Lipoxygenase in the Expressed Juice of Alfalfa Leaves

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The activity of lipoxygenase found in the expressed green juice of *Medicago sativa* leaves was greater than amounts previously reported for other sources. The effect of various antioxidants on oxygen consumption and carotene destruction attributable to lipoxygenase in crude leaf extracts (green juice) was investigated. *tert*-Butylhydroquinone and butylated hydroxytoluene inhibited lipoxygenase 100% under the conditions of the experiments. Protein concentrates derived from green juice having reduced lipoxygenase activity due to addition of antioxidants should be of superior quality.

Leaf protein concentrates (LPC's) offer potential as food sources for animals and humans. A commercial process (Pro-Xan) to produce a protein–xanthrophyll concentrate from *Medicago sativa* (alfalfa) leaves that is used as a poultry feed has been reported (Kohler and Knuckles, 1977; Knuckles et al., 1972). A modified process is being developed for production of a soluble white alfalfa protein concentrate suitable for human consumption (de Fremery et al., 1973; Miller et al., 1975; Edwards et al., 1975; Knuckles et al., 1975). It has been observed, however, that during storage the white LPC develops an "oxidized" odor and flavor which might make it unacceptable for use in food products. When chopped alfalfa leaves are pressed, cell integrity is destroyed, and the resulting juice is a biochemical jumble in which a myriad of chemical reactions, both enzymatic and nonenzymatic, occur. An important degradative reaction may be the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids. The resulting hydroperoxides cause carotene bleaching and react further with lipids and sulfur amino acids to cause oxidized flavors in, and a lower nutritive value of, the final LPC product [see Gardner (1979) and Galliard (1975) for review articles]. This paper describes a preliminary study of lipoxygenase in crude, green alfalfa juice.

EXPERIMENTAL SECTION

Alfalfa tops were harvested before the onset of flowering and immediately dropped into liquid nitrogen. Large pieces of stem were removed and the leaves were ground in a Tekmar (Cincinnati, OH) Model SD-45 homogenizer under liquid N_2. The ground alfalfa leaves were placed in nylon bags, thawed, and pressed with a hydraulic press (~1000 psi). The resulting green juice was either frozen in small containers for future use or stored on ice and used within 2 h for experiments. Lipoxygenase was assayed with an oxygen monitoring system (Yellow Springs Instrument Co., Yellow Springs, OH) as described previously (Wallace and Wheeler, 1975) but with the following modifications: 500 mg of linoleic or linolenic acid (Applied Science Labs, Inglewood, CA) was dissolved in 50 mL of pH 11 borate buffer, yielding clear stock substrate solution (36 mM); all buffers were ionic strength 0.2. When 10–25 μL of green juice was injected into 0.6 mM substrate mixture (50 μL of stock substrate solution in 3.0 mL of air-equilibrated buffer at pH 6.9), all of the available O_2 (0.72 μmol) was consumed in 3 min or less. Reproducibility was ca. ±10%. Linolenic acid was routinely used as the substrate because it comprises ~60% of the total fatty acids of alfalfa lipids (Van der Veen and Olcott, 1967). Protein was determined by the method of Lowry et al. (1951).

Measurement of β-carotene destruction through a coupled oxidation with lipoxygenase and linolenic acid was carried out as follows. The rate of loss of β-carotene absorbance at 465 nm was determined in a mixture of 2 mL of phosphate buffer, pH 6.9, 20 μL of a saturated β-carotene solution in acetone, and 10 μL of stock linolenic acid solution. Reaction was initiated by adding 10 μL of green juice.

RESULTS AND DISCUSSION

The lipoxygenase(s) in the crude green juice has (have) several characteristics similar to those of lipoxygenases from other sources as follows: it is not immediately inhibited by concentrations of CN^- as high as 20 mM; preincubation of the juice for 30 min with 20 mM CN^-, however, causes a 50% loss in O_2 uptake activity.

Oxygen uptake in the assay reaction mixture ceases before all the substrate is consumed when a lower (~5 μL of juice instead of the normal 10–25 μL) enzyme concentration is used for reaction. This reaction inactivation has also been noted when using soybean (Smith and Lands, 1970, 1972) and wheat lipoxygenases (Wheeler and Wallace, 1973).

The activity vs. enzyme concentration curve does not go through the origin and falls off at higher enzyme concentrations (Figure 1). The former effect has been observed with soybean lipoxygenase (Allen, 1968) and was then attributed to adsorption by lipoxygenase, at low concentrations, to glass surfaces. Using a concentrated wheat germ lipoxygenase, however, Wallace and Wheeler (1979) demonstrated that both effects were attributable to the physical state of the insoluble substrate.

Lipoxygenases, in either a crude or purified state, generally exhibit a lag period, τ, defined as the time from inhibition of reaction to arrival at a steady-state rate of O_2 uptake. In general, valuable kinetic information can be obtained from a study of the dependence of τ on *substrate* concentration for various enzymes (Walter, 1966), including lipoxygenase from soybeans (Smith and Lands, 1972), but highly purified enzymes (unpublished observations) and substrates free of hydroperoxides are necessary. It is interesting, however, that even in the crude state, τ[E] is a constant for lipoxygenase, so that a plot of $1/\tau$ vs. *enzyme* concentration yields a straight line (Figure 2). If one uses substrate essentially free of hydroperoxide impurities, we feel that this test is the definitive means of distinguishing lipoxygenase action in crude systems from other oxidative enzymes such as peroxidases.

The specific activity and the stability of lipoxygenase in freshly prepared juice varied widely from one batch to the next. The half-life varied from extremes of 2 h to 1 week in batches of green juice held at ~3 °C. The factors responsible for such marked difference in enzyme stability

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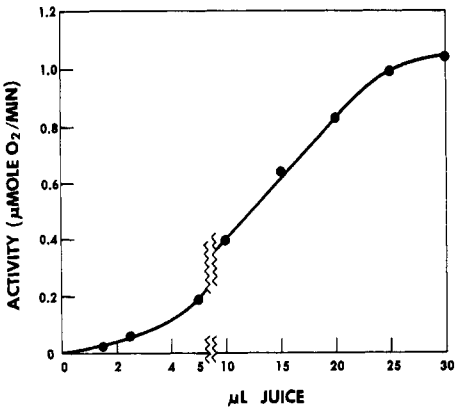

Figure 1. Rate of O_2 consumption vs. enzyme concentration using 0.6 mM linolenic acid substrate.

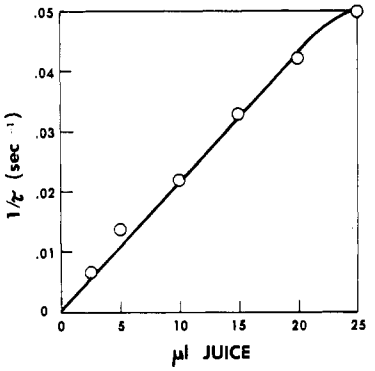

Figure 2. Inverse of the induction time, τ, vs. enzyme concentration using 0.6 mM linolenic acid substrate.

are not known but could be related to levels of proteases, polyphenol oxidases, natural inhibitors, etc. which vary according to weather, time of day, and year, etc. when the alfalfa is harvested. The specific activity of lipoxygenase in the juice was strikingly high, varying from 1.03 to 2.2 μmol of O_2 min^{-1} (mg of protein)$^{-1}$ in various batches tested. In comparison, the specific activity in 24 soybean genotypes tested for lipoxygenase activity (at pH 6.0) varied from 0.34 to 1.02 μmol of O_2 min^{-1} (mg of P)$^{-1}$ (Chapman et al., 1976), and that of wheat germ was only 0.06 μmol of O_2 min^{-1} (mg of P)$^{-1}$ or ~20 times less than the value for alfalfa juice (Wallace and Wheeler, 1975).

Grossman et al. (1972) measured the lipoxygenase activity, using linoleic acid substrate, of various subcellular fractions of alfalfa leaf homogenates, but (when converted to micromoles of O_2 per minute per milligram of protein) the specific activity of the highest (chloroplast) fraction was only ~0.07. This is only ca. one-twentieth of the value we found for alfalfa juice. Although alfalfa lipoxygenase is more active toward linolenic than linoleic acid (Figure 3), the difference is far too small to account for this difference in determined specific activities. It appears that the homogenization of the leaves with liquid N_2, followed by high-pressure expression of green juice from the leaves, may disrupt subcellular organelles more than is possible using traditional extraction techniques such as the Triton X extraction used by Grossman et al. (1972) and thus releases a greater fraction of the tissue lipoxygenase into solution.

Inherent O_2 Consumption in Green Juice. The oxidizing enzymes and their naturally occurring substrates are presumably mixed together in the juice as a result of grinding and pressing the alfalfa leaves. We followed the O_2 consumption resulting from inherent (no added substrate) oxidation reactions with the oxygen monitor simply

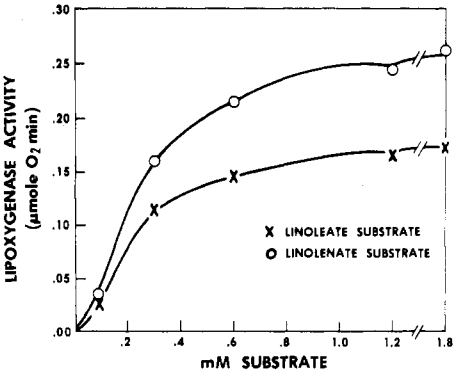

Figure 3. Lipoxygenase activity vs. substrate concentration for two substrates. Aliquots of the stock substrates were air-equilibrated with pH 6.9 buffer (3 mL total), and the reaction was initiated by injection of 25 μL of green juice.

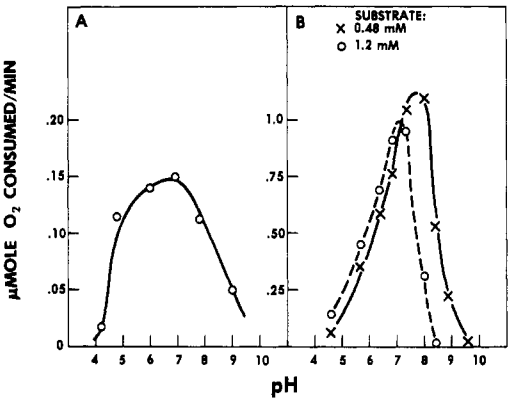

Figure 4. (A) Inherent O_2 consumption in green juice as a function of pH. 200 μL of juice was added to 2.8 mL of buffer. The rate shown is the tangent to the O_2 consumption curve 1 min after juice was added to buffer. (B) pH profile of lipoxygenase in crude green juice (25 μL) assayed with added 0.48 and 1.2 mM linolenic acid.

by injecting 0.1–0.2 mL of green juice into air-equilibrated buffer. The O_2 uptake rates in such experiments are typically ~0.75 μmol of O_2 min^{-1} mL^{-1} or ~50 times slower than determinations of juice lipoxygenase activity using added linolenate and so do not interfere with lipoxygenase activity determinations. The presence of 10 mM CN^- reduces the inherent O_2 consumption of the juice by ~65% (phenol oxidases, respiratory enzymes, and peroxidases, for example, are inhibited by CN^-). The remaining CN^--insensitive O_2-consuming reactions could be due to lipoxygenase acting on linolenic and linoleic acid released by juice esterases.

In the pilot plant process for production of LPC's, the expressed green juice (usually ca. pH 6) is quite foamy (highly oxygenated) and may stand 1 h or more before coagulation of the protein is begun. For this reason, the juice is often adjusted to pH 8.5 in order to retard enzymatic oxidation reactions. The pH profiles of both inherent juice oxygen consumption (due to lipoxygenase, polyphenol oxidases, and other enzymes) and lipoxygenase using the standard enzyme assay at two substrate concentrations are shown in Figure 4. Inherent O_2 consumption is reduced by adjusting the juice from its natural pH 6 to pH 8.5 (Figure 4A), but significant O_2 uptake activity remains. Lipoxygenase is also quite active at 0.48 mM linolenate at pH 8.5 (Figure 4B) but has virtually no activity at 8.5 when using 1.2 mM linolenate. This shift in pH optimum was noted previously (Wallace and Wheeler, 1975) and was attributed to pH-sensitive substrate inhibition. The signifigance of this effect is that as

Table I. Inhibition of Lipoxygenase Activity in Green Juice by Antioxidants

antioxidant (0.5 mg/mL)	lipoxygenase act.,[a] μmol of O_2/min	% inhibition
control	0.49	
BHT	0.39	20
ethoxyquin	0.29	41
BHA	trace	~100
TBHQ	trace[b]	~100
HQ	0.39	20

[a] 25 μL of green juice was injected into 0.6 mM linolenic acid in pH 6.9 phosphate buffer (3 mL total) containing 30 μL of antioxidant stock solution at 50 mg/mL in ethanol. [b] Corrected for O_2 consumption due to TBHQ itself.

Table II. Effect of Antioxidants of the Inherent Oxygen Consumption of Green Juice

antioxidant (0.5 mg/mL)	rate of oxygen uptake,[a] μmol of O_2/min	% inhibition
control	0.071	
BHA	0.037	48
HQ	0.043	40
TBHQ	0.095	34% increase

[a] 150 μL of green juice was injected into 85 mL of pH 6.8 phosphate buffer. Oxygen uptake was nonlinear; rates were determined by taking the tangent to the curve 1 min after addition of juice to the buffer.

esterases begin to liberate free linolenate in fresh juice, the concentration will be low so that lipoxygenase may be quite active at pH 8.5.

Effects of Antioxidants. The addition of antioxidants is a potential means of reducing the amount of lipid oxidation occurring in leaf juice prior to coagulation of the protein. At 0.5 mg/mL, both butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) eliminated lipoxygenase activity under the conditions of the experiment (Table I). Hydroquinone (HQ), ethoxyquin, and butylated hydroxytoluene (BHT) were less effective lipoxygenase inhibitors. TBHQ and BHA, with one *tert*-butyl group each, may be attracted to the hydrophobic binding site of lipoxygenase (Wheeler and Wallace, 1978) and thus be more effective inhibitors than HQ (no *tert*-butyl group) and BHT (two *tert*-butyl groups). Steric hinderance due to the second *tert*-butyl group may prevent BHT from reaching the hydrophobic binding site.

Both BHA and HQ inhibit inherent O_2 consumption in the juice by 40–50% (Table II), but TBHQ, which completely inhibits lipoxygenase activity, causes an *increase* in inherent O_2 consumption by the juice. TBHQ may act as a substrate for a diphenol oxidase enzyme present in the juice, but because HQ does not cause the same phenomenon to occur, the putative diphenol oxidase would have to require the *tert*-butyl group for activity.

β-Carotene Bleaching. It is well-known that carotenes, xanthophylls, and chlorophyll are bleached through a coupled oxidation with lipoxygenase and its substrates. Under the conditions described under Experimental Section, all the absorbance by β-carotene at 465 nm disappeared within 6 min when a mixture of crude green juice, linolenate, and β-carotene were incubated together. Juice and linolenate were both required for bleaching to occur. TBHQ, BHA, and ethoxyquin (all at 0.25 mg/mL) completely inhibited this bleaching, while BHT was only ~75% effective. Ramadoss et al. (1978), working with soybean extracts, showed that two lipoxygenase isoenzymes acting in combination are necessary for β-carotene to be effectively bleached. Any one isoenzyme by itself is relatively ineffective. This may explain why the antioxidant ethoxyquin only inhibited lipoxygenase O_2 uptake activity by 41% (Table I) but inhibited carotene bleaching 100%. It may only inhibit one isoenzyme.

Because BHA effectively inhibits both lipoxygenase activity and β-carotene bleaching, it should be tested in a pilot plant scale experiment to determine if it will improve the qualities of both Pro-Xan and white LPC for human consumption. TBHQ might also work for the same reasons, but the possibility that it is utilized as a substrate for a phenol oxidase enzyme in the juice might make it too short-lived to be effective.

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