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SOYABEAN LIPOXYGENASE: AN IRON-CONTAINING ENZYME

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SUMMARY

Both flameless atomic absorption spectrometry and a spectrophotometric determination (Fe^{2+} (*o*-phenanthroline)$_3$) have demonstrated the presence of iron in the extensively purified classical Theorell soyabean lipoxygenase, (linoleate: oxygen oxidoreductase, EC 1.13.11.12) in a 1:1 ratio. Direct removal of the metal by means of well-known complexing agents, under a variety of conditions, does not seem possible. The enzyme is inhibited by L(+)-cysteine under aerobic conditions only. This inhibition is accelerated dramatically by *o*-phenanthroline, probably due to an increase of the autoxidation of cysteine catalysed by a Cu–cysteine–*o*-phenanthroline complex, copper originating from the enzyme preparation.

The inhibition by cysteine *plus* *o*-phenanthroline is accompanied by the formation of Fe^{2+} (*o*-phenanthroline)$_3$, although the time necessary for the stoichiometric conversion of the iron into that complex is considerably longer than that for the inactivation proper.

INTRODUCTION

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) from various sources is not inhibited by CN^-, azide, diethyldithiocarbamate, F^-, pyrophosphate and EDTA[1]. From this lack of inhibition by a wide variety of efficient metal-complexing agents, it has been concluded that plant lipoxygenases do not possess essential metals. Some five years ago, X-ray fluorescence spectrometry of a partially purified commercial soyabean lipoxygenase (Sigma) indicated to us the presence of iron and manganese. However, the data could not be interpreted quantitatively so that no conclusive picture could be established. The presence of manganese was confirmed by ESR spectroscopy but no iron signal could be observed so that the iron, if present, was likely to occur as Fe^{2+}. Manganese was lost (ESR) upon further purification without affecting the enzyme activity. However, the amount of purified enzyme available

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at that time was too small for X-ray fluorescence spectrometry and no definite conclusion concerning the presence of iron in the purified material could be drawn.

According to Chan[2], a recent atomic absorption spectrometric analysis of a purified commercial soyabean lipoxygenase preparation demonstrated the presence of iron in a 1:1 ratio. This prompted us to re-study the iron content of extensively purified soyabean lipoxygenase and the necessity of iron in the oxygenation of linoleate. In this paper the isolation and purification of the enzyme, the determination of iron by means of flameless atomic absorption spectrometry, and some inhibition studies are described.

MATERIALS

Soyabeans (Yellow II) were obtained from Unimills B.V., Zwijndrecht, The Netherlands. $(NH_4)_2SO_4$ ("for biochemical purposes"), L(+)-cysteine·HCl, sodium diethyldithiocarbamate, *o*-phenanthroline·HCl ("zur Analyse"), $Na_2HPO_4 \cdot H_2O$ and Na_2HPO_4 ("nach Sörensen") from E. Merck A.G., Darmstadt, Germany; isopentane (b.p. 28.1 °C) and NaCN ("analysed reagent") from J. T. Baker Chemicals N.V., Deventer, The Netherlands. In all experiments demineralized water was used, unless otherwise stated.

METHODS

Extraction of soyabeans

400 g soyabeans were ground in a cooled analytical mill (Janke and Kunkel, Germany) at $\leqslant$ 30 °C, the meal was defatted by Soxhlet extraction with isopentane, two times 8 h, and the solvent removed *in vacuo*. The defatted meal (325 g) was stirred with 1600 ml 0.2 M acetate buffer, pH 4.5, for 30 min at 4 °C.

Fractionation

The suspension was centrifuged in a Sorvall RC-2 for 20 min at 10 000 × g, and the supernatant (1250 ml; protein content 25 g) fractionated with solid $(NH_4)_2SO_4$. The precipitate formed between 33 and 60% saturation was collected and suspended in 50 ml water (protein content: 15 g). The resulting suspension was dialyzed against water for 1 h at 4 °C until the solution was practically clear. The $(NH_4)_2SO_4$ concentration in this stage was approx. 30% saturation.

Zone precipitation

Zone precipitation was performed on Sephadex G-100 (5 cm × 80 cm) according to the principle described by Porath[3]. Column preparation: Sephadex G-100 (150 g) was equilibrated in 70% satd $(NH_4)_2SO_4$ in water for 16 h and the suspension transferred to the column. After settling of the Sephadex particles, the solution above the sediment was drained into the Sephadex bed. 1 l of $(NH_4)_2SO_4$ solution with linearly decreasing concentration (70 to 30% satn) was washed into the column. After application of the enzyme solution (60 ml), elution was performed with 2 l of $(NH_4)_2SO_4$ solution the concentration of which was continuously lowered from 30 to 0% saturation. 15-ml fractions were collected and tested for lipoxygenase activity (see below). Half the activity was present in five consecutive fractions which were pooled (protein

content: 800 mg). The other half was recovered in another 1000 ml which were not used in this experiment. The enzyme was precipitated by adding solid $(NH_4)_2SO_4$ to 60% saturation .The residue obtained by centrifugation was dissolved in 15 ml water and the solution dialyzed for 5 h against a total volume of 5 l 0.125 M acetate buffer, pH 5.5.

Chromatography on CM-Sephadex C-50

The dialyzed solution was brought on a CM-Sephadex C-50 column (2.5 cm × 35 cm), equilibrated in 0.125 M acetate buffer, pH 5.5, eluted with a linear NaCl gradient (0–0.2 M) in the same buffer, and collected in 11-ml fractions. For elution pattern see Fig. 1.

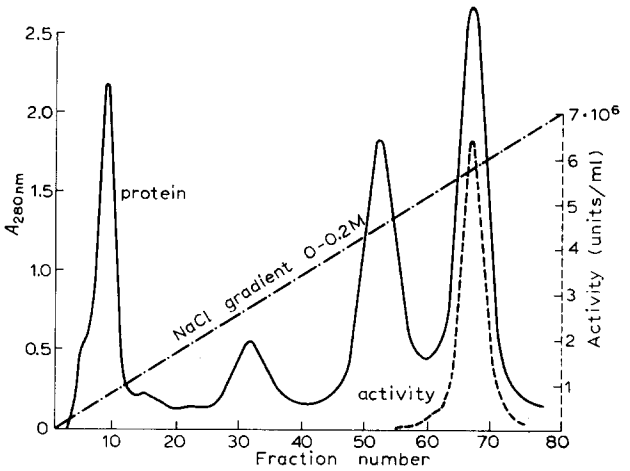


Fig. 1. Elution pattern of lipoxygenase fraction from zone precipitation on CM-Sephadex C-50.

Fractions 64–70 were pooled and the enzyme precipitated by adding solid $(NH_4)_2SO_4$ to 60% saturation. The precipitate was dissolved in 5 ml of demineralized water which additionally had been distilled. In all subsequent isolation steps the same type of water was used. The solution was dialyzed for 4 h against a total volume of 4 l 0.01 M $(NH_4)_2SO_4$.

Gel filtration on Sephadex G-25

To remove any adhering iron, the resulting enzyme solution was passed through a 2.5 cm × 35 cm bed of Sephadex G-25 (medium), equilibrated in 0.01 M $(NH_4)_2SO_4$. The enzyme was eluted with the same $(NH_4)_2SO_4$ solution and collected in fractions of 2.5 ml. The enzyme was obtained in 11 fractions which were stored separately. All glassware was carefully treated with dil. HNO_3 to dissolve any solid iron present, and rinsed well.

Recovery and enzyme activity

The total amount of lipoxygenase isolated was 90 mg, calculated from absorbance at 280 nm of the various fractions, taking $E^{0.1\%}_{280\,nm} = 1.43$[4]. The specific activity was 3 500 000 U/mg.

Purity control

Electrofocusing on polyacrylamide gel[5] (pH 5–7) showed the enzyme as one distinct band contaminated with traces of two other components (Fig. 2).

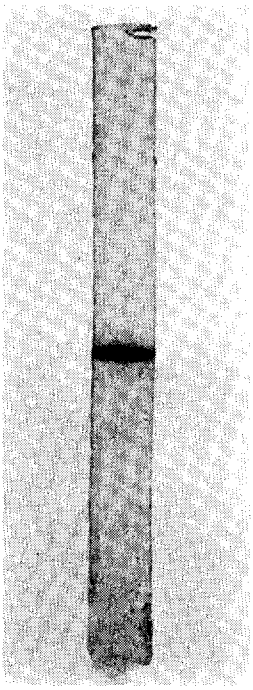


Fig. 2. Isolated lipoxygenase focused on polyacrylamide between pH 5 and 7.

Protein and enzyme activity determination

Protein was determined according to Lowry *et al.*[6]. Lipoxygenase activity was measured as described in Ref. 5 under "Assay of "acid" enzyme".

Iron determination

The iron content of the purified lipoxygenase was determined by flameless atomic absorption spectrometry. A heated graphite furnace (H.G.A. 70) was used as atomizer in a Perkin–Elmer 303 atomic absorption spectrometer. Unwanted absorption was corrected for with a deuterium background corrector.

Two fractions from the Sephadex G-25 filtration experiment containing 9.1 and 3.3 mg lipoxygenase per ml, were injected in 20-μl portions into the furnace. The atomizer was programmed as follows: 40 s drying at 100 °C, followed by 30 s charring at 1100 °C; atomisation for 20 s at 2600 °C. The absorption was measured at 344.06 nm (iron absorption line of low sensitivity). Standards of 1 μg Fe/ml and 2.5 μg Fe/ml were prepared in 0.01 M $(NH_4)_2SO_4$ solution. With the spectrometer settings used, the blanks gave no detectable iron absorption, *i.e.* iron content of the blank < 0.1 μg Fe/ml.

Inhibition experiments

Unless otherwise stated inhibitors were added to 0.5 ml 10^{-5} M lipoxygenase solution, in the appropriate buffer of the desired pH. Preincubation was performed at 0 °C, unless stated otherwise; anaerobic preincubations in Thunberg tubes. After evacuation, the tubes were fluxed with N_2 (O_2-free) and evacuated again. This procedure was done twice. After the preincubation, the residual activity was determined, as indicated above, against the corresponding controls.

Oxidation of cysteine

Oxidation of cysteine was followed in a Gilson KM oxygraph. The cell was filled with 0.05 M phosphate buffer, pH 7.0. After stabilisation, the various components, dissolved in 0.1 ml water and adjusted to pH 7.0 were, added and the oxygen uptake was measured. Temperature: 20 °C.

RESULTS AND CONCLUSION

The isolated enzyme constitutes the major lipoxygenase component of the soyabeans, which implies that it is the classical Theorell enzyme[7], specified as lipoxygenase I by Christopher *et al.*[8] and as "acid" enzyme A_1 by Verhue and Francke[5].

Iron determination

The iron contents of the two lipoxygenase fractions were 5.15 and 1.85 μg Fe/ml, respectively. On the basis of a molecular weight of 102 000 (ref. 9) for the lipoxygenase, molar ratios (Fe:enzyme) of 0.092:0.089 and 0.033:0.032 are found. This points to the presence of one iron atom per molecule of enzyme, which agrees with the work reported by Chan[2].

Flameless atomic absorption spectrometry analysis also showed the presence of copper in a ratio of 1:20 (copper:enzyme).

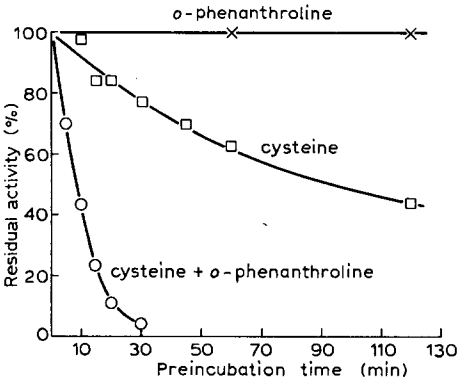
Effects of complexing agents

o-Phenanthroline (10^{-3} M) does not inhibit the enzyme (10^{-5} M) in the pH range of 3–12 for 24 h at 0 °C, nor at room temperature at pH 9–12 for 2 h. At pH 9 and room temperature neither cyanide (10^{-3} M), EDTA (10^{-3} M) and α,α'-bipyridyl (10^{-3} M) show any inhibition in a 4-h period. Treatment of the enzyme (10^{-6} M) with the denaturing agents sulphobetaine (10^{-3} M), sodium dodecyl sulphate (10^{-3} M), cetidylammonium bromide (10^{-3} M), acetone (10%, v/v) or 4 M urea failed to induce any inhibition by *o*-phenanthroline at pH 9 and 0 °C for 20 h. Heating the enzyme in the presence of *o*-phenanthroline for 2 h at 40 °C and pH 7 was without effect. Direct removal of the iron by the well-known complexing agents does, therefore, not seem possible. This may be due either to the iron being inaccessible for the complexing agent or to the complexing constant of the iron–enzyme complex being very high (for Fe^{2+}(*o*-phenanthroline)$_3$:β_3 approx. 21 (ref. 10)).

Inhibition by L(+)-cysteine and effect of o-phenanthroline

The enzyme is clearly inhibited by L(+)-cysteine (10^{-3} M) (Fig. 3). *o*-Phenanthroline accelerates this inhibition drastically, whereas alone it has no influence.

The inhibition by L(+)-cysteine combined with *o*-phenanthroline is accompa-



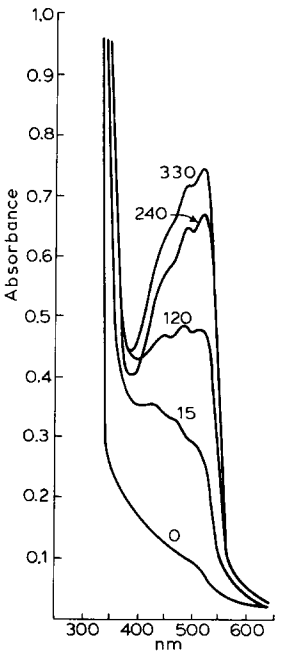


Fig. 3. Inhibition of soyabean lipoxygenase by L(+)-cysteine and effect of *o*-phenanthroline. Enzyme concentration, 10^{-5} M; cysteine concentration, 10^{-1} M; *o*-phenanthroline concentration, 10^{-3} M in 0.02 M phosphate buffer, pH 7.5. ×—×, *o*-phenanthroline; □—□, cysteine; ○—○, cysteine *plus* *o*-phenanthroline.

Fig. 4. Spectra of soyabean lipoxygenase inhibited by L(+)-cysteine *plus* *o*-phenanthroline as a function of time (min). Enzyme concentration, 10^{-4} M; L(+)-cysteine concentration, 10^{-2} M; *o*-phenanthroline concentration, 10^{-2} M in 0.05 M phosphate buffer, pH 7.5.

nied by red colouring of the solution, which points to formation of Fe^{2+}(*o*-phenanthroline)$_3$. However, at 90% inhibition of the enzyme only 20% of the iron is present as the phenanthroline complex. The stoichiometric conversion is not reached until after about 6 h. The gradual formation of the complex is shown in Fig. 4.

Anaerobically neither cysteine nor a combination of cysteine and *o*-phenanthroline inhibit the lipoxygenase, which implies that oxygen plays a role in the inactivation of the enzyme. It is known that cysteine can autoxidize, especially in the presence of heavy metal ions, in particular Cu^{2+} and Fe^{2+} (ref. 11). The enzyme preparation used still contained traces of copper. In this connection it is important that both CN^- and diethyldithiocarbamate prevent the inhibition of our enzyme by cysteine (aerobic conditions). It was, therefore, assumed that the inactivation of lipoxygenase by cysteine under aerobic conditions is caused by autoxidizing cysteine catalyzed by traces of metal ions. This is in agreement with the findings described by Mitsuda *et al.*[12]. The reason for the acceleration of the inhibition by cysteine caused by *o*-phenanthroline follows from a study of the autoxidation of cysteine in the Gilson Oxygraph (Fig. 5). L(+)-Cysteine as such is autoxidized only slowly. Addition of lipoxygenase does not influence this oxidation whereas subsequent addition of diethyldithiocarbamate even stops the oxidation completely. In that stage, *o*-phenanthroline has no effect at all (Fig. 5a). When, on the other hand, *o*-phenan-

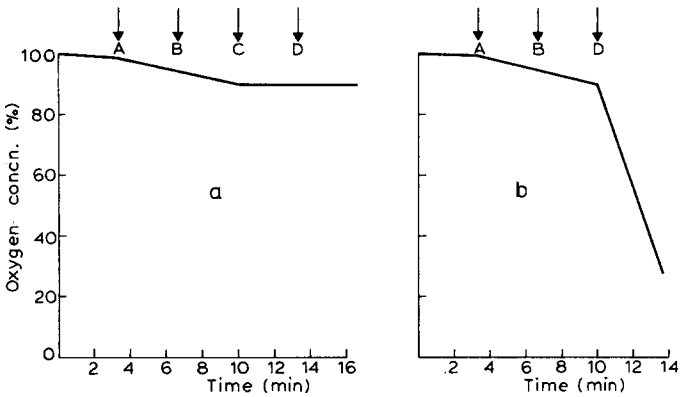


Fig. 5. (a) Oxygen consumptoni of autoxidizing L(+)-cysteine. Starting from 0.02 M phosphate buffer pH 7.5, cysteine (10^{-3} M) is added at point A, lipoxygenase (10^{-5} M) at B, diethyldithiocarbamate (10^{-3} M) at C and *o*-phenanthroline (10^{-3} M) at D. (b) Effect of *o*-phenantroline on the autoxidation of L(+)-cysteine in the presence of lipoxygenase. Starting from the same system as under A, cysteine (10^{-3} M) is added at point A, lipoxygenase (10^{-5} M) at B and *o*-phenanthroline at D.

throline is added to a combination of cysteine and lipoxygenase, the oxygen consumption is increased enormously (Fig. 5b). The rate of oxidation parallels in that case the rate of inactivation of the enzyme by a combination of cysteine and *o*-phenanthroline (*cf.* Fig. 3). The rapid inactivation of our enzyme by the combination indicated above seems to be due to an accelerated oxidation of cysteine, probably caused by a mixed complex of, possibly, copper with cysteine and *o*-phenanthroline. This is substantiated by the finding that a mixture of 10^{-4} M Cu^{2+}, 10^{-3} M cysteine and 10^{-3} M *o*-phenanthroline in phosphate buffer, pH 7, which is deep violet-brownish, decolourizes within a few minutes on shaking with air. Similarly, addition of Cu^{2+} (10^{-6} M) to a mixture of L(+)-cysteine (10^{-3} M) and *o*-phenanthroline (10^{-3} M) increases the oxygen consumption in the Oxygraph greatly, whereas Fe^{2+} does not.

DISCUSSION

Both flameless atomic absorption spectrometry and the spectrophotometric determination clearly indicate the presence of iron in our enzyme in a 1:1 ratio. Whether the copper found by atomic absorption spectrometry is an essential part of the enzyme or just a contamination cannot be established yet. Taking into account the purity of our enzyme preparation (Fig. 1), we can exclude the presence of other proteins containing the iron. Direct removal of the iron by well-known complexing agents does not seem possible under a variety of conditions.

The inhibition of our enzyme by a combination of cysteine and *o*-phenanthroline is rather complex. The actual inactivating agent is not clear. H_2O_2 does not seem very likely, since it could not be detected. It is still the question as to whether the inactivation is the direct consequence of oxidation and/or removal of the iron. An alternative could be modification (*e.g.* oxidation, hydroxylation) of one or more of the ligands and a subsequent release of iron. The latter does not seem impossible since the time necessary to convert the iron stoichiometrically into the *o*-phenanthroline

complex is much longer than that for the incativation proper. The formation of the red-coloured Fe^{2+}(*o*-phenanthroline)$_3$ during the inactivation by the combination of cysteine and *o*-phenanthroline does not necessarily imply that the iron in the enzyme is in the ferrous state, since any Fe^{3+} present could have been reduced by cysteine prior to the complex formation.

Pistorius and Axelrod[13] arrived at the conclusion that the iron in soyabean lipoxygenase is present in the ferric form. This conclusion was mainly based on the inhibition of Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid) as far as can be read from the paper. It is well known (ref. 14) that polyphenolic antioxidants can inhibit strongly the lipoxygenase-catalysed oxidation, and can also be oxidized themselves. Although the typical phenolic character in Tiron may be weakened by the presence of the sulphonic acid groups, inhibition of the type mentioned cannot be excluded. So, in that case removal of the iron may be a secondary effect (*cf.* inhibition by cysteine), associated with oxidation of Fe^{2+} to Fe^{3+} (the oxidation potential is lowered drastically in the presence of Tiron). Therefore, it is still questionable whether the iron is in the ferric form.

So, we must conclude that direct proof of the essentiality of iron in the oxygenation of linoleate is still lacking. However, considering the activation of oxygen in the lipoxygenase reaction as an essential step and the knowledge of the properties of metal ions like Fe^{2+} in this connection, a function of the iron in the enzymic catalysis seems very likely.

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