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LIPOXYGENASE FROM *MEDICAGO SATIVA*: PURIFICATION ON HYDROXYAPATITE

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Abstract—Three lipid-oxidizing fractions obtained from an alfalfa extract by stepwise elution from a CM-cellulose column were further purified on hydroxyapatite columns. Although substantial separation between lipoxygenase and peroxidase activities was achieved, only one of the protein fractions migrated as a single band on polyacrylamide gel electrophoresis and exhibited the properties of pure lipoxygenase

INTRODUCTION

IT WAS shown previously[1,2] that chromatography of an aq. Triton X100 extract, prepared from alfalfa leaves, on carboxymethylcellulose (CM-cellulose) separated four distinct protein peaks (A, B, C and D) which coincided with peaks of lipid oxidizing activities. Fractions A, B and C were eluted from the CM-cellulose column with 0·05, 0·1 and 0·15 M acetate buffer, pH 5·5, respectively, whereas fraction D was eluted with 0·1 M Na_2HPO_4. Although lipoxygenase (E.C. 1.13.1.13) activity was found in fractions B and C, these fractions were still impure. The present report describes attempts to purify fractions B, C and D by means of chromatography on hydroxyapatite.

RESULTS AND DISCUSSION

Chromatography on hydroxyapatite

The main fractions obtained by chromatography on hydroxyapatite are listed in Table 1, together with their specific activities with respect to linoleate oxidation, carotene bleaching and peroxidase-like action. Fraction A, which had been shown to contain peroxidase,[2] could not be purified on hydroxyapatite, since all activities were eluted with dilute (1 mM) phosphate buffer, pH 6·8. Fractions B, C and D were well adsorbed, and stepwise elution with increasing concentrations of phosphate buffer, pH 6·8, produced discrete protein peaks.

✠ Died in the Yom Kippur War, October 1973

[1] GROSSMAN, S., BEN-AZIZ, A., BUDOWSKI, P., ASCARELLI, I., GERTLER, A., BIRK, Y. and BONDI, A. (1969) *Phytochemistry* **8**, 2287

[2] BEN-AZIZ, A., GROSSMAN, S., BUDOWSKI, P. and ASCARELLI, I. (1971) *Phytochemistry* **10**, 1823

Two subfractions B_1 and B_2, were obtained on elution of fraction B from hydroxyapatite. B_1 displayed strong peroxidase activity, but caused only moderate oxidation of carotene and little oxidation of linoleate. B_2 was relatively more active in carotene bleaching and linoleate oxidation, but showed less peroxidase activity.

TABLE 1 SPECIFIC ACTIVITIES OF SUBFRACTIONS OBTAINED BY CHROMATOGRAPHY OF FRACTIONS B, C AND D, ON HYDROXYAPATITE*

Fraction	Phosphate buffer (M)	Linoleate oxidation (μl O_2/min/mg protein)	Carotene oxidation (μg/min/mg protein)	Peroxidase activity ($-\Delta E_{430}$/min/mg protein)
B_1	0 10	15	82	171
B_2	0 15	96	148	84
C_1	0 15	150	308	9
C_2	0 20	204	925	ND†
D_1	0 15	340	485	ND
D_2	0 20	220	131	ND

* Total activities roughly follow the order of specific activities, but are not included in the table, because fractions B, C and D, from which the subfractions were prepared, were not from the same starting material (see Experimental) and therefore not directly comparable

† Not detectable

Subfractions C_1 and C_2, obtained from fraction C, had high specific activities with respect to carotene bleaching and linoleate oxidation, but negligible peroxidase activity. A small amount of peroxidase activity was eluted from the column before the emergence of C_1 and is not included in the table

Subfraction D_1 emerged as a broad peak with shoulders upon elution with 0 25 M phosphate buffer, pH 6·8. Increasing the buffer concentration to 0·2 M removed additional protein (peak D_2). Considerable carotene and linoleate oxidizing activities were present, especially in D_1, but no detectable peroxidase activity was found

The abrupt change in buffer concentration is liable to cause the appearance of slowly eluted residual protein in the following fraction Inspection of the protein elution profiles revealed a marked similarity between peaks B_2 and C_1, probably due to a carry-over effect during CMC chromatography. Nevertheless, as seen from Table 1, the specific activities were not only different in the various subfractions, but they also varied in relation to each other, so that additional purification could be achieved

Polyacrylamide gel electrophoresis

The subfractions obtained from hydroxyapatite were tested for purity by polyacrylamide gel electrophoresis. This technique revealed the presence of 3 to 5 protein bands in most of the fractions, some of the bands obtained from different fractions appearing in the same position. Only fraction C_2 yielded a single band.

The reason for the lower specific activity of C_2 with respect to linoleate oxidation, compared to the apparently less pure fractions D_1 and D_2, is not known Possibly the large amount of fraction D submitted to chromatography on hydroxyapatite created conditions under which the impure fractions were more stable than the homogeneous subfraction C_2, but the possibility that the D subfractions contained very active isoenzymes cannot be discounted.

Properties of fraction C_2

Since fraction C_2 alone appeared to be pure by polyacrylamide gel electrophoresis, it was submitted to a series of tests. The following compounds were found to be without effect on its linoleate-oxidizing activity: 10^{-2} M sodium azide, sodium cyanide and EDTA, and 10^{-3} M sodium fluoride and lead acetate. The absence of inhibition by these reagents indicates that the enzyme is devoid of prosthetic metal groups, and that no SH groups are involved in its activity. These results point to C_2 being lipoxygenase.

The substrate specificity of C_2 was tested with oleic and linoleic acids. Linoleate was rapidly oxidized, whereas oleate was resistant to oxidation by C_2, in accordance with our previous results[2] obtained with the impure fraction C. The Lineweaver–Burk plot for linoleate oxidation by fraction C_2 yielded a K_m value of $1{\cdot}2 \times 10^{-3}$ M. This is slightly smaller than 5×10^{-3} M previously reported[2] for the less pure fraction C, indicating increased affinity of the purer enzyme for linoleic acid.

EXPERIMENTAL

Most of the procedures were described previously,[1,2] with the following exceptions Fractions A to D, obtained by stepwise elution from CM-cellulose, were prepared separately from fresh alfalfa leaves at different times In the case of fraction D, the amount of starting material was 10 times greater than the amounts taken for preparing fractions A–C All fractions were dialyzed against 1 mM phosphate buffer, pH 6 8, before being chromatographed on hydroxyapatite Hydroxyapatite columns, 2 × 25 cm, were prepared according to the technique of Tiselius *et al*[3] Electrophoresis on polyacrylamide gel was as described by Ornstein and Davis[4] Since no migration occurred when the medium was acid, the pH was adjusted to 8 3

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[4] Ornstein, L and Davis, B J (1962) *Disc Electrophoresis*, Distillation Products Industries, New York