intramammary infusion of the penicillin–novobiocin oil suspension containing chlorobutanol during their prepartum nonlactating period approximately 30 days before her expected calving date.

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Carotene and Chlorophyll Bleaching by Soybeans with and without Seed Lipoxygenase-1

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Carotene and chlorophyll bleaching activities of whole mature seed extracts of two soybean genotypes that lack lipoxygenase-1 (L-1) activity were compared with two soybean genotypes with normal L-1 activity. Assays were conducted with three substrates: (1) methyl linoleate, pH 7.0; (2) linoleic acid, pH 7.0; (3) linoleic acid, pH 9.0. No carotene or chlorophyll bleaching occurred with substrate 1. The two soybean genotypes without L-1 activity showed no conjugated diene formation with substrate 2 or 3 although carotene and chlorophyll were bleached with substrate 2. The presence of L-1 in seed extracts stimulated carotene and chlorophyll bleaching some at pH 7.0 and much at pH 9.0. The presence of L-1 stimulated chlorophyll cooxidation more than carotene cooxidation. L-1 purified from soybean seeds by ammonium sulfate fractionation and ion-exchange chromatography bleached both carotene and chlorophyll. However, the cooxidation of carotene and chlorophyll relative to the peroxidation of linoleic acid (cooxidation potential) is lower for purified L-1 than for whole mature seed extracts. The relevance of this information to food processing is discussed.

It has long been known that soybean seed lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) can cause coupled oxidation of carotene and unsaturated lipids (Sumner and Sumner, 1940). A number of investigators (Weber et al., 1974; Grosch et al., 1977) have presented evidence that soybean lipoxygenase type 2 [lipoxygenase isozymes 2 (L-2) and 3 (L-3) of Christopher et al. (1972)] are responsible for the coupled oxidation of carotene and that lipoxygenase type 1 (L-1) exhibits only slight carotene cooxidation activity. Ramadoss et al. (1978) demonstrated that purified soybean L-2 and L-3 in the presence of methyl linoleate or linoleic acid and oxygen were not effective catalysts of carotene bleaching. However, combinations of either L-1 and L-3 or L-2 and L-3 had a synergistic effect on carotene bleaching. Also, L-3 readily bleached carotene when L-1 or L-2 was replaced with the 13-hydroperoxide isomer from the peroxidation of linoleic acid.

The results of Grosch and Laskawy (1979) indicate that the lipoxygenase isozymes do not act synergistically in carotene oxidation. They also found that the 13-hydroperoxide generated from the action of L-1 and L-2 on linoleic acid effectively stimulated the oxidation of linoleic acid as well as the bleaching of polyenes such as carotene. Ikediobi and Snyder (1977) found that L-1 effectively cooxidized β-carotene while catalyzing the oxidation of linoleic acid.

Holden (1965) reported that lipoxygenase from soybean seeds also was involved in the bleaching of chlorophyll. However, another heat-labile factor involved in the destruction of the lipid hydroperoxide appeared to be necessary for chlorophyll bleaching.

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Additional studies confirmed that the lipid hydroperoxides resulting from lipoxygenase action on linoleic acid accelerate the bleaching of chlorophyll, but other enzymes which are possibly involved have yet to be identified (Imamura and Shimizu, 1974; Peiser and Yang, 1978).

Hildebrand and Hymowitz (1981) screened the U.S. Department of Agriculture soybean germ plasm collection for genotypes with greatly reduced or missing L-1, L-2, or L-3 activity. They found two soybean genotypes [Plant Introduction (P.I.) 133226 and P.I. 408251] that lacked detectable L-1 activity. The study reported herein was undertaken to determine if the lack of L-1 activty has an effect on the carotene and chlorophyll bleaching activities from seed extracts of these L-1 variants relative to "normal" soybeans and if purified L-1 is effective in carotene and chlorophyll bleaching.

EXPERIMENTAL SECTION

Materials and Reagents. Seeds analyzed were harvested from four soybean genotypes (P.I. 133226, P.I. 408251, cv. Amsoy 71, and cv. Williams) grown in the greenhouse in 1980 at Urbana, IL. Lysozyme, Folin reagent, β-carotene, chlorophyll *a*, Tween 20, Tween 80, linoleic acid, and methyl linoleate were obtained from Sigma Chemical Co. (St. Louis, MO). Chromatography equipment and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals (Piscatawa, NJ). All elutions were collected by an ISCO Model 328 fraction collector (Instrument Specialties Co., Lincoln, NE).

Carotene and Chlorophyll Solutions. Aqueous solutions of carotene and chlorophyll were prepared by using the method of Ben-Aziz et al. (1971) as modified by Reynolds (1981). The carotene solution was prepared by mixing 2 mg of β-carotene with 0.09 g of Tween 80 and dissolving in 2 mL of chloroform, which was then evaporated under a stream of nitrogen. The resulting residue

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Table I. Carotene and Chlorophyll Bleaching by Soybeans with and without Lipoxygenase-1 at pH 7.0 with Linoleic Acid as the Substrate[a]

genotype	carotene bleaching, A_{460} min^{-1} (mg of protein)$^{-1}$	chlorophyll bleaching, A_{430} min^{-1} (mg of protein)$^{-1}$	conjugated diene formation, A_{234} min^{-1} (mg of protein)$^{-1}$
Amsoy 71[b]	−0.027[d] ± 0.001[e]	−0.042 ± 0.002	0.31 ± 0.02
Williams	−0.052 ± 0.003	−0.102 ± 0.007	0.61 ± 0.09
P.I. 133226	−0.021 ± 0.002	−0.015 ± 0.003	−0.013 ± 0.003
P.I. 408251	−0.021 ± 0.001	−0.013 ± 0.001	−0.24 ± 0.01
lipoxygenase-1[c]	−2.2 ± 0.2	−7.3 ± 0.3	−123.3 ± 0.4

[a] Substrate 2. [b] Seeds of cultivars Amsoy 71 and Williams have lipoxygenase-1; seeds of P.I. 133226 and P.I. 408251 do not have detectable lipoxygenase-1. [c] Lipoxygenase-1 purified from Williams. [d] Data presented are the means of two sets of six samples except for lipoxygenase-1 where the data presented are the means of three assays. [e] ±SD.

Table II. Carotene and Chlorophyll Bleaching by Soybeans with and without Lipoxygenase-1 at pH 9.0 with Linoleic Acid as the Substrate[a]

genotype	carotene bleaching, A_{460} min^{-1} (mg of protein)$^{-1}$	chlorophyll bleaching, A_{430} min^{-1} (mg of protein)$^{-1}$	conjugated diene formation, A_{234} min^{-1} (mg of protein)$^{-1}$
Amsoy 71[b]	−0.19[d] ± 0.03[e]	−0.73 ± 0.11	2.0 ± 0.3
Williams	−0.18 ± 0.07	−0.83 ± 0.08	2.2 ± 0.3
P.I. 133226	−0.007 ± 0.007	−0.02 ± 0.01	0.08 ± 0.08
P.I. 408251	−0.005 ± 0.004	−0.008 ± 0.005	0.06 ± 0.07
lipoxygenase-1[c]	−1.1 ± 0.02	−2.25 ± 0.06	220.1 ± 0.2

[a] Substrate 3. [b-e] The same as in Table I.

was dissolved in 4 mL of water. The chlorophyll solution was prepared by dissolving 1 mg of chlorophyll *a* and 0.1 g of Tween 80 in 10 mL of acetone and drying dried under nitrogen, and the residue was redissolved in 2 mL of water.

Seed Extracts. The seed extracts were prepared as described by Hildebrand and Hymowitz (1981). For all assays, seed extracts were prepared immediately prior to assaying, and all seed extracts were kept on ice during the assays. Extracts from six individual seeds from plants of all four genotypes were included in each assay. All experiments were performed twice, and the results reported are the means of the 12 determinations.

Substrates. Assays were conducted by using three substrate solutions: (Substrate 1) Methyl linoleate (0.2 mL) was dissolved in 50 mL of 99% ethanol and diluted with 200 mL of 0.1 M phosphate buffer, pH 7.0. (Substrate 2) Linoleic acid (0.2 mL) and 0.2 mL of Tween 20 were dissolved in 50 mL of 99% ethanol. This was diluted with 200 mL of 0.1 M phosphate buffer, pH 7.0. (Substrate 3) Linoleic acid (0.2 mL), 0.2 mL of Tween 20, and 0.6 mL of 1 M KOH were added to 50 mL of water. The solution was shaken until the linoleic acid went into solution and then diluted with 200 mL of 0.2 M borate buffer, pH 9.0.

All substrate solutions were prepared just before assaying and kept on ice during the assays. Linoleic acid and methyl linoleate were stored frozen under nitrogen and only opened in an atmosphere of nitrogen.

Cooxidation and Peroxidation Assays. Immediately prior to assaying, 0.02 mL of aqueous carotene or chlorophyll solution was added to 2 mL of aerated substrate solution. Just after the addition of an appropriate volume of seed extract (usually 0.05 mL to substrates 1 and 2 and 0.01 mL to substrate 3), the solutions were rapidly mixed, and the changes in absorbance were recorded at 460 nm (carotene bleaching), 430 nm (chlorophyll bleaching), or 234 nm (conjugated diene formation). The results are reported as the initial rate of change in absorbance per minute per milligram of protein corrected for a control, which was an identical assay using a seed extract (cv. Williams) which had been boiled for 1 h. All assays were conducted on a Hitachi 110 double-beam recording spectrophotometer at 25 °C (Nissei Sangyo Instruments, Inc., Mountain View, CA).

Lipoxygenase-1 Purification. Lipoxygenase-1 was isolated from soybean seeds (cv. Williams) and purified essentially by the method of Christopher et al. (1972). The lipoxygenase isozymes were separated by ion-exchange chromatography by DEAE-Sephadex A-50 in a 1.6 × 40 cm column. L-3 was washed off the column with the equilibrium buffer (0.02 M sodium phosphate, pH 6.8). L-2 and then L-1 came off the column with the 0.02–0.2 M sodium phosphate buffer, pH 6.8, gradient. The L-1 fractions were pooled, concentrated by lyophilization, and then rechromatographed on a second DEAE-Sephadex A-50 column. The flow rate was 9 mL h^{-1} for both columns. Fractions of 3 mL were collected from the first column and 4.5 mL for the second column. The fractions having peak L-1 activity from the second exchange column were pooled and concentrated. This L-1 preparation was used in the assays for carotene and chlorophyll bleaching as described above using substrates 2 and 3.

Soluble Protein. Protein contents of seed extracts were determined by the biuret method (Clark and Switzer, 1977) and of L-1 by the Lowry method (Lowry et al., 1951) using lysozyme as the standard.

RESULTS

Oxidation and Peroxidation Activity. The assays using substrate 1 showed no difference in carotene or chlorophyll bleaching relative to that of the boiled control for any of the four genotypes used in this experiment. However, conjugated diene formation with substrate 1 was readily detected with all seed extracts showing changes in absorbance at 234 nm min^{-1} (mg of protein)$^{-1}$ ranging from 0.01 to 0.04.

With substrate 2, seed extracts of all genotypes bleached carotene and chlorophyll even though no conjugated diene formation was detected with seed extracts from P.I. 133226 or P.I. 408251 (Table I). All genotypes showed about the same carotene bleaching with substrate 2 except Williams which had somewhat higher activity. Cultivars Amsoy 71 and Williams showed about 3–5 times as much chlorophyll bleaching compared to P.I. 1333226 or P.I. 408251.

With substrate 3, seed extracts of Amsoy 71 and Williams had very active carotene and chlorophyll bleaching activities as well as conjugated diene formation (Table II).

Table III. Cooxidation Potentials of Carotene and Chlorophyll of Seed Extracts and Purified Lipoxygenase-1 at pH 7.0 and pH 9.0

genotype	$-\Delta A_{460}/\Delta A_{234}$ of carotene	$-\Delta A_{430}/\Delta A_{234}$ of chlorophyll
	pH 7.0	
Amsoy 71	0.087	0.135
Williams	0.085	0.167
lipoxygenase-1	0.018	0.059
	pH 9.0	
Amsoy 71	0.095	0.365
Williams	0.082	0.377
lipoxygenase-1	0.005	0.010

P.I. 133226 and P.I. 408251 had no detectable carotene or chlorophyll bleaching activity or conjugated diene formation with substrate 3.

Purified L-1 showed very high carotene and chlorophyll bleaching activity with both substrates 2 and 3 (Tables I and II). Both carotene and chlorophyll bleaching activities were higher at pH 7.0 (substrate 2) than at pH 9.0 (substrate 3) even though conjugated diene formation was greater at pH 9.0.

In all assays there was a slight delay in the cooxidation of carotene and chlorophyll relative to conjugated diene formation.

Cooxidation Potential. Extracts of Amsoy 71 and Williams had about the same carotene cooxidation potential (change in absorbance at 460 nm per milligram of protein divided by change in absorbance at 234 nm mg per milligram of protein) at pH 7.0 as at pH 9.0 but greater chlorophyll cooxidation potential ($\Delta A_{430}/\Delta A_{234}$) at pH 9.0 (Table III). Seed extracts had at least 5 times the carotene cooxidation potential and 2 times the chlorophyll cooxidation potential as purified L-1 at pH 7.0 (substrate 2). Seed extracts had at least 16 times the carotene cooxidation potential and 36 times the chlorophyll cooxidation potential as purified L-1 at pH 9.0 (substrate 3) (Table III).

DISCUSSION

The results reported here clearly demonstrate that soybean seed lipoxygenase-1 can increase the bleaching of both carotene and chlorophyll. At pH 7.0 the presence of L-1 does not appear to have much effect on carotene bleaching even though purified L-1 does bleach carotene. Apparently there is adequate activity of other enzymes in seeds such as L-2 and L-3 to effectively bleach the carotene. The decline in 234-nm absorption with extracts from seeds of the two genotypes that lack L-1 activity is probably due to the large peroxidative activity of the ester isozymes (Ramadoss et al., 1978) and/or due to the presence of other peroxidatic enzymes in soybean seed such as peroxidases (Sessa and Anderson, 1981). This high peroxidative activity could have resulted in more rapid destruction than formation of the linoleic acid hydroperoxides (some hydroperoxides are produced by autoxidation as well as by lipoxygenase catalysis). A much larger effect of L-1 on carotene bleaching is seen at pH 9.0 due to the high activity of L-1 and low activity of L-2 and L-3 (Christopher et al., 1970, 1972).

The greater effect of L-1 on chlorophyll bleaching than carotene bleaching seen in this study may be due to different mechanisms of cooxidation of chlorophyll and carotene. A number of studies have indicated that peroxy radicals resulting from lipoxygenase action can directly oxidize carotene (Arens et al., 1973; Weber et al., 1974; Weber and Grosch, 1976). However, Ikediobi and Snyder (1977) suggested that free radical intermediates could not directly cause the cooxidation of β-carotene. They proposed instead that the peroxy radicals oxidize lipoxygenase-1 to an active (oxidized) enzyme which in turn oxidized β-carotene. Peiser and Yang (1979), on the other hand, presented evidence that sulfite-mediated destruction of β-carotene occurred by a free radical mechanism. The results of Reynolds (1981) indicate different mechanisms of carotene and chlorophyll bleaching by pea lipoxygenase isozymes. The differences in carotene and chlorophyll bleaching in this study may be due to physical differences in the carotene and chlorophyll micelles in the aqueous substrates as well as due to differences of the molecular cooxidation mechanism in this reaction system.

More work is needed to determine the precise mechanism of carotene and chlorophyll bleaching both in vivo and in plant products. It does appear that at least in vitro, the mechanism of carotene and chlorophyll bleaching is different.

A number of investigators (Weber et al., 1974; Ramadoss et al., 1978) have presented evidence that L-1 and L-3 or L-2 and L-3 act synergistically in carotene cooxidation. However, Grosch and Laskawy (1979) found that L-1, L-2, and L-3 did not act synergistically in the carotene cooxidation process. The individual isozymes were as effective as a combination of the isozymes. Reynolds (1981) also found that with lipoxygenase isozymes of pea seed, combinations of the isozymes were not more effective in carotene cooxidation than single isozymes.

Holden (1965) and Ikediobi and Snyder (1977) found that purified L-1 can cause carotene bleaching. However, Holden (1965) found that purified L-1 (commercial) did not bleach chlorophyll. In the study reported herein, purified L-1 caused both carotene bleaching and chlorophyll bleaching and the presence of L-1 in crude seed extracts stimulated both carotene bleaching and chlorophyll bleaching. Perhaps the L-1 peparation used in this study had lost less activity at the time of assaying than those used by Holden (1965). The effectiveness of L-1 in causing carotene and chlorophyll bleaching is most likely due to the fact that L-1 produces the 13-hydroperoxy linoleic acid isomer (Roza and Franke, 1973; Vliegenthart and Veldink, 1977) which is the most effective isomer in carotene bleaching (Ramadoss et al., 1978).

The cooxidation potential of purified L-1 is less than that of crude extracts from soybean seed, indicating perhaps that L-1 is less effective in polyene cooxidation than other enzymes in soybean seeds (such as L-2 and L-3), consistent with other reports (Weber et al., 1974; Ramados et al., 1978). The stimulation of carotene and chlorophyll bleaching at pH 9.0 by the presence of L-1 in extracts of soybean seeds is probably due to the high activity of L-1 in soybean seeds at pH 9.0 generating the 13-hydroperoxy linoleic acid isomer that directly or indirectly stimulates polyene cooxidation.

The results reported here support the results of Grosch and Laskawy (1979) and Reynolds (1981) that there is probably no real synergism of the lipoxygenase isozymes in carotene and chlorophyll cooxidation. The results also show that purified L-1 bleaches carotene and chlorophyll at pH 7.0 and 9.0 but the presence of L-1 in soybean seeds results in only a slight increase in carotene and chlorophyll bleaching at pH 7.0. L-1 is far more stable to heat than L-2 and L-3 (Christopher et al., 1970), and with heat inactiviation of L-2 and L-3 the presence or absence of L-1 in soybean seeds may assume greater significance in carotene and chlorophyll bleaching. Soybean derivatives are increasingly being added to a number of processed food products. Where bleaching of these products is undesir-

able, it may be useful for the processors to use soybeans that lack L-1. In the case of bread making soybean lipoxygenase is effective as a bleaching agent, it increases the mixing tolerance, and it improves dough rheology (Frazier, 1979; Faubion and Hoseney, 1981). Use of soybean genotypes without lipoxygenase-1 may therefore be undesirable in bread making.

Greater knowledge of chemical and biochemical differences of seeds of different soybean genotypes will likely enhance the capacity of soybean derivatives to increase the nutritional and organoleptic properties of processed food products.

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Degradation Kinetics of Betanine in Solutions As Influenced by Oxygen

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The degradation kinetics of betanine were determined for nitrogen-flushed solutions. Trace levels of residual oxygen were found to influence pigment degradation unless a large molar excess of betanine was present. In the absence of oxygen, betanine stability was greatly enhanced and degradation occurred by a 0.5 reaction rate order. An activation energy of 30.7 ± 1.0 kcal/mol was calculated for this reaction.

Determination of stability of quality factors in foods requires an understanding of rate orders and the parameters that influence them. First-order reactions are most common, but other rate orders are often encountered. If first-order kinetics were used to describe a reaction proceeding by a different rate order, major errors can result. When materials are subject to oxidation, the level of oxygen present may affect the rates of destruction.

The oxidative deterioration of fats and oils is well characterized. The kinetics and mechanisms of oxidation for organic materials in aqueous solution have not been as thoroughly explored. Numerous pigments, vitamins, and flavor compounds are susceptible to reaction with oxygen in solution. In this study, the role of oxygen on the degradation rate of betanine in solution was explored.

Betanine is the primary pigment of red beets. Three carboxyl groups, a quaternary amine, and a glucose moiety impart hydrophilic character to the conjugated chromophore. The sensitivity of beet pigments to oxygen has long been known (Vilece et al., 1955; Habib and Brown, 1956). More recently, the effects of various parameters on betanine stability have been researched. In a study of the effects of pH, temperature, light, and air exposure of betanine solution at pH 7.0, oxygen appeared to increase the rate of degradation by 15% at 15 °C (von Elbe et al., 1974). Pasch and von Elbe (1979) measured betanine degradation rates at 75 °C as influenced by organic acids, multivalent cations, antioxidants, and sequestrants. These authors found that both Fe^{2+} and Cu^{2+} at 100 ppm increased rates of betanine loss while 10 000 ppm of citric acid or EDTA prolonged half-life values by 1.5 times. These results are consistent with an oxidative mechanism. The effectiveness of the antioxidants α-tocopherol and ascorbic acid in this study could be attributed to the high level of oxygen that was maintained. Bilyk et al. (1981) found that both ascorbic acid and isoascorbic acid stabilize betanine when used at levels greater than 0.1%.

In the presence of sufficient oxygen, betanine loss in solution follows first-order kinetics. Saguy et al. (1978) observed first-order kinetics with an activation energy of 20.4 kcal/mol for heated betanine solutions. Beet juice in unsealed vials showed similar pigment stability with an

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