BBA 66745

PURIFICATION OF SOYBEAN LIPOXYGENASE BY AFFINITY CHROMATOGRAPHY

SHLOMO GROSSMAN, MOSHE TROP, SANDRA YARONI AND MEIR WILCHEK[a]

Department of Life Sciences, Bar-Ilan University, Ramat-Gan and [a]Department of Biophysics, The Weizman Institute of Sciences, Rehovoth (Israel)

(Received April 26th, 1972)

SUMMARY

A rapid chromatographic procedure for the purification of soybean lipoxygenase (linoleate:O_2 oxidoreductase, EC 1.13.1.13) is described. The separation was achieved by passing the crude extract through a linoleyl aminoethyl agarose column. The unadsorbed proteins were eluted with 0.005 M acetate buffer (pH 5.0).

Lipoxygenase was eluted from the adsorbent with 0.2 M acetate buffer at the same pH. Both the yield and purity of the lipoxygenase purified by the affinity column were facilitated by prior removal of contaminant proteins on CM-cellulose.

The final product is almost homogeneous.

INTRODUCTION

Lipoxygenase (linoleate:O_2 oxidoreductase, EC 1.13.1.13) catalyzes the oxidation by molecular oxygen of unsaturated fatty acids interrupted by *cis*-methylene[1]. This enzyme is not inhibited by pyrophosphate, fluoride, cyanide, azide, *p*-chloromercuribenzoate[1]. It was concluded that the enzymatic activity is not dependent on metal ions or sulphydryl groups and does not contain a prosthetic group[1]. Many of the studies on lipoxygenase have been carried out on crude extracts or commercial preparations which are heterogeneous.

Recently, conventional methods for the purifications of the crude enzyme have been reported[2,3]. In this study the purification of soybean lipoxygenase to homogeneity by affinity chromatography on linoleyl aminoethyl agarose is described. A 46-fold purification was achieved with this simple and rapid procedure.

MATERIALS AND METHODS

Lipoxygenase, linoleic acid, cyanogen bromide, ethylene diamine and acrylamide were obtained from Fluka, CM-cellulose was from Serva, and the agarose from Sigma.

Preparation of linoleyl aminoethyl agarose

The aminoethyl agarose was prepared as described previously[4]. 1 ml of linoleic acid was coupled to 2 g of this agarose derivative by the carbodiimide method[5]. The reaction mixture was stirred for 4 h at room temperature under N_2, and washed extensively with 0.005 M acetate buffer (pH 5.0) until no absorption at 280 and 234 nm was detected.

CM-cellulose purification of lipoxygenase

A soybean homogenate (30%, w/v) was prepared in 0.005 M phosphate buffer, pH 7.0. After centrifugation at 20 000 × *g* for 10 min, the supernatant was adjusted to pH 4.5 with 1 M HCl, centrifuged under the same conditions, and the pH brought to 5.5 with 1 M NaOH. This solution was dialyzed for 16 h at 4 °C against 0.005 M acetate buffer at the same pH, and applied to a CM-cellulose column equilibrated with the same buffer.

Protein determination

The protein was determined according to the method of Lowry *et al.*[6] when homogenates or crude extracts were used. The purified protein was estimated from $E^{0.1\%}_{280\ nm} = 1$. The calculation of the specific activity was based on the absorbance at 280 nm.

Enzyme assays

Linoleate oxidation was assayed by two different techniques: (a) oxygen absorption, measured polarographically[7], and (b) measurement of conjugated diene formation[8].

The specific activity was calculated as (a) μl O_2 consumed per mg enzyme per min or (b) μmoles conjugated diene formed per mg enzyme per min.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed (using 7.5% gels with Tris–glycine buffer, pH 8.6), as described by Davis[9]. The electrophoresis was performed at room temperature for 3 h, at 250 V and 5 mA per tube in 7 cm × 0.5 cm tubes.

The proteins were stained with coomassie brilliant blue[10].

RESULTS AND DISCUSSION

A typical chromatographic elution pattern of the lipoxygenase extract on CM-cellulose is presented in Fig. 1. Lipoxygenase activity appeared in two peaks, which may be two isoenzymes. The first peak (A) was eluted with 0.05 M acetate buffer (pH 5.5) and the second peak (B) with a 0.1 M solution of the same buffer. The enzyme activities were assayed at pH 6.6–6.7 and at pH 9.0 for Fractions A and B, respectively (the optimum pH of the respective fractions). The nature of the early peak was not investigated further since it represented less than 5% of the total activity.

Fraction B was precipitated with 60% (w/v) $(NH_4)_2SO_4$, dissolved in 0.005 M acetate buffer (pH 5.0) and adsorbed onto the linoleyl aminoethyl agarose column. After extensive washing with the same buffer, the enzyme was eluted with 0.2 M

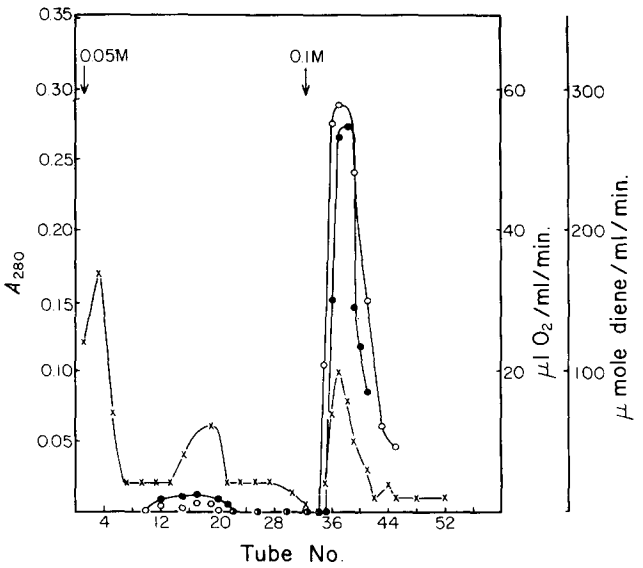

Fig. 1. CM-cellulose purification of lipoxygenase. Soybean homogenate was applied to a (2 cm × 5 cm) CM-cellulose column in 0.005 M acetate buffer (pH 5.5). The first peak was eluted with 0.05 M acetate buffer. The second peak was eluted with a 0.1 M solution of the same buffer. Fractions of 6 ml were collected. Fraction A was collected from tubes 14–20 and Fraction B from tubes 30–40. ×—×, absorbance at 280 nm; ●—●, linoleate oxidation assayed spectrophotometrically; ○—○, linoleate oxidation assayed polarographically.

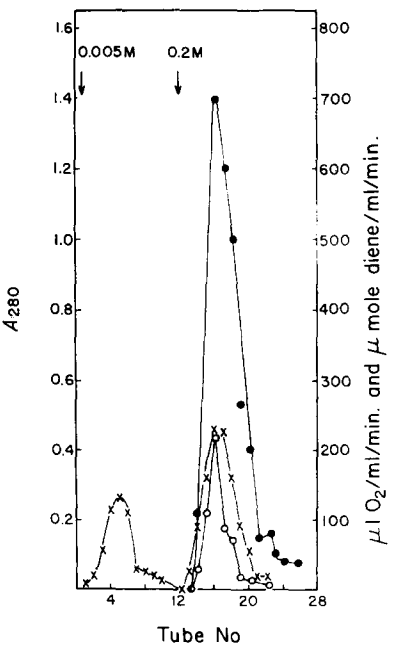

Fig. 2. Affinity chromatography of lipoxygenase on a linoleyl aminoethyl agarose column. The enzyme was adsorbed with 0.005 M acetate buffer (pH 5.0) and was eluted with a 0.2 M solution of the same buffer. Fractions of 5 ml were collected. ×—×, absorbacne at 280 nm; ●—●, linoleate oxidation assayed spectrophotometrically; ○—○, linoleate oxidation assayed polarographically.

TABLE I

PURIFICATION OF LIPOXYGENASE FROM SOYBEAN

The enzyme was assayed at pH 6.6–6.7 with $7.5 \cdot 10^{-3}$ M linoleate in 0.25% (w/v) Tween 20.

	Specific activity (μl O_2 *per mg protein per min*)	*Yield* (%)	*Enrichment*
1. Soybean homogenate	25.0	100.0	1.0
2. pH fractionation	60.0	50.0	2.4
3. Dialysis	60.0	46.0	2.4
4. CM-cellulose B	740.0	21.3	29.0
5. Agarose linoleate (B)	1155.0	14.0	46.0
6. Agarose blank (B)*	629.0	13.0	25.0

* In this case a column of aminoethyl agarose containing no linoleic acid was used.

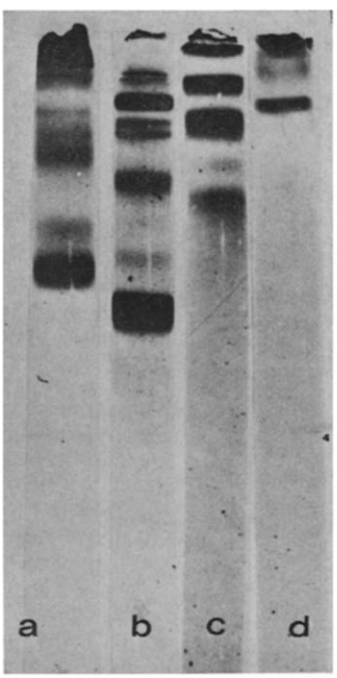

Fig. 3. Polyacrylamide gel electrophoretic pattern of soybean lipoxygenase at different steps of purification. The electrophoresis was performed in 7.5% polyacrylamide gels for 3 h under 250 V and 5 mA per tube (7 cm × 0.5 cm tubes). The gels were stained with coomassie brilliant blue. a, soybean homogenate; b, homogenate after various pH fractionation; c, preparation after CM-cellulose chromatography; d, preparation after affinity chromatography.

acetate buffer at the same pH in more than 90% yield (Fig. 2). The overall purification from the starting preparation was about 46-fold (Table I).

Fig. 3 shows the electrophoretic patterns in acrylamide gel at various stages of purification. It can be seen that the crude extract and the various pH fractionations contained at least 8 different proteins, whereas after CM-cellulose chromatography the number was reduced to 4. The affinity-purified lipoxygenase had only one band and was assumed to be a single protein.

In this study the yield is considerably high and the column can be used many times without loss of efficiency. The critical stage in the separation, however, is the CM-cellulose step, since attempts to purify commercial lipoxygenase directly by affinity chromatography were unsatisfactory.

ACKNOWLEDGEMENTS

Part of this research was supported by Bar-Ilan Research Fund No. 161-81-05-5.

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