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Purification and Some Properties of Rubber Seed Lipoxygenase

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ABSTRACT
Lipoxygenase was extracted from rubber seed and purified by ammonium sulphate precipitation, gel filtration on Sephadex G – 25 and ion – exchange chromatographies on DEAE – cellulose columns. The enzyme was purified 78 fold and 38.56% of the enzyme activity was recovered in the purification. The molecular weight of the enzyme was 102,000 daltons and there were two subunits of the enzyme with molecular weights of 49,000 and 107,000 daltons. The rubber seed lipoxygenase had two ionizing groups at the pK values of 4.8 and 7.3. It also had pK values of 5.2 and 7.5 as the ionization constants of the free enzyme. The activation energy (E_a) of the rubber seed lipoxygenase was 7.2 kcal/mole and the enthalpy (ΔH) was 5.3 kcal/mole.

Key Words: *Lipoxygenase, Rubber Seed*

INTRODUCTION
Lipoxygenase (Linoleate: oxygen oxidoreductase EC 1.99. 2.1) is an enzyme which, in the presence of oxygen, specifically catalyzes the unsaturated fatty acids containing 1, 4 – cis, cis – pentadiene structures and produces cis, trans – conjugated monohydroperoxides as primary products (Tappel *et al.,* 1953; Beneytout *et al.,* 1989). Examples of its substrates are linoleic, linolenic and arachidonic acids (Holman, 1960; Al – Obaidy and Siddiqi, 1981; Douma, 2001).

The primary products of the enzyme, namely the monohydroperoxides, and the breakdown of these primary products, for example, aldehydes, ketones, alcohols, and short hydrocarbons are the causes of either desirable flavours such as fresh vegetables, oxidized soybean flavours, and the good scents of fresh flowers or undesirable off-flavours that occur in stored and processed foods (Sessa, 1979; Frankel, 1984; Gurr and Harwood, 1991; Sicdow, 2000).

Lipoxygenase was initially found in soybean and later in wheat, potatoes, some peas and beans, lentiles, white lupine, radishes, alfalfa, and green algae (Al-Obaidy and Siddiqi, 1981; Beneytout *et al.,* 1989) and subsequently characterized. The enzyme had not been extracted from rubber seed, purified, and characterized. Likewise the catalytic groups and thermodynamics of lipoxygenase had not been studied.

In this work, lipoxygenase was extracted from rubber seeds and purified. The properties of the enzyme were found but were based mainly on finding the enzyme's subunits, its pK values and its thermodynamic characteristics.

MATERIALS AND METHODS

Plant material

The seed of *Hevea brasiliensis* (rubber seeds) were obtained at the Faculty of Agriculture Demonstration Farm, University of Nigeria Nsukka. They were dehulied manually, sundried, and stored at 25 ± 2 ^{0}C.

Chemicals

DEAE – cellulose was Sigma product, Sephadex G –25 and G – 150 were from Pharmacia. All other chemicals were of analytical grades.

Extraction of lipoxygenase

The extraction of the enzyme was a modified method of Minguez – Mosquera *et al* (1993). The rubber seeds were homogenized in a warring blender with 50 mM phosphate buffer, pH 7.0. The homogenate was filtered through a cheese-cloth. The filtrate was centrifuged at 2000 rpm for 30 min and the supernatant, which was the crude enzyme, was stored at- 20 ^{0}C.

Purification of lipoxygenase

Modified method of Roy and Kornigsberg (1972) was used in the purification of the enzyme as described below.

Ammonium sulphate precipitation

Crystallized ammonium sulphate was added slowly to the crude enzyme solution with stirring to 20-30% saturation. The mixture was placed in a refrigerator at O ^{0}C for 1hr to ensure maximum precipitation. The precipitated protein was removed by centrifuging the mixture at 10,000 g for 15 min and decanting the supernatant .

Gel filtration

The precipitate containing the enzyme was dissolved in 50 mM phosphate buffer pH 7.0 . To desalt the protein, the mixture was applied to a column of 2.5 x 72 cm containing Sephadex G-25 previously equilibrated with the phosphate buffer. Fractions of 6ml were collected at a flow rate of 30 ml/hr and assayed for enzyme activity and protein content. Those fractions that showed enzyme activity were pooled, concentrated to 7 ml by vacuum drying and used for ion exchange chromatography.

Ion exchange chromatography

The enzyme solution from gel filtration was applied to a column of 2.5 x 42 cm containing DEAE-cellulose preequilibrated with the same buffer. The enzyme solution was eluted with the phosphate buffer. Fractions of 6ml were collected at the flow rate of 30ml/hr; enzyme activity and protein content were determined. Fractions that showed enzyme activity were pooled as before and concentrated to 5ml as mentioned earlier. To improve the purity of the enzyme, second ion exchange chromatography was done by repeating the ion exchange chromatography with the pooled concentrated enzyme solution

Determination of protein content

A volume of 3ml of diluted enzyme solution with the phosphate buffer (1:10 v/v) was applied in the cuvette of a spectrophotometer and the absorbance was read at 260 and 280nm. The formula used to determine protein content was

Protein (mg/ml) = 1.45 $O.D._{280}$-0.76 $O.D._{260}$ (Al-Obaidy and Siddiqi,1981).

The formula for enzyme concentration was

Enzyme concentration or unit of enzyme (units/ml) = Specific activity x protein content

where Specific activity (units/mg protein) = enzyme activity/protein content (Segel, 1976).

Substrate preparation

Substrate was prepared using a modified method of Surrey (1964). Linoleic acid (0.5 ml) and 0.5ml of Tween 20 were dissolved in 2ml of distilled water. To clear the turbidity of the mixture,a few drops of 2M NaOH were added until complete transparency was obtained. Distilled water was added to make up the volume to 25ml. This solution was stored at 5 ^{0}C.

Assay of enzyme activity

The substrate (2.9ml) was flushed with oxygen gas for 2 min and allowed to stand for 10 min. This was poured into the cuvette in the spectrophotometer and 0.1ml of the enzyme solution was added to start the reaction. The absorbance of the mixture was read every 30 sec at a wavelength of 234 nm.

The enzyme activity was calculated as the change in absorbance per unit time at 25 ^{0}C. One unit of enzyme activity was defined as the amount of enzyme giving an initial velocity value corresponding to a change in absorbance of 0.001 min^{-1} (Anosike and Ayaebene, 1982).

Molecular weight determination

The determination of molecular weight of the enzyme was by gel filtration on a Sephadex G-150 column (2.5 x 48 cm) using the method of Axelrod *et al* (1981). The protein markers used were 2.5 mg of cytochrome C (MW 12,400), 1.5 mg of myoglobin (MW 17,800), 2 mg of bovine serum albumin (MW 65, 000), and 1.5 mg of phycoerythrin (MW 250,000) with 1 ml of rubber seed lipoxgyenase solution.

Determination of subunit molecular weight

The subunits of the enzyme were determined by using the method of Weber and Osborn (1969). Sodium dodecyl sulphate- (SDS) - polyacrylamide gel electrophoresis (PAGE) was used in the method and the SDS-denatured protein markers were fumarase (MW 49,000), pyruvate kinase (MW 57, 000), catalase (MW 60,000), β -galactosidase (MW 130,000) and myosin (MW 220, 000).

In each gel of 6 gel test tubes, 1 ml of tracking dye (0.05% Bromophenol blue) was used. The staining solution was a mixture of 1.25g of Coomassie blue, 454 ml of 50% methanol and 46 ml of glacial acetic acid while the destaining solution was a mixture of 25 ml of acetic acid, 30 ml of methanol and 400 ml of distilled water. The experiments were carried out at room temperature.

The formula for the calculation of the mobility was

Mobility = distance of protein migration/ length after destaining X length before staining/ distance of dye migration (Weber and Osborn, 1969).

Effect of pH on K_m and V_{max}

The method was a modified method of Cleland (1982). Substrate solution ranging from 1 to 10 ml were prepared and buffers of different pH (3.0 to 9.0) were also prepared with 0.1M sodium acetate buffer for pH 3 to 6 and 50mM phosphate buffer for pH 7 to 10.

A volume of 1 ml of the enzyme preparation was poured into each of the test tubes containing 1 ml of buffer of the different pH and the mixtures were incubated for 10 min at 25 ^{0}C. Then 1 ml was taken from each of the mixtures and added to each of the test tubes containing the different substrate solutions. Control without the enzyme was also prepared.

Absorbances at 234 nm and at 30 sec intervals were determined and their enzyme activities were found. The Lineweaver – Burk (1934) plots were used to determine the V_{max} and Km at each pH value. The plot of log V_{max} and pH was done to determine the effect of pH on log V_{max} and to find the pK value. The plot of log V_{max}/K_m versus pH was also done to determine the effect of pH on log V_{max}/K_m and to determine the ionization constant (pK) of the amino acids on the enzyme, enzyme-substrate complex, and substrate.

Effect of temperature on enzyme activity

Substrate solution ranging from 1 to 10 ml were prepared in 10 test tubes. To 10 other test tubes that were placed in various constant-temperature bath of 10 – 80 ^{0}C for 5 min (for adequate temperature equilibrium) were applied 5 ml of the enzyme solution and 5 ml of 50mM phosphate buffer pH 7.0. These mixtures, containing the enzyme solution and buffer, were incubated for 10 min at 25 ^{0}C after which 1 ml from each mixture was applied to substrate solutions (1 – 10 ml) to start the reaction. Their absorbances were read and enzyme activities determined as mentioned earlier.

The plot of log V_{max} versus $^1/T$ ^{0}K was used to construct the Arrhenius plot. Km and V_{max} were calculated from Lineweaver-Burk (1934) plots.

RESULTS AND DISCUSSION

Table 1. Purification profile of rubber seed lipoxygenase

Purification step	Total protein (mg/ml)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude lipoxygenase Extract	18.64	0.08	100	1.00
20-30% Ammonium sulphate precipitation	14.53	0.41	74.15	1.64
Gel filtration on Sephadex G-25	6.43	1.20	51.31	25.89
Ion-exchange chromatography on DEAE-cellulose	2.75	1.81	44.09	48.14
Second ion-exchange chromatography on DEAE-cellulose	1.98	2.06	38.56	78.03

Table 1 shows the purification profile of rubber seed lipoxygenase. There was a 78-fold purification of the enzyme with 38.56% recovery of the enzyme activity. There was increase in specific activity and purification fold but decrease in total protein and percent yield from extraction to second ion exchange chromatography. These increase and decrease as described above in the purification steps were similar to the results obtained by Christopher *et al* (1970) and Ikediobi and Obasuyi (1982).

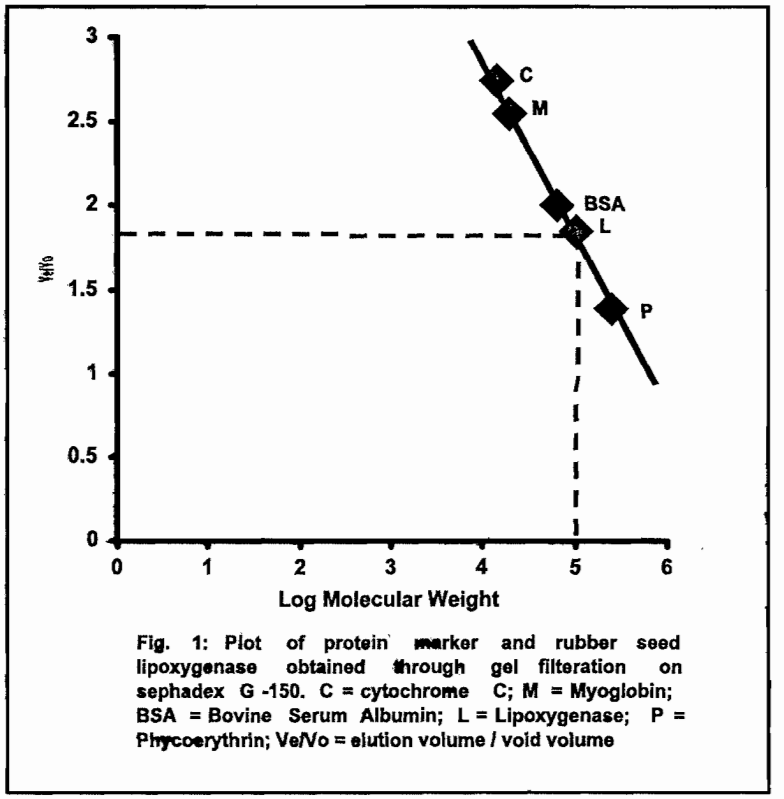

Fig. 1: Plot of protein marker and rubber seed lipoxygenase obtained through gel filteration on sephadex G -150. C = cytochrome C; M = Myoglobin; BSA = Bovine Serum Albumin; L = Lipoxygenase; P = Phycoerythrin; Ve/Vo = elution volume / void volume

Fig. 1 is the plot of molecular weights of the protein markers and rubber seed lipoxygenase versus elution volume/void volume. The molecular weight of rubber seed lipoxygenase obtained from the plot was 102,000 daltons. This is similar to 102,000 daltons obtained by Christopher *et al* (1972) for soybean lipoxygenase but higher than 100,000 ± 5,000 daltons obtained by Axelrod *et al* (1981) for soybean lipoxygenase using strokes method. Moreover, the result is lower than 108,000 ± 2,000 daltons obtained by Stevens *et al* (1970) for soybean lipoxygenase and 124 ,000 daltons for lipoxygenase from green algae by Beneytout *et al* (1989).

Subunit molecular weight

Two protein bands were obtained when the rubber seed lipoxygenase was introduced into 6 SDS-polyacrylamide gels. It could be concluded therefore that the two bands were two different polypeptide chains of the enzyme. From the SDS-PAGE of rubber seed lipoxygenase with the protein markers, the molecular weights of the enzyme were 49,000 and 107,000 daltons. Stevens *et al* (1970), in using ultracentrifugation method, obtained 58,000 and 112,000 daltons for soybean lipoxygenase. It seemed therefore that rubber seed lipoxygenase has two subunits as suggested by

Stevens *et al* (1970) for soybean lipoxygenase. Rubber seed lipoxygenase could then be an oligomeric protein with two subunits.

The results of the electrophoretic mobility (in cm) of rubber seed lipoxygenase at 6 gel tubes ranged from 0.28 to 0.31 and 0.84 to 0.87 while the average mobility (in cm) calculated from the 6 gel tubes were 0.30 ± 2 and 0.85 ± 3 respectively.

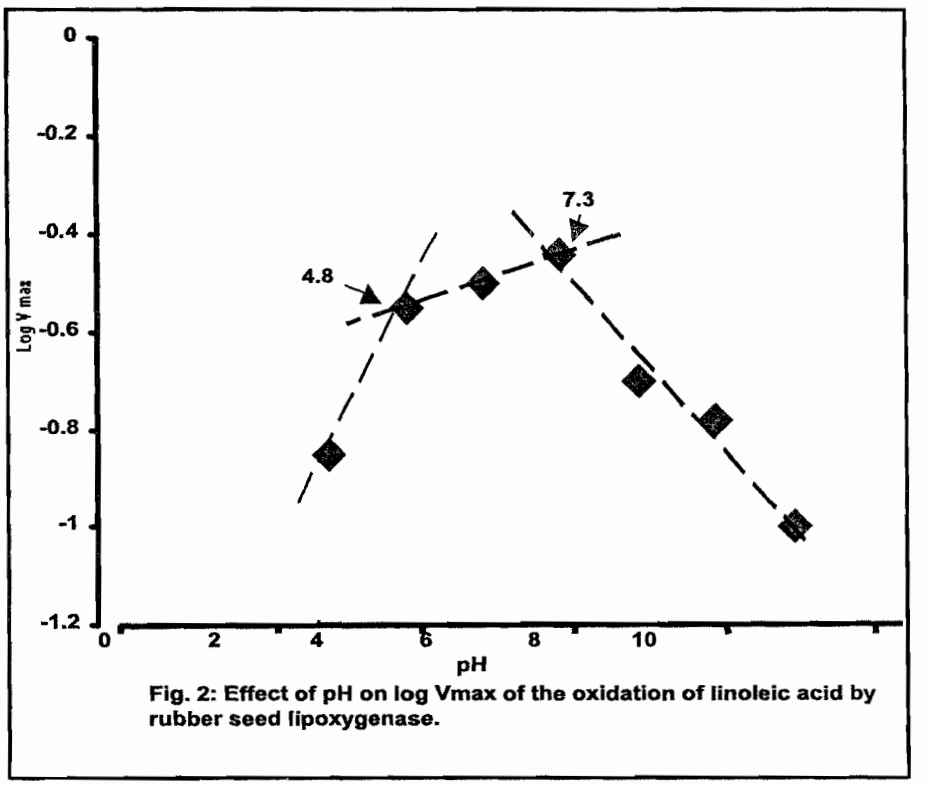

Fig. 2: Effect of pH on log Vmax of the oxidation of linoleic acid by rubber seed lipoxygenase.

The plot of log V_{max} against pH (Fig. 2) showed pK values of 4.8 and 7.3. The results showed that the catalysis of the rubber seed lipoxygenase on the substrate, linoleic acid, depended on the ionization of two groups on the enzyme substrate complex.

The plot of pH versus log V_{max}/K_m (Fig. 3) gave the pK values of 5.2 and 7.5. These pk values could be two ionizing groups on the free enzyme necessary for catalytic activity. Since linoleic acid does not ionize at these pK values (but at 6.5), the results of the pK values could be the ionization constants of the free enzyme only.

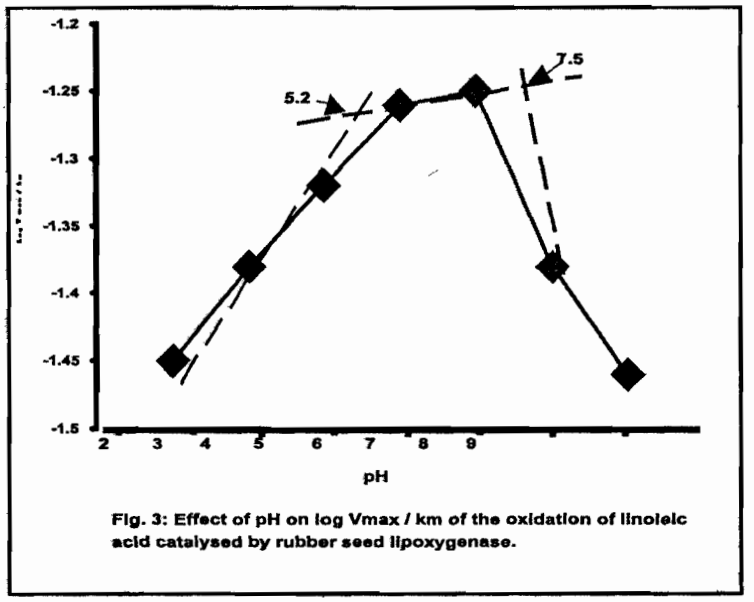

Fig. 3: Effect of pH on log Vmax / km of the oxidation of linoleic acid catalysed by rubber seed lipoxygenase.

Since the pK value of 7.5 from plot of log V_{max}/K_m against pH is close to pK value of 7.3 from the plot of log V_{max} against pH, it shows that the ionization of these groups represented by these two pK values are unaffected by the combination of the enzyme with the substrate. It follows therefore that these two groups are concerned with the mechanism of catalysis and may not be involved in the binding of the substrate. The pK values of 4.8 and 5.2 may be involved in the binding of the substrate to the active site since the two pK values are not close numbers.

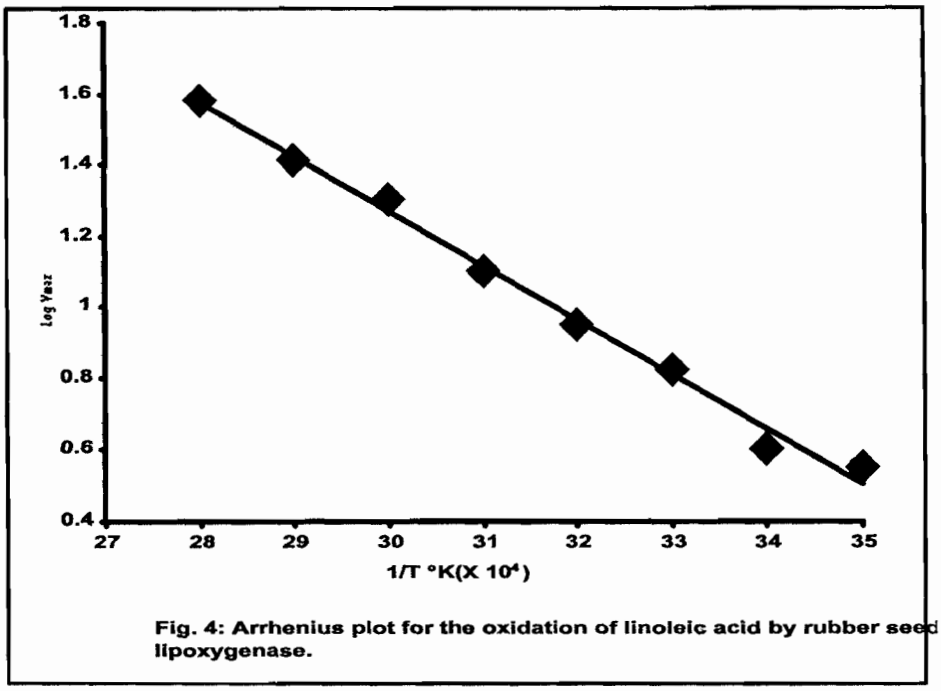

Fig. 4: Arrhenius plot for the oxidation of linoleic acid by rubber seed lipoxygenase.

For the Arrhenius plot of Fig. 4, the line obtained at pH 7.0 and at temperature range of 10 to 80 ^{0}C was linear.

Table 2. Thermodynamic characterization of thermal activation of rubber seed lipoxygenase at pH 7.0 and at several temperatures

Temperature (^{0}C)	Activation Energy (E_a) (Kcal/mol)	ΔH (Kcal/ mol)	ΔG (Kcal/mol)	ΔS (cal/mol/^{0}k)
10	7.2	5.3	1.4	66.3
20	7.2	5.3	1.6	64.4
30	7.2	5.3	1.9	64.2
40	7.2	5.3	1.9	63.8
50	7.2	5.3	1.8	64.3
60	7.2	5.3	1.6	64.6
70	7.2	5.3	1.5	64.1
80	7.2	5.3	1.5	64.5

The thermodynamic characterization of rubber seed lipoxygenase, calculated from the different temperatures of 10 to 80 ^{0}C, is shown at Table 2 .The activation energy (Ea) was 7.2 kcal/mole in all the temperatures. The result of Al-Obaidy and Siddiqi (1981) was 7.1 kcal/mole. Enthalphy (ΔH) was 5.3 kcal/mole in all the temperatures. Free energy change (ΔG) and entrophy change (ΔS) were almost constant in all the temperatures. In enzyme reaction, primary bond rearrangements, when they occur (that is S ® P), are often so clearly balanced that their enthalphy changes are not greater than those of secondary bonds, and are small in many cases (Clark and Switzer, 1977). Probably, that was why the ΔH was small and constant in all the temperatures.

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