dissolved in 1.5 mL of 0.05 M phosphate buffer (pH 7.8), 0.3 mL of emulsion containing β-carotene (30 mM), and different PUFA substrates (180 μM) for a total reaction volume of 1.8 mL. The time course of bleaching at 25 °C was recorded on a Cary 219 spectrophotometer (Varian, CA) by measuring the decrease in absorbance at 460 nm, the wavelength of maximum absorbance of β-carotene, following initiation of the reaction by addition of the PUFA substrates.

RESULTS AND DISCUSSION

Initially, bleaching activity was calibrated by determining the amounts of the soybean and trout gill lipoxygenase preparations that caused similar maximum velocities of bleaching of β-carotene with linoleic acid as the substrate. Thus, 100 μg of soybean LO and 50 μg of

Institute of Food Science, Cornell University, Ithaca, New York 14850.

0021-8561/89/1437-0866$01.50/0