Enzymic Pathway to Ethyl Vinyl Ketone and 2-Pentenal in Soybean Preparations

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Previous work by this laboratory showed that under anaerobic conditions and the presence of a polyunsaturated fatty acid, soybean (*Glycine max* L.) lipoxygenase isoenzymes converted a lipoxygenase-catalyzed oxidation product of linolenic acid, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid, into 1-penten-3-ol, 2(*Z*)-penten-1-ol, and 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid. It seemed plausible that the "raw bean odor", ethyl vinyl ketone, previously isolated from soybean homogenates by other workers could arise from oxidation of 1-penten-3-ol by alcohol dehydrogenase. It is shown here that both ethyl vinyl ketone and 2-pentenal are produced by a soybean preparation after anaerobic incubation with 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and linolenic acid and that NAD^+ stimulated the formation of 2-pentenal. In the presence of NAD^+, two separable isoenzymes of soybean alcohol dehydrogenase were capable of utilizing as substrates both 1-penten-3-ol and 2(*Z*)-penten-1-ol, as well as (2*E*)-hexen-1-ol. In terms of substrate preference indicated by K_m, the order was 2(*E*)-hexen-1-ol > 2(*Z*)-penten-1-ol > 1-penten-3-ol. Because ethyl vinyl ketone formed in the presence of only 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and linolenic acid in the absence of NAD^+, another pathway also seemed possible.

Keywords: *Lipoxygenase; 1-penten-3-ol; 2(Z)-penten-1-ol; alcohol dehydrogenase; ethyl vinyl ketone; 2-pentenal; linolenic acid; hydroperoxide; flavor*

INTRODUCTION

Ethyl vinyl ketone (EVK) was detected in homogenized soybean (*Glycine max* L.) by Mattick and Hand (1969) but not in heat-inactivated homogenates, indicating its enzymic origin. According to these workers, EVK imparted a decided "raw bean odor" to the soybean homogenates. Although they postulated a lipoxygenase-mediated pathway to EVK through free radical cleavage of the 13-hydroperoxide of linolenic acid, no research was completed to prove their hypothesis. Among other aldehydes, 2(*E*)-pentenal was identified as a quantitatively important volatile originating from oxidation of linolenic acid by soybean lipoxygenase isoenzymes (Grosch and Laskawy, 1975); however, its mechanism of origin was not explored.

Recently, Kondo et al. (1995) and Salch et al. (1995) demonstrated the formation of 2-penten-1-ol and 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid in soybean homogenates from cleavage of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HPOT). Additionally, Salch et al. (1995) found that the cleavage was catalyzed by lipoxygenase isoenzymes promoted by anaerobic conditions and the presence of a polyunsaturated fatty acid. The latter workers found that 1-penten-3-ol and pentene dimers additionally were products. A similar anaerobic reaction of lipoxygenase with linoleic acid and its 13-hydroperoxide affords pentane (Garssen et al., 1971; Johns et al., 1973) and 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid (Garssen et al., 1971).

It seemed plausible that the 2-penten-1-ol and 1-penten-3-ol produced by the anaerobic action of lipoxygenase on 13*S*-HPOT and linolenic acid could be metabolized by soybean alcohol dehydrogenase and NAD^+ to the corresponding 2-pentenal and EVK, thus establishing a possible metabolic route to the latter two compounds. This paper describes two isoenzymes of soybean alcohol dehydrogenase and their comparative ability to oxidize 2-penten-1-ol, 1-penten-3-ol, and 2(*E*)-hexen-1-ol in the presence of NAD^+.

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EXPERIMENTAL PROCEDURES

Materials. 2(*Z*)-Penten-1-ol, 1-penten-3-ol, 2(*E*)-hexen-1-ol, EVK, 2(*E*)-hexenal, tetracosane, and *O*-benzylhydroxylamine were from Aldrich Chemical Co., Milwaukee, WI. Piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (Pipes), *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (Epps), 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches), [bis[2-hydroxyethyl]-amino]tris[hydroxymethyl]methane (Bis-Tris), lipoxygenase (lipoxidase type 1), and NAD^+ were purchased from Sigma Chemical Co., St. Louis, MO. *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes) and tris[hydroxymethyl]-aminomethane (Tris) were from Research Organics, Cleveland, OH. Linolenic acid was 99+% pure from NuChek Prep., Elysian, MN. 13*S*-HPOT was prepared from lipoxygenase oxidation of linolenic acid as described previously (Gardner et al., 1991). 2-Pentenal was prepared by periodinane oxidation of 2(*Z*)-penten-1-ol according to the procedure of Dess and Martin (1983).

Enzyme Preparation. A 12.5 g quantity of soybean seeds (*G. max* L. cv. Century) was water-imbibed for 2 h with aeration. The imbibed seeds were homogenized in 200 mL of ice-cold 20 mM Tris-HCl, pH 8.0, at 4 °C for 1 min at full speed with a Brinkmann Polytron homogenizer. The homogenate, filtered through two layers of cheesecloth, was centrifuged at 25000*g* for 30 min. The floating lipid pellet was removed, and the supernatant was fractionated by $(NH_4)_2SO_4$ precipitation between 30 and 60% saturation. The 60% $(NH_4)_2SO_4$ pellet was resuspended in 20 mL of 50 mM Hepes, pH 7.5, 10 mM dithiothreitol (DTT) and dialyzed overnight in 4 L of the same buffer at 4 °C. The dialyzed preparation was concentrated to about 15 mL, filtered with a Nalgene SFCA 45 μm syringe filter, and loaded onto a DEAE-Sephacel (Pharmacia) anion-exchange column (2.5 × 30 cm). Elution was with 60 mL of

50 mM Hepes, pH 7.5, and 10 mM DTT, followed by a linear gradient of 0.08−0.4 M NaCl in 940 mL of the same buffer. Fractions of 10 mL were collected, and each was assayed for alcohol dehydrogenase activity using 2 mM NAD^+ and 5 mM 2(*E*)-hexen-1-ol according to the procedure described below.

Assay Procedure. Alcohol dehydrogenase activity was measured by the NAD^+ to NADH conversion, an increase in absorbance at 340 nm, with a Beckman DU-8U kinetic spectrophotometer at 25 °C. Unless stated otherwise, the reaction mixtures were 50 mM Ches, pH 9.0, 2 mM NAD^+, and the alcohols. Except for assay of individual fractions from the DEAE-Sephacel column, all activities determined with pooled column fractions were concentrated 5−6-fold with Centriprep 50 from Amicon, Beverly, MA.

The pH optimum was determined in one of the following buffers: acetate (pH 5), Bis-Tris (pH 6), Pipes (pH 6.5−7), Hepes (pH 7.5), Epps (pH 8−8.5), and Ches (pH 8.5−9).

Product Analyses. Experiments to test the biogenesis of EVK and 2-pentenal in the presence of 13*S*-HPOT and NAD^+ were completed with a 30−60% $(NH_4)_2SO_4$ precipitated soybean preparation that had been dialyzed overnight (total volume of 20−24 mL from 12.5 g of soybeans, see above). Incubations were with 2.5 mL of soybean dialysate in a total volume of 5 mL containing 25 mM Hepes, pH 7.5, 5 mM DTT, either 0 or 1 mM of both 13*S*-HPOT and linolenic acid, and either 0 or 5 mM NAD^+. Incubations were also completed with soybean dialysate (2.5 mL in 5 mL total) containing 25 mM Hepes, pH 7.5, 5 mM DTT, either 0 or 0.125 mM of both 1-penten-3-ol and 2(*Z*)-penten-1-ol, and either 0 or 5 mM NAD^+. After incubation under a stream of nitrogen with stirring for 15 min at 25 °C, the reaction was terminated with 1 mL of *O*-benzylhydroxylamine reagent (50 mM *O*-benzylhydroxylamine, 100 mM Pipes, pH 6.5) and 5 mL of methanol. After the *O*-benzyloximes had formed for several hours, the derivatives were extracted with 10 mL of $CHCl_3$. The $CHCl_3$ layer was evaporated and the residue taken up in hexane for gas chromatography−mass spectrometry (GC−MS) analyses.

A Centriprep 50 concentrate from DEAE-Sephacel peaks 1 and 2 was used to determine by GC−MS the oxidation products of 1-penten-3-ol, 2(*Z*)-penten-1-ol, and 2(*E*)-hexen-1-ol as their *O*-benzyloximes. The reaction mixture was 250 μL of enzyme concentrate in a total volume of 5 mL containing 5 mM of the test alcohol, 10 mM NAD^+, and 50 mM Ches, pH 8.5. The mixture was incubated for 10 min at 25 °C, after which time 1 mL of benzylhydroxylamine reagent and 5 mL of methanol was added; 10 μg of tetracosane was added as an internal standard. After 10 min, the *O*-benzyloximes were extracted with 10 mL of $CHCl_3$. The extract was evaporated and taken up in hexane for GC−MS. Standard *O*-benzyloximes were prepared from 10 mg each of the authentic aldehydes/ketone, 2(*Z*)-pentenal, 2(*E*)-hexenal, or EVK, then reacted with 1 mL of *O*-benzylhydroxylamine reagent, and extracted into $CHCl_3$ by addition of 1 mL of ethanol and 2 mL of $CHCl_3$. GC−MS analyses of the *O*-benzyloximes were completed using a Hewlett-Packard Model 5890 GC (capillary column: Hewlett Packard HP-5MS cross-linked 5% phenyl methyl silicone, 0.25 mm × 30 m, film thickness 0.25 μm) interfaced with a Model 5971 mass selective detector operating at 70 eV. The temperature was programmed from 65 to 260 °C at 10 °C/min and held for 5 min at 260 °C (He flow of 0.670 mL/min; injector temperature, 260 °C). Flame ionization detection gas chromatography (GC) was used to estimate the quantity of product using tetracosane as an internal standard. Analyses were completed with a Hewlett-Packard GC, Model 5890, equipped with a fused silica capillary column, SPB 1 (0.32 mm × 30 m; film thickness 0.25 μm) from Supelco, Bellefonte, PA. The temperature was programmed from 65 to 260 °C at 15 °C/min and held for 5 min at 260 °C (He flow of 2 mL/min; injector temperature, 260 °C).

RESULTS

Previously, it was determined that 13*S*-HPOT was anaerobically cleaved by lipoxygenase into 1-penten-3-ol and 2(*Z*)-penten-1-ol to the extent of about 12.5% each

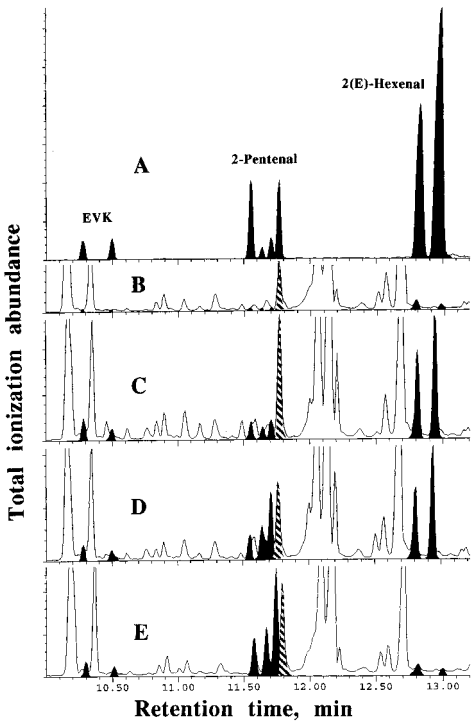

Figure 1. Biogenesis of ethyl vinyl ketone and 2-pentenal by a soybean preparation [30−60% saturation of $(NH_4)_2SO_4$ precipitate] with or without 13*S*-HPOT plus linolenic acid (1 mM each), with or without NAD^+ (5 mM), compared with reacting 0.125 mM 2(*Z*)-penten-1-ol plus 1-penten-3-ol with the soybean preparation in the presence of 5 mM NAD^+. Products (aldehydes/ketones) were reacted with *O*-benzylhydroxylamine to give *syn* and *anti* *O*-benzyloximes, which were analyzed by GC−MS total ionization. A, standard *O*-benzyloximes; B, without 13*S*-HPOT and linolenic acid and with NAD^+ (without 13*S*-HPOT, linolenic acid, and NAD^+ was essentially the same as B, data not shown); C, with 13*S*-HPOT and linolenic acid and without NAD^+; D, with 13*S*-HPOT, linolenic acid, and NAD^+; E, with 2(*Z*)-penten-1-ol, 1-penten-3-ol, and NAD^+ [with 2(*Z*)-penten-1-ol and 1-penten-3-ol and without NAD^+ gave negligible yields of the appropriate *O*-benzyloximes, data not shown]. Shading indicates appropriate *O*-benzyloxime peaks, and cross-hatching indicates *O*-benzyloxime isomer of 2-pentenal coeluting with an unknown.

(Salch et al., 1995). Thus, we tested the hypothesis that alcohol dehydrogenase and NAD^+ might oxidize these alcohols to the corresponding ketone or aldehyde. For this experiment a dialyzed $(NH_4)_2SO_4$ precipitate (30−60% saturation), containing both lipoxygenase and alcohol dehydrogenase activities, was used. Despite the claim by Tihanyi et al. (1989) of $(NH_4)_2SO_4$ inactivation, this procedure recovered 73% of the original activity. After overnight dialysis, the activity was reduced further to 37% but was only reduced to 63% when compared with overnight storage of the crude extract at 4 °C. This crude enzyme preparation was reacted under a stream of nitrogen with or without 13*S*-HPOT and linolenic acid (linolenic acid serves as a reductant required to cycle lipoxygenase to the reduced ferrous form) and with or without NAD^+. The aldehydes/ketones were trapped as nonvolatile *O*-benzyloximes for GC and GC−MS analyses (Figure 1). Compared to the *O*-benzyloxime derivatives of the aldehydes the yield for the *O*-benzyloxime of EVK was only 8%; thus, EVK would be expected to give comparatively smaller peak areas as seen in Figure 1. The *O*-benzyloxime derivatives separate by GC into *syn* and *anti* isomers. The 2-pentenal *O*-benzyloxime separated into four isomers indicating the additional presence of *Z* and *E* double bonds. Selected ion monitoring and comparison with a

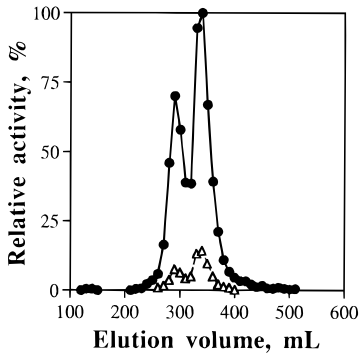

Figure 2. Separation of soybean alcohol dehydrogenases after DEAE-Sephacel chromatography. Relative activity (Δ absorbance$_{340}$/min) was measured in the presence of NAD^+ (5 mM) and 20 mM Tris, pH 8; a preliminary fractionation was completed with $(NH_4)_2SO_4$ (30−60% saturation) precipitation. Substrates (5 mM) were 2(*E*)-hexen-1-ol (●) and 1-penten-3-ol (△).

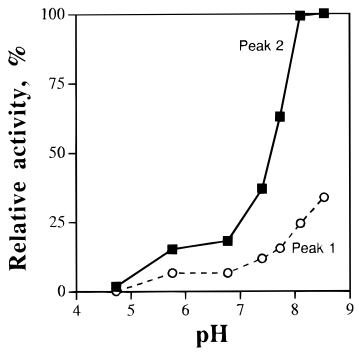

Figure 3. Alcohol dehydrogenase activity dependence on pH for peaks 1 and 2 of DEAE-Sephacel separated activities (see Figure 2). Substrate was 5 mM 2(*E*)-hexen-1-ol in the presence of 2 mM NAD^+.

Table 1. Relative V_{max} and K_m Values for Soybean Alcohol Dehydrogenase Activity Peaks Obtained from DEAE-Sephacel Chromatography Using Various Alcohol Substrates[a]

	ADH peak 1		ADH peak 2	
substrate	rel V_{max}	K_m	rel V_{max}	K_m
(2*E*)-hexen-1-ol	100	0.945 ± 0.47	100	3.77 ± 0.61
(2*Z*)-penten-1-ol	15 ± 0	1.76 ± 0.23	16 ± 0	6.92 ± 1.10
1-penten-3-ol	17.5 ± 4.9	4.74 ± 0.48	37.5 ± 24.7	23.9 ± 18.5

[a] Values determined from reciprocal plots (1/*v* vs 1/*S*) using the following substrate concentrations: 1, 1.25, 1.67, 2.5, 5, 10, and 20 mM; at least two velocities were determined at each substrate concentration. NAD^+ concentration was 2 mM. Values represent the mean and standard error obtained from the plots of two separate experiments.

standard showed that the last eluting peak of the four 2-pentenal derivatives was mixed with an unknown. Also, 2(*E*)-hexenal was formed presumably by action of hydroperoxide lyase on 13*S*-HPOT followed by conversion of the hydroperoxide lyase product, (3*Z*)-hexenal, by a (3*Z*:2*E*)-enal isomerase. As seen by Figure 1, EVK, 2-pentenal, and especially 2(*E*)-hexenal were produced by the addition of 1 mM each of 13*S*-HPOT and linolenic acid. The addition of 5 mM NAD^+ markedly stimulated the production of 2-pentenal but did not appear to stimulate formation of EVK. Since 2(*E*)-hexenal does not originate from the corresponding alcohol, the presence of NAD^+ did not significantly affect the formation of 2(*E*)-hexenal compared to without NAD^+, as expected. Incubation of a theoretical amount (0.125 mM) of both 1-penten-3-ol and 2(*Z*)-penten-1-ol with 5 mM NAD^+ gave yields of both EVK and 2-pentenal comparable to the condition with 1 mM each of 13*S*-HPOT and linolenic acid plus 5 mM NAD^+. The *O*-benzyloximes are illustrated as total ionization curves (Figure 1), but they were identified by both their retention time and GC−MS relative to standards. The significant MS ions of the *O*-benzyloximes are reported below.

For further purification, the $(NH_4)_2SO_4$-precipitated and dialyzed preparation was applied to DEAE-Sephacel chromatography, giving separation of two activity peaks as detected using 2(*E*)-hexen-1-ol and 1-penten-3-ol substrates (Figure 2). Ethanol and 2(*Z*)-penten-1-ol as substrates gave the same two peaks of activity (data not shown). Because of some overlap in the two peaks, the peak shoulders with the most separation were pooled for assay.

When the activity dependence on pH was examined for the two peaks from DEAE-Sephacel, the optimum for both was about pH 8.6 or greater (Figure 3). According to Tihanyi et al. (1989), the optimum for soybean alcohol dehydrogenase is pH 9−10.

A comparison was made of K_m and relative V_{max} values obtained for the two separated alcohol dehydrogenase activities with three substrates, 2(*E*)-hexen-1-ol, 1-penten-3-ol, and 2(*Z*)-penten-1-ol (Table 1). The K_m values were 1-penten-3-ol > 2(*Z*)-penten-1-ol > 2(*E*)-hexen-1-ol, and relative V_{max} values were 2(*E*)-hexen-1-ol > 1-penten-3-ol ≥ 2(*Z*)-penten-1-ol. Also, DEAE-Sephacel peak 1 consistently gave lower K_m values than peak 2.

To ascertain that the oxidation of alcohols was taking place under conditions of the assay, the products were examined as their *O*-benzyloximes by GC−MS (Figure 4). MS of *O*-benzyloximes was as follows for 2(*E*)-hexenal: [*m*/*z* (percent relative intensity, ion structure)] 203 (4, M^+), 160 (9, $[M - CH_3(CH_2)_2]^+$), 91 (100, $C_6H_5CH_2^+$), 77 (4, $C_6H_5^+$), 65 (5); the MS of the second isomer was similar, except the intensities were somewhat different for *m*/*z* 203 (0.3) and 160 (19). The MS of four 2-pentenal *O*-benzyloximes [presumably *syn* and *anti*

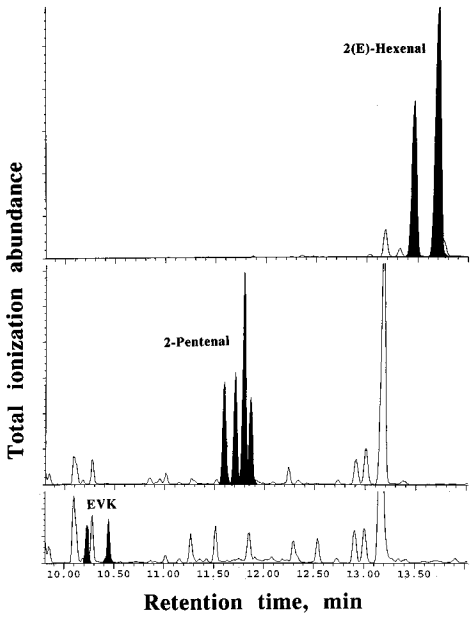

Figure 4. Total ionization GC−MS of *O*-benzyloximes (*syn* and *anti* isomers) obtained by reaction of aldehydes/ketones with *O*-benzylhydroxylamine after oxidation of 5 mM of the following alcohols: 2(*E*)-hexen-1-ol (top), 2(*Z*)-penten-1-ol (middle), or 1-penten-3-ol (bottom) in the presence of 10 mM NAD^+ and DEAE-Sephacel separated soybean alcohol dehydrogenase (peak 2, Figure 2). Shaded peaks indicate *O*-benzyloximes of interest.

plus 2(*E*) and 2(*Z*) isomers] was similar as follows, except the relative intensities of *m*/*z* 189 and 160 varied considerably: [*m*/*z* (percent relative intensity, ion structure)] 189 (0.3−9, M^+), 160 (1−20, [M − CH_3-$(CH_2)_2]^+$), 91 (100, $C_6H_5CH_2^+$), 77 (4−6, $C_6H_5^+$), 65 (5−7). The MS of two EVK *O*-benzyloximes (*syn* and *anti* isomers) was similar as follows: [*m*/*z* (percent relative intensity, ion structure)] 189 (5−6, M^+), 91 (100, C_6H_5-CH_2^+), 77 (4, $C_6H_5^+$), 65 (5). GC−MS of standard *O*-benzyloximes compared with *O*-benzyloximes obtained after alcohol bioconversion ascertained that the alcohols were being converted to their respective aldehydes/ketones. By GC−flame ionization detection the yield in micrograms (and estimated percent molar yield in parentheses) of the *O*-benzyloximes obtained from reactions catalyzed by DEAE-Sephacel peaks 1 and 2 were as follows: 2(*E*)-hexenal, peak 1 = 46 μg (0.91%) and peak 2 = 134 μg (2.6%); 2-pentenal, peak 1 = 4.4 μg (0.10%) and peak 2 = 21 μg (0.44%); EVK, peak 1 = trace and peak 2 = 1.2 μg. Since the formation of *O*-benzyloximes of EVK is only about 8% as efficient as that of the aldehydes, the peak 2 EVK yield may be closer to 15 μg (0.32%). As expected, the yields were generally reflective of an inverse relationship with the K_m values; however, peak 1 cannot be compared to peak 2 as the activity of the enzyme in peak 1 was significantly lower.

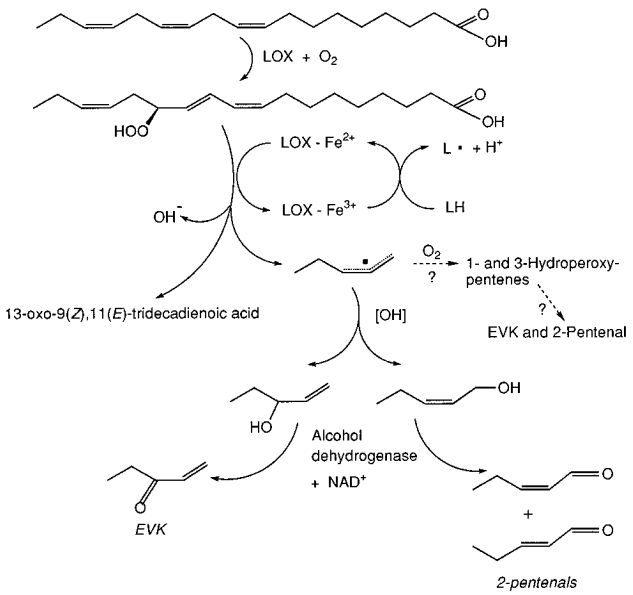

Figure 5. Pathway to ethyl vinyl ketone and 2-pentenal from linolenic acid through oxidation and anaerobic cleavage by lipoxygenase followed by alcohol dehydrogenase oxidation of the pentenols. LOX-Fe^{2+} and LOX-Fe^{3+} represent reduced and oxidized forms of lipoxygenase, respectively; LH and L• polyunsaturated fatty acid and its pentadienyl radical, respectively.

DISCUSSION

Both EVK (Mattick and Hand, 1969) and 2(*E*)-pentenal (Grosch and Laskawy, 1975) have been identified in soybean preparations without a demonstration of their biogenesis. In this paper we report that 13*S*-HPOT and linolenic acid can serve as precursors to EVK and 2-pentenal and that NAD^+ stimulated the formation of at least 2-pentenal. In a previous study, some of us (Salch et al., 1995) showed that 13*S*-HPOT cleaved into 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid, 1-penten-3-ol, and 2(*Z*)-penten-1-ol under anaerobic conditions. It seemed plausible that soybean alcohol dehydrogenase and NAD^+ could oxidize 1-penten-3-ol and 2(*Z*)-penten-1-ol into EVK and 2-pentenal, respectively. This oxidation has been confirmed, and the soybean alcohol dehydrogenases responsible have been characterized.

Although soybean alcohol dehydrogenase has been examined previously (Brzezinski et al., 1986; Kimmerer, 1987; Matoba et al., 1989; Tihanyi et al., 1989), these workers examined neither 1-penten-3-ol, 2(*Z*)-penten-1-ol, nor 2(*E*)-hexen-1-ol as substrate. Tihanyi et al. (1989) did not achieve separation of alcohol dehydrogenase isozymes by DEAE-Sephacel chromatography, but they separated four isozymes by isoelectric focusing. In this work two peaks of alcohol dehydrogenase activity were separated by DEAE-Sephacel chromatography. Both active peaks afforded a pH optimum of about 9. The two isozymes (or a mixture of isozymes) were active with three substrate alcohols tested, 2(*E*)-hexen-1-ol, 1-penten-3-ol, and 2(*Z*)-penten-1-ol. Our data indicated that 2(*Z*)-penten-1-ol may have been converted to a mixture of 2(*Z*)- and 2(*E*)-pentenal, which is a predictable result considering the ease of isomerizing α,β-unsaturated aldehydes.

To compare the affinity of alcohol dehydrogenase to the various substrates, K_m values were determined. Since K_m is defined as the substrate concentration that permits half-maximum rate of reaction, comparatively lower K_m values are a measure of maximum catalytic rates occurring at lower substrate concentrations and generally indicate a tighter binding of substrate to the enzyme. It follows then from the values obtained in this study that the order of catalytic substrate affinity is 2(*E*)-hexen-1-ol > 2(*Z*)-penten-1-ol > 1-penten-3-ol.

Considering the poor substrate affinity of 1-penten-3-ol, it could be argued that little conversion to ketone could be expected, and indeed, little or no enhancement in EVK production from 13*S*-HPOT and linolenic acid was seen in the presence of NAD^+. However, considering the potent odor of EVK, traces of this compound would make a significant flavor impact. In our hands, EVK has an extremely unpleasant, penetrating odor. It should be pointed out that three common products of 13*S*-HPOT decomposition are aldehydes, 2(*E*)-hexenal, 12-oxo-9(10)-dodecenoic acid (from hydroperoxide lyase; Gardner et al., 1991), and 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid (Salch et al., 1995), which could serve to recycle NADH to NAD^+, a necessary condition to oxidize alcohols by alcohol dehydrogenase.

The data obtained in this study permitted the construction of a complete biochemical pathway to EVK and 2-pentenal from linolenic acid (Figure 5). In Figure 5 there is an apparent conflict between O_2 requirement (first step) and an anaerobic cleavage to 13-oxo-9(*Z*),-11(*E*)-tridecadienoic acid, 1-penten-3-ol, and 2(*Z*)-penten-1-ol. In practice, the cleavage of 13*S*-HPOT occurred aerobically (Kondo et al., 1995; Salch et al., 1995), probably owing to depletion of O_2 by lipoxygenase oxidation of endogenous polyunsaturated fatty acids. The endogenous polyunsaturated fatty acids also undoubtedly served to reduce the ferric state of lipoxygenase to maintain the redox cycle. It was shown, however, that saturating the enzymic reaction with pure O_2 does inhibit cleavage (Salch et al., 1995). The latter findings are in agreement with those of Garssen et al. (1971), who found that anaerobic conditions were required to cleave the 13-hydroperoxide of linoleic acid in the presence of unoxidized linoleic acid.

As seen in Figure 1, only 13*S*-HPOT and linolenic acid were required to form EVK, and NAD^+ did not appear

to stimulate its production; thus, another route of EVK formation was indicated. Also, it is noted that a small amount of 2-pentenal formed even in the absence of NAD^+. Grosch and Laskawy (1975) additionally obtained 2(*E*)-pentenal as a product from oxidation of linolenic acid by soybean lipoxygenase isozymes in the apparent absence of alcohol dehydrogenase and NAD^+. Another plausible route would require the pentene radical (Figure 5) to react with O_2, forming 1- and 3-hydroperoxypentenes. Free radical decomposition of the hydroperoxides possibly would lead to hydroxypentenes as well as their corresponding ketone/aldehyde, EVK and 2-pentenal. Because anaerobic conditions were maintained during the reaction by stirring under a stream of nitrogen, there is an apparent conflict with the proposed oxidation of the pentene radical to afford hydroperoxypentenes. Since the aqueous solutions were not rigorously purged prior to reaction, the total absence of O_2 in the initial reaction period cannot be assumed. Nevertheless, the question of how EVK and 2-pentenal might also form in the absence of NAD^+ has not been completely resolved.

In conclusion, we have presented evidence for a metabolic pathway to 2-pentenal and the raw-beany odor, EVK, by the scheme shown in Figure 5.

ABBREVIATIONS USED

Bis-Tris, [bis[2-hydroxyethyl]amino]tris[hydroxymethyl]methane; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; Epps, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; EVK, ethyl vinyl ketone; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; 13*S*-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; Tris, tris[hydroxymethyl]aminomethane.

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