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Oxidation of β-Carotene by Bovine Milk Lactoperoxidase–Halide–Hydrogen Peroxide Systems

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The halide-mediated peroxidase-catalyzed oxidation of β-carotene was studied as a model system for lipid peroxidation. Of the halide ions tested, Br^-, I^-, and SCN^- all caused some degree of β-carotene oxidation at pH 7.0 and 4.4. No oxidation was observed with Cl^-, however, which is consistent with Cl^- not being a substrate for lactoperoxidase. The β-carotene oxidation caused by the lactoperoxidase–I^-–H_2O_2 system was pH dependent, the rate of oxidation being 100-fold higher at pH 4.4 than at pH 7.0. When both I^- and SCN^- were present, the oxidation rate was reduced. At least partially, this interference between the two substrates was due to competition for the substrate-binding sites on the enzyme.

The enzyme lactoperoxidase (LP) (EC 1.11.1.7), which occurs in milk from several species, forms an antibacterial system together with the substrates thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2) (Reiter et al., 1963; Björck et al., 1975; Reiter, 1978a, 1979). The general product of the peroxidase-catalyzed reaction is presumed to be hypothiocyanite ($OSCN^-$) (Hoogendoorn et al., 1977), but other compounds might also be formed (Hogg and Jago, 1970; Björck and Claesson, 1980; Pruitt et al., 1982). Hypothiocyanite reacts with protein sulfhydryl groups, and the antibacterial effect is inhibited by sulfhydryl-containing reducing compounds, like cysteine and dithiothreitol (Aune and Thomas, 1978; Thomas and Aune, 1978). LP can also catalyze the peroxidation of the halides bromide and iodide (Morrison and Schonbaum, 1976).

Lipid peroxidation might be mediated by peroxidase-catalyzed oxidation of halides (Benenson et al., 1980). A direct bleaching of β-carotene was observed with horseradish peroxidase (Ben Aziz et al., 1971). Kanner and Kinsella (1983a,b) have developed a model system for the study of the peroxidation of β-carotene and linoleate, mediated by the LP-catalyzed peroxidation of various halides. By dissolving β-carotene in the presence of a detergent it is possible to get a stabilized water solution in which it is possible to directly follow its oxidation.

This paper deals with the halide-mediated oxidation of β-carotene by lactoperoxidase and SCN^-, I^-, and Br^- at pH 4.4 and 7.0 and the interaction between SCN^- and I^- at different concentrations.

MATERIALS AND METHODS

Chemicals. LP was purchased from Boehringer Mannheim (Mannheim, West Germany). H_2O_2 (30%, analytical grade) and β-carotene (synthetic, 97%) were from Merck (Darmstadt, West Germany). All other chemicals used were of analytical grade, and double-distilled water was used in all solutions. According to the specifications given by the manufacturer the sodium chloride contained less than 0.005% Br^- and 0.001% I^-.

Aqueous β-Carotene Solution. A 25-mg portion of β-carotene and 0.9 mL of Tween 80 were dissolved in 25 mL of chloroform. A 1-mL portion of this solution was evaporated to dryness under vacuum and the residue dissolved immediately in 10 mL of 0.25% EDTA solution and filtered through filter paper. Thereafter, 40 mL of 0.01 M sodium acetate buffer (pH 4.6) was added. The β-carotene solution was prepared on the day of the experiment (Ben Aziz et al., 1971). The final concentration of β-carotene was adjusted to ~14 μM, as determined spectrophotometrically.

β-Carotene Oxidation Assay. The amount of unreacted β-carotene was measured as the absorption at 460 nm. The absorbance measurements were carried out on

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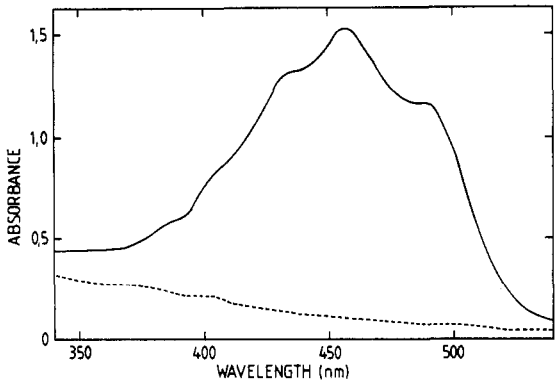

Figure 1. Absorption spectra of β-carotene in reduced (—) and oxidized (---) form. The β-carotene was dissolved as described in Materials and Methods (final concentration 18 μM).

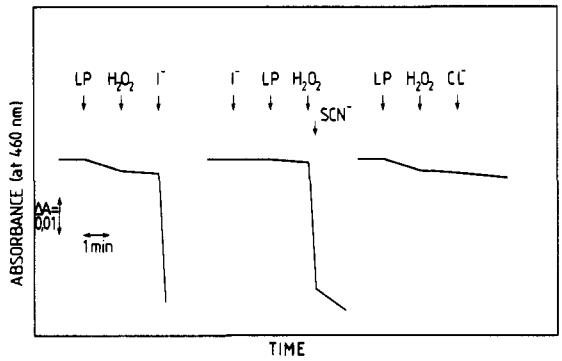

Figure 2. Time course of β-carotene oxidation in the presence of LP, H_2O_2, and various halides at pH 4.4 (0.01 M sodium acetate buffer). Final concentrations: [β-carotene] = 14 μM; [LP] = 0.4 μg mL^{-1}; $[I^-]$ = 20 μM; $[H_2O_2]$ = 400 μM; $[SCN^-]$ = 100 μM; $[Cl^-]$ = 300 mM.

a Perkin-Elmer Hitachi 200 double-beam spectrophotometer. The reaction mixture contained 2.5 mL of aqueous β-carotene solution in 0.01 M sodium acetate buffer (pH 4.4) or in 0.01 M imidazole–HCl buffer (pH 7.0). A 20-μL portion of LP solution (100 μg mL^{-1}) and 100 μL of 10 mM H_2O_2 solution were added to give final concentrations of 0.8 μg mL^{-1} (~10 nM) and 0.4 mM, respectively. The experiments were carried out at 30 °C. The concentrations of SCN^- and I^- were varied in the range 0–100 μM.

RESULTS AND DISCUSSION

The spectra of reduced and partly oxidized β-carotene (Figure 1) show the decrease in absorbtion in the band at 450–460 nm following oxidation. If a time course of the reaction was followed (Figure 2), some features were repeatedly observed. A tendency for β-carotene to autoxidate was shown in the presence of LP before addition of any substrate. A long-term change in the β-carotene spectrum might also depend on a changing environment in the solution. If the water content increases, it is known that the extinction in the visible region is reduced and that peaks near the ultraviolet region appear (Hager, 1970). Addition of halide nearly always abolished this autoxidation, and it was also substantially decreased by the presence of hydrogen peroxide. A direct reaction between LP and lipid compounds mediated by iron-catalyzed production of reactive oxygen radicals has been reported in the case of denatured lactoperoxidase (Eriksson, 1970) and horseradish peroxidase (Eriksson et al., 1971).

As soon as the reaction mixture contained all the components in the peroxidase system—enzyme, halide, and hydrogen peroxide—the oxidation of β-carotene started (Figure 2).

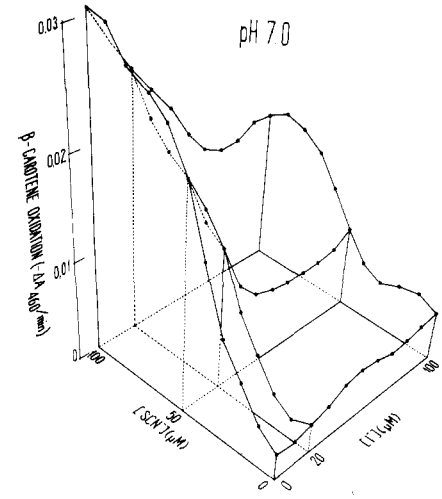

Figure 3. β-Carotene oxidation in the presence of LP, H_2O_2, I^-, and SCN^- at pH 7.0 (0.01 M imidazole–HCl buffer). Reaction mixture contained LP (0.4 μg mL^{-1}), H_2O_2 (400 μM), and various concentrations of SCN^- and I^-.

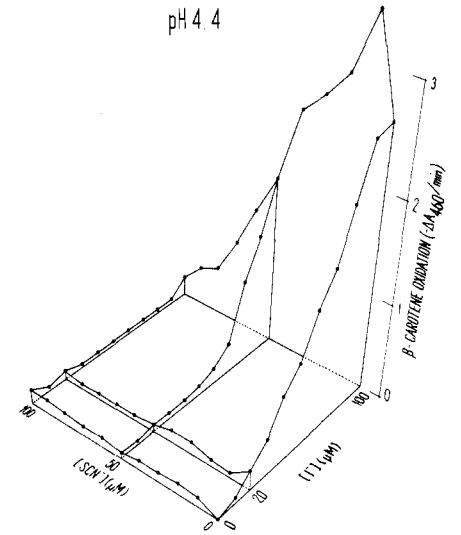

Figure 4. β-Carotene oxidation in the presence of LP, H_2O_2, I^-, and SCN^- at pH 4.4 (0.01 M sodium acetate buffer). Conditions are as in Figure 3.

In a survey experiment the effect of thiocyanate was compared to the effect of the halides I^-, Br^-, and Cl^-. The optimal concentrations of halide and H_2O_2 found by Kanner and Kinsella (1983a,b) were confirmed. Kanner and Kinsella (1983a) also reported that β-carotene oxidation was mediated by Cl^- at concentrations about 0.1–0.2 M. For the LP concentration used in this experiment, it has not been possible for us to obtain similar results (various concentrations of Cl^- between 0.1 and 0.3 M were tested). Even the small autoxidation in the presence of LP was unaffected by the addition of Cl^- to the reaction mixture. This is the case of both pH 4.4 and 7.0. A certain level of other halides is found even in the purest qualities of NaCl, as has been discussed for the case of stimulation of chloroperoxidase catalatic activity by Br^- (Griffin, 1983).

A study of the interference between I^- and SCN^- in the concentration ranges 0–100 μM at pH 4.4 and 7.0 (Figures 3 and 4) showed that the simultaneous presence of both I^- and SCN^- reduced the oxidation rate of β-carotene. At pH 4.4 the I^--mediated oxidation was nearly 100 times larger than at pH 7.0 (note the different scales in Figures 3 and 4). The addition of SCN^- inhibited the reaction, and as can be seen from a comparison with the values at pH 7.0, SCN^- possessed only a slight β-carotene bleaching

ability in itself which was of the same order of magnitude both at pH 4.4 and at pH 7.0.

The three-dimensional diagrams show that the stoichiometric relation required for maximal inhibition is coarsely 1:1. The interference between I^- and SCN^- was probably at least partially related to their competition for the substrate site on LP. The inhibitory effect was immediate and, as shown in complementary experiments, independent of the order in which the components were added.

The mechanism for β-carotene oxidation with iodide as substrate at low pH might be connected with the production of I_2 as the immediate reaction product as suggested for the antibacterial effect of the LP–I^-–H_2O_2 system (Thomas and Aune, 1977). SCN^- is reported to inhibit the iodination of tyrosine competitively (Tenovuo, 1978) but stimulates the coupling reaction leading to the formation of thyroid hormone (Virion et al., 1980). The presence of a regulatory binding site for SCN^- might also explain the small increase in activity at $[I^-] = 100$ μM and $[SCN^-] = 10$–20 μM (Figure 4), which is also observed in classical LP activity assays using chromogene substrates in the presence of SCN^- (Ekstrand, unpublished results). The kinetics of tyrosine iodination is complex and is regulated by both the substrates and the products mono- and diiodotyrosine (Taurog et al., 1974; Taurog and Nakashima, 1978; Virion et al., 1981).

β-Carotene is commonly considered as a scavenger of oxygen radicals, e.g. singlet oxygen, $^1O_2$* (Foote and Denny, 1968). That $^1O_2$* is formed is generally accepted in the myeloperoxidase–Cl^-–H_2O_2 system (Rosen and Klebanoff, 1977), where OCl^- is thought to react with H_2O_2 to form $^1O_2$* and Cl^-. Formation of $^1O_2$* has also been shown to occur in the LP–Br^-–H_2O_2 system (Piatt and O'Brien, 1979; Kanofsky, 1983). It is possible that the effective β-carotene oxidation mediated by I^- involves $^1O_2$* formation. That acid pH favors the oxidation of H_2O_2 by Br_2 is shown by the virtually catalatic effect of chloroperoxidase (Griffin, 1983). However, the specificity of β-carotene as a singlet oxygen scavenger in water emulsions has been disputed (Lindig and Rodgers, 1981). Another possible mechanism is the ability of I_2 to add to double bonds in lipid molecules (Boeynaems et al., 1981). The primary peroxidase-catalyzed oxidation product of I^- appears to be I^+, which has been shown both in the case of iodination of tyrosine catalyzed by thyroid peroxidase (Ohtaki et al., 1982) and for the pseudocatalatic effect of lactoperoxidase (Huwiler and Kohler, 1984). For further discussion of the mechanism of halide-mediated oxidation, see Kanner and Kinsella (1983c) and Neary et al. (1984). That carotenoids might act as target substances protecting against the antibacterial peroxidase–halide systems is indicated by the nonresistance of carotenoid deficient mutants of *Sarcina lutea* (Krinsky, 1974; Reiter 1978b).

In the LP–SCN^-–H_2O_2 system, $OSCN^-$ (Hoogendoorn et al., 1977; Pruitt and Tenovuo, 1982) and presumably higher oxyacids of SCN^- (Hogg and Jago, 1970; Björck and Claesson, 1980; Pruitt et al., 1982) are the main oxidative agents. So far there is no evidence that oxygen radicals are involved in the antibacterial action of this system (Pruitt et al., 1982).

From the present studies of the LP-catalyzed halide peroxidation and its effect on β-carotene, it is possible to conclude that the I^--mediated oxidation of β-carotene observed at pH 4.4 is not shown at neutral pH or when I^- is replaced by SCN^-. This confirms the observed specificity of the LP–SCN^-–H_2O_2 system, which mainly acts on sulfhydryl groups either free or in cysteine side chains of target proteins (Thomas and Aune, 1978). When both SCN^- and I^- are present, they interfere with each other and the oxidation rate is decreased. It has also been impossible to detect any oxidation of β-carotene with Cl^- as the halogen substrate (concentrations ranging up to 0.3 M).

The lactoperoxidase–thiocyanate–hydrogen peroxide system is an active antibacterial factor in several physiological secretions, e.g. milk and saliva. Its mode of action against bacteria has been explained in several ways, but the current opinion is that it very specifically acts on sulfhydryl groups and not by formation of reactive oxygen radical species and general membrane destruction. This is supported by the results presented here. The lack of oxidation of β-carotene indicates that no reactive radical species is formed by the lactoperoxidase–thiocyanate–hydrogen peroxide system. This has implications even for the general peroxidase effects in phagocytosis and extracellular lysis of bacteria in inflammatory situations. The lactoperoxidase system might become increasingly nutritionally and technologically important, and therefore the question of its toxicity and mode of action will be of great interest in the future.

Further studies are necessary to elucidate whether SCN^- might interfere with the halide-mediated lipid peroxidation in other systems, e.g. with cholesterol, and whether lipid peroxidation in general is an essential feature of the antibacterial effect of some of the peroxidase–halide systems.

Registry No. Bromide, 24959-67-9; iodide, 20461-54-5; thiocyanide, 302-04-5; β-carotene, 7235-40-7; lactoperoxidase, 9003-99-0.

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Fatty Acid Composition of Developing Soybeans

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Fatty acid composition and total free fatty acid content of seven soybean [*Glycine max* (L.) Merr.] cultivars at various stages of bean development from the 45th day after flowering up to the 75th day, at 10-day intervals, were determined. In almost all the cultivars amounts of palmitic, stearic, and oleic acids and total free fatty acids decreased while those of linoleic and linolenic acids increased during seed maturation. Minor variability in the pattern of these components in different cultivars of this legume was observed.

Soybean [*Glycine max* (L.) Merr.] is used mainly for oil extraction and meal production as it is a chief source of oil and protein (Smith and Circle, 1972). Extensive investigations on complete chemical composition of developing seeds of this legume have been carried out (Yazadi-Samadi et al., 1977; Yao et al., 1983). However, detailed information on its fatty acid composition is rather scanty (Roehm and Privett, 1970; Rubel et al., 1972; Privett et al., 1973; Manek, 1975). Further, investigations on the comparative changes in fatty acid composition of different cultivars of soybean during bean maturation have so far not been conducted. The objective of this paper is, thus, to examine the extent of variability in fatty acid composition of different cultivars of soybean during seed development. Such information on genetic variability in fatty acid composition may be useful in future efforts to effect quantitative and qualitative improvement in oil content of this legume.

MATERIALS AND METHODS

Seven commercial cultivars of soybean, namely Alankar, Ankur, Black tar, Bragg, Cobb, HM-1 and SH-3 were used in the present studies. The soybean crop was raised in the experimental farm of Haryana Agricultural University, Hissar, India, during the 1982 growing season. The flowers were tagged immediately after their emergence. The pod samples were harvested at intervals of 10 days from the 45th day after flowering, and the sampling was continued up to the 75th day when the crop matured.

Immediately after harvest, the pods were shelled and the collected seed samples were dried at 50 °C in a hot-air oven for 48 h. The dried seeds were ground to a fine powder and extracted with petroleum ether at ambient temperature. The petroleum ether extract was filtered and concentrated in vacuo below 40 °C. The extracted oil was kept in air-tight bottles and stored in a refrigerator until further analysis.

Total free fatty acid content was estimated by titrating the oil sample against standard alkali by AOAC method No. 28.032 (1984), and the results are expressed as equivalent to oleic acid.

Methyl esters of fatty acids were prepared from the oil by transesterification in methanol using sodium methoxide as catalyst (Luddy et al., 1968). Fatty acid methyl esters were separated by GLC using an Aimil Nucon gas chromatograph series 5500 fitted with flame ionization detector and stainless-steel column ($^1/_8$ in. o.d. × 8 ft) packed with 15% polydiethylene glycol succinate on Chromosorb W under standard operating parameters (Gupta and Dhindsa, 1982). The components were identified by comparison of their retention times with those of authentic samples recorded under the same operating parameters. The peak areas were calculated by multiplying peak height with width at half-height and were normalized to relative area percents (McNair and Bonelli, 1969).

RESULTS

The changes in the relative area percents of methyl esters of constituent fatty acids during seed development in the different cultivars of soybean are presented in Table I. A perusal of the data indicates that saturated fatty acids, palmitic and stearic acids, decreased as the seed matured in all the cultivars except Bragg and SH-3, which showed slight increase in palmitic acid during advanced stages of maturity. However, the rate of decrease in palmitic acid content in other cultivars at advanced stages of maturity was much lower as compared to that at earlier stages. Oleic acid content showed a slight increase at initial stages in Alankar, Ankur, and Black tar and then decreased at later stages. In other cultivars studied oleic acid content decreased progressively with seed development. Linoleic acid content increased in all cultivars with advancement of seed maturity. Except in Alankar and Ankur, linolenic acid increased in all other cultivars with seed maturation. In Alankar, it initially increased and later on decreased while in Ankur it remained almost unchanged. Total free fatty acid content in all cultivars decreased with advancing maturity of seeds.

DISCUSSION

The fatty acid composition of soybean oil has been reported by other workers as follows (percent): palmitic,

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