THE FORMATION OF FREE RADICALS DURING THE REACTION OF SOY BEAN LIPOXIDASE

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Introduction

Lipoxidase has been obtained from many plant sources but it is uncertain whether it occurs in animal tissues (Holman and Bergström, 1951). The enzyme attacks lipids with methylene-interrupted multiple unsaturation in which the double bonds have cis configuration. In general, the products of enzyme catalyzed oxidation are similar to those produced by autoxidation and it has been suggested that the enzyme acts by abstracting a hydrogen atom to initiate a chain reaction (Holman and Bergström, 1951). However, the work of Tappel, Boyer, and Lundberg (1952) and Khan (1961) suggested that an enzyme-substrate-oxygen complex forms and that the reaction proceeds under the directive influence of the enzyme.

The nature of the site of catalysis is not understood. No co-factors are known, and metals appear not to be involved in catalysis since metal chelating agents are without effect. Siddiqui and Tappel (1957) observed that lipoxidases from urd and mung beans were inhibited by the thiol inhibitor p- mercuribenzoate and the inhibitions were reversed by glutathione, but no inhibition of alfalfa, pea, peanut, soy bean, and wheat lipoxidases has been demonstrated.

Thus while the reactions of the active centre of lipoxidase are obscure, it has been postulated that free radical changes occur (Holman and Bergström, 1951; Tappel, Berger and Lundberg, 1952; Siddiqui and Tappel, 1957). The present work investigates such changes during the reaction of linoleic acid with purified soy bean lipoxidase.

Materials and Methods

Highly purified soy bean lipoxidase was obtained from Sigma Chemical Co., St. Louis, U.S.A.

Linoleic acid (94-96% pure), obtained from Fluka, Buchs, Switzerland was prepared for use as a dispersion in phosphate buffer by blending in a Virtis '45' homogenizer.

Free radicals were determined by electron paramagnetic resonance using a Varian V4501 spectrometer with 100 Kc modulation. Spectra were recorded as first derivative curves on a Varian G10 recorder. The sample was contained in an aqueous sample holder with 0.16 ml in the measuring field. Samples were maintained at -100°C by cold nitrogen gas.

Results

The anaerobic incubation of linoleic acid with lipoxidase resulted in the generation of the complex EPR spectrum shown in Fig. 1. A six line signal developed, with two hyperfine groups anisotropic about a spectroscopic splitting factor (g) of 2.03. The line width of 210 gauss was shown by spectrum measurements at other power levels to be due to hyperfine structure and not to power saturation. The signal reached a maximum after 2 min incubation at 0°C and persisted even after 5 min. No signal was observed when linoleic acid or lipoxidase was incubated alone.

When linoleic acid and lipoxidase were incubated in air, with or without a preceding anarobic phase, the same six-line spectrum was obtained, but in addition a double peak was present. The double peak was grouped around g = 2.008, with hyperfine splitting of 20 gauss as shown in Fig. 2. The two-band spectrum reached a maximum after 90 sec incubation at 0°C and persisted during further incubation up to 5 min.

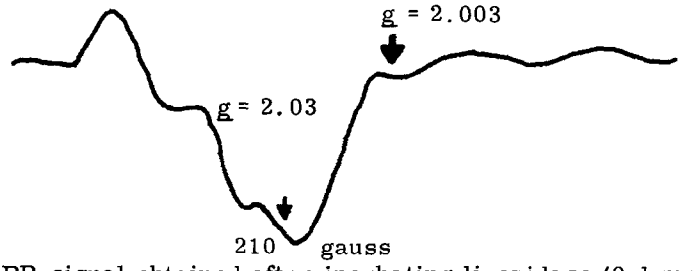

Fig. 1. EPR signal obtained after incubating lipoxidase (0.1 mg) and linoleic acid (5 mg) anaerobically for 2 min at 0°C. The reactants were dispersed in 1.2 ml 0.1M phosphate buffer (pH 6.9) contained in a Thunberg Tube which was evacuated and filled with nitrogen five times.

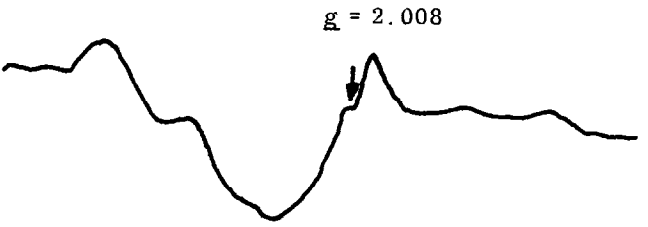

Fig. 2. EPR signal obtained after incubating lipoxidase and linoleic acid in air for 90 sec at 0°C. The reactants were used as in Fig. 1.

Discussion

The six-line spectrum obtained when lipoxidase and linoleic acid were incubated aerobically or anaerobically is markedly anisotropic about g = 2.03. This large shift in g value from the free spin value of 2.0023 indicates appreciable spin-orbit coupling and may be characteristic of free radicals in which the odd electron is primarily located on sulfur (Gardner and Fraenkel, 1956). Marked asymmetry of the hyperfine groups has been observed in solutions of sulfur in oleum at low temperatures (Ingram and Symons, 1957). Broad spectra anisotropic about high g values, similar to those observed in the present work, have also been observed in X-ray irradiated cystine and some fibrous proteins, and have been interpreted as due to localization of the unpaired electron on the S-S bond (Gordy, Ard and Shields, 1955). While it should be noted that the high electron affinity of the oxygen atom may cause the localization of a free electron, with anisotropic g splitting, the shift in g from the free spin value is unlikely to exceed 0.004 (Gardner and Fraenkel, 1956)

Thus this evidence indicates that sulfur free radicals were generated when soy bean lipoxidase was incubated with linoleic acid. Whether these radicals have an essential function is not clear, since thiol inhibitors do not affect this enzyme, in contrast to urd and mung bean lipoxidases which appear to have essential thiol groups. These differences may result from different susceptibilities of the thiol groups to chelation rather than from functional differences. Certainly the low activation energies of thiol free radicals (Bickel and Kooijman, 1952; Harris and Winter, 1952) makes them ideally suited to accept reversibly a hydrogen radical during lipoxidation.

It is probable that the double peak observed when linoleic acid and lipoxidase were incubated in air was due to peroxy free radicals the formation of which is in accord with the postulated reaction mechanisms of the enzyme.

The hyperfine splitting of 20 gauss about g = 2.008 is similar to that produced by peroxy free radicals generated when irradiated Teflon was aged in air (Ard, Shields and Gordy, 1955). Although the latter spectrum shows a triplet, the third peak is small and could well be obscured by the thiol radical during the reaction of lipoxidase with linoleic acid.

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