Properties of Agarose-Bound Soya-Bean Lipoxygenase

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Studies on lipoxygenase (EC 1.13.1.13) usually involve aqueous solutions of the enzyme. Recent techniques of enzyme insolubilization (Silman & Katchalsky, 1966) do not appear to have been applied to lipoxygenase. The present paper describes some properties of an insoluble lipoxygenase preparation obtained by binding the enzyme to agarose.

Soya-bean lipoxygenase (Fluka A.G., Buchs, Switzerland) was purified by chromatography on CM-cellulose (Grossman *et al.*, 1969). The eluate obtained with 0.1 M-sodium acetate buffer, pH 5.5, was dialysed against water and freeze-dried. Insolubilization was achieved by making the purified enzyme react with CNBr-treated agarose (Axen *et al.*, 1967; Cuatrecasas *et al.*, 1968). The insoluble product was washed with 0.1 M-NaHCO₃ to remove any remaining soluble lipoxygenase, and with water to remove the NaHCO₃. Preparations were obtained containing 1–12 mg of protein/g of agarose. The products retained their activity for at least 1 month when stored wet at room temperature.

Activity was determined by polarographic measurement of oxygen uptake by linoleate, as described for soluble lipoxygenase (Grossman *et al.*, 1969). Additional criteria used were diene conjugation of linoleate and bleaching of a carotene–linoleate solution. These techniques were applied by forming the agarose–lipoxygenase gel into a column 3 cm high and 1 cm in diameter and allowing 5–10 ml of linoleate solution (7.5 mM) (Grossman *et al.*, 1969) or 20 ml of linoleate (750 µg/ml)–β-carotene solution (10 µg/ml) (Ben Aziz *et al.*, 1971) to percolate through the column over a time-interval of 7–10 min. Conjugated dienes in the effluent were measured spectrophotometrically at 234 nm (Ben Aziz *et al.*, 1970) and the carotene remaining in the effluent was determined spectrophotometrically at 460 nm (Ben Aziz *et al.*, 1971).

Both soluble and agarose-bound forms of the enzyme rapidly oxidized linoleate, whereas they were completely inactive towards oleate. The specific activities of the soluble and insoluble preparations were 200 and 190 µl of O₂/min per mg of protein respectively. A similar substrate specificity was observed when the substrate solution was percolated through agarose-bound lipoxygenase: a pronounced diene peak was formed from linoleate, whereas no peak appeared with oleate as a substrate.

Percolation of an aqueous carotene–linoleate

solution through a column of agarose-bound lipoxygenase caused the bleaching of 70% of the carotene. No autooxidation of carotene was observed during the test period, and when linoleic acid was omitted from the test solution the entire amount of carotene was recovered in the effluent.

The pH optima for the soluble and insoluble preparations were 7.0 and 8.0 respectively. The agarose-bound enzyme appeared to exhibit little activity below pH 6.5, whereas the soluble lipoxygenase was still markedly active at pH 6.0.

Nordihydroguaiaretic acid [2,3-dimethyl-1,4-bis-(3',4'-dihydroxyphenyl)butane], which is known as an effective antioxidant and inhibitor of lipoxygenase, completely inhibited both soluble and insoluble lipoxygenase in the linoleate-oxidizing system, at a concentration of 0.1 mM. At a concentration of 35 µM nordihydroguaiaretic acid caused 62 and 84% inhibition of soluble and insoluble lipoxygenase respectively.

Michaelis constants were determined from Lineweaver–Burk plots over the range 1–0.01 mM-linoleate. *K_m* values were 1.8×10^{-4} M and 3.0×10^{-4} M for the soluble and insoluble enzyme respectively.

The effluent obtained when an aqueous solution of linoleate was percolated through agarose-bound lipoxygenase exhibited a pronounced peak close to 234 nm characteristic of a conjugated diene hydroperoxide. A similar effect was observed for agarose-bound haemoglobin prepared in the same fashion as insoluble lipoxygenase. Haemoproteins, like lipoxygenase, are known to catalyse the peroxidation of linoleate, but the primary reaction catalysed by haemoproteins is believed to be the homolytic scission of preformed hydroperoxide (Tappel, 1961), a process resulting in the setting up of radical-propagated autooxidation chains. When pre-oxidized linoleate was percolated through agarose-bound haemoglobin, the diene peak originally present disappeared, to be replaced by a maximum at 222 nm and several minor peaks between 250 and 300 nm. Insoluble lipoxygenase, on the other hand, did not alter the diene absorption spectrum of pre-oxidized linoleate. The destruction of conjugated diene hydroperoxide in the presence of haemoglobin is similar to the effect of a lipohydroperoxidase present in lucerne (*Medicago sativa*) (Gardner, 1965) and strengthens the assumption that haemoproteins act primarily on hydroperoxides.

Table 1. *Formation of substrate-enzyme complex with two preparations of agarose-bound lipoxxygenase*

Agarose-bound lipoxxygenase was first washed anaerobically with deaerated 0.2M-sodium phosphate buffer, pH 7.5, at 25°C. Linoleate solution (5ml) (Grossman *et al.*, 1969), labelled with 2.7×10^6 c.p.m. of [U - 3H] linoleate (4.3 Ci/mmol; Israel Nuclear Research Center, The Negev, Israel), was then added and stirred anaerobically with the enzyme preparation for 10 min. After removal of excess of substrate by exhaustive washing with deaerated buffer, the enzyme-substrate complex was decomposed by stirring with the phosphate buffer for 1 h under air, and this was followed by washing with the buffer until no conjugated dienes or radioactivity could be detected in the washings. Molar amounts were computed from the molar extinction coefficient of 25 600 for linoleic acid hydroperoxide (Holman, 1947) and the molecular weight of 100 000 for soya-bean lipoxxygenase (Tappel, 1963). Protein content was determined as the difference between the amount of protein added and that recovered from the supernatants after completion of the binding reaction, the actual determination being by the method of Lowry *et al.* (1951). Radioactivity was measured by counting that of 1.0 ml of effluent in 10 ml of Bray's (1960) solution in a Packard model 3003 scintillation counter, all values being corrected for internal quenching.

Enzyme preparation	Protein content (mg/g of agarose)	Substrate-enzyme molar ratios computed from measurement of	
		Radioactivity	Conjugated dienes
A	3.5	1.7	2.1
B	11.2	1.6	1.7

The above results illustrate the similarity between soluble and insoluble lipoxxygenase, as well as the different actions of lipoxxygenase and haemoglobin on pre-oxidized linoleate. The insoluble lipoxxygenase also permits an elegant demonstration of the existence of an enzyme-substrate complex. It was found that insoluble lipoxxygenase was able to bind linoleic acid to form a complex, stable under anaerobic conditions. When this complex was subsequently brought in contact with air, conjugated diene hydroperoxide was formed and released into solution, and this could be determined both by spectrophotometry and by the amount of radioactivity released when labelled linoleic acid was used. The results of a typical experiment with two different preparations are presented in Table 1. It was found that 1.6–2.1 mol of oxidized linoleate was released from 1 mol of enzyme, with reasonably good agreement between the two assay techniques. In the present experiments a large excess of substrate was present: the molar ratio of substrate to enzyme, as measured by the amount of reaction product released, was not significantly affected by increasing the amount of enzyme introduced into the reaction system, indicating that the insoluble enzyme was saturated with substrate.

Attempts to study the enzyme-substrate complex by the technique of equilibrium dialysis were

unsuccessful because it was not possible to prevent adsorption of linoleic acid on the dialysis membrane.

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