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LIPOXYGENASE ISOENZYMES FROM *SOLANUM TUBEROSUM*

A. PINSKY, J. SPORN, S. GROSSMAN and M. RIVLIN
Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

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Abstract—Two lipoxygenase isoenzymes were separated from potato tubers (*Solanum tuberosum*). Experiments with chemical modifications showed that tryptophan is essential for enzyme activity and that one or more tryosine residues was involved. On the other hand, no lysine or sulfhydryl groups were necessary. Both enzymes had an optimum pH of 5·5. They were not affected by calcium ions but were inhibited by cysteine.

INTRODUCTION

A SURVEY of lipoxygenase activity in plant tissues revealed considerable activity in the potato tuber *Solanum tuberosum*.[1,2] Galliard[3] and Galliard and Phillips[4] described the partial purification of a specific lipoxygenase from potato tubers and some of its properties.

The present work deals with the isolation and properties of two potato lipoxygenase isoenzymes together with experiments on the identity of the active center of one of the isoenzymes.

RESULTS

Purification

Chromatography on ecteola cellulose with 0·05 and 0·1 M phosphate buffer pH 6·5 as eluents enriched fraction-*A* and -*B* 10·6- and 2·2-fold respectively (Table 1). Fraction-*A* had a high specific activity in oxidizing linoleate and resembled the soybean lipoxygenase in this respect.

Since most of the activity was in fraction-*A*, further purification was done with this fraction. It could be precipitated by adding ammonium sulphate to 80% saturation. The precipitate when dissolved in 5 ml 0·1 M phosphate buffer could be further purified by chromatography on a Sephadex G200 column (100 × 4 cm) with 0·1 M phosphate buffer pH 6·5 as eluent. A typical purification experiment with the yield and specific activity at each stage, is given in Table 1.

Determination of Purity by Electrophoresis

Polyacrylamide disc electrophoresis of fraction-*A* at various stages of purification showed that this contained 5–6 protein bands after Ecteola cellulose chromatography, and

[1] RHEE, K. S. and WATTS, B. W. (1966) *J. Food Sci.* **31**, 664.
[2] PINSKY, A., GROSSMAN, S. and TROP, M. (1971) *J. Food Sci.* **36**, 571.
[3] GAILLARD, T. (1970) *Phytochemistry* **9**, 1725.
[4] GAILLARD, T. and PHILLIPS, D. R. (1971) *Biochem. J.* **124**, 431.

one major band and a weak component, after molecular filtration on Sephadex G200. Cellulose acetate strip electrophoresis at pH 6·5 of fraction-*A* gave 4 active fractions after the Ecteola cellulose elution and 2 after molecular filtration on Sephadex G200, as determined

TABLE 1. YIELDS AND ENRICHMENT OF POTATO LIPOXYGENASE AT VARIOUS STAGES OF PURIFICATION

Stage	Protein (mg)	Total activity (μl/O_2/min)	Specific activity (μl O_2/min/mg protein)	Enrichment	Yield (%)
Dialysed homogenate	100	6600	66	1	100
Fraction-*A* after ecteola cellulose	8·3	5798	702	10·6	87
Fraction-*A* after precipitation by 80% saturation with ammonium sulphate	6·6	4300	651	9·8	65
Fraction-*A* after molecular filtration (Sephadex G200)	3·5	3870	1107	16·2	58
Fraction-*B* after ecteola cellulose	6·8	990	148	2·2	15

according to Grossman *et al.*[5] Using both electrophoretic methods fraction-*B* contained one active fraction only. Measurement of the migration distance indicated that fraction-*A* still contained traces of fraction-*B*. Electrophoresis of the fractions at pH 4·6 gave the same pattern except that all fractions migrated to the cathode.

TABLE 2. SPECIFICITY OF FRACTIONS-*A* AND -*B*

Substrate	Relative activity as compared to linoleic acid as 100%: Fraction-*A* after Ecteola cellulose chromatography	Fraction-*A* after molecular filtration	Fraction-*B*
Linoleic acid	100	100	100
Linolenic acid	80	88	105
Trilinolein	25	17	10
Dilinolein	50	50	25
Linoleic acid *cis*methylester	6	5	4
Ethyl ester	—	7	—

Properties of the Isoenzymes

pH activity curve. Both isoenzymes had the same pH profile with high activity from pH 5·0 to 6·5, the optimum being at pH 5·5. Some activity was still observed down to pH 3·5 and up to pH 7·0.

[5] GROSSMAN, S., PINSKY, A. and GOLDWEITZ, Z. (1971) *Anal. Biochem.* **44**, 642.

Substrate specificity. Both enzymes had the same specificity (Table 2) and they were more active on the free acid than on the glycerides. Both also had reduced activity on the various linoleic acid esters.

Michaelis constant. A Lineweaver–Burk plot gave K_m values of 3×10^{-4} M and 8×10^{-4} M, for fractions-*A* and -*B* respectively.

Inhibitors. Neither fraction was inhibited by cyanide, *p*-chloromercuribenzoate (*p*CMB) or ethylenediaminetetra-acetic acid (EDTA) at 10^{-3} M. Preincubation of fraction-*A* with cysteine resulted in 50 and 85% inhibition when the molar ratio of the enzyme to the cysteine was 1:25 and 1:63 respectively. A similar result was obtained for fraction-*B*. A 7 M concentration of urea caused complete inhibition of both enzymes whereas 2 M urea had no effect.

Chemical Modification

Nitration. A molar ratio of tetranitromethane (TNM) to fraction-*A* enzyme of 265:1, caused 90% inhibition of linoleate oxidation and a ratio of 52:1 gave 40% inhibition. Incubation of the reagent with the enzyme for 2 hr resulted in 75% inhibition with the inhibition gradually increasing with time up to 6 hr. An investigation of the optimal pH of nitration showed that in the acid range (pH 5·5–6·5), there was only a 30% loss of activity, whereas the inhibition reached 90% at pH 8.

Acetylation. Acetylation with acetic anhydride did not cause any significant change in the activity of fraction-*A*. Even when the acetic anhydride-enzyme ratio was 20 000:1 there was only a 30% loss of activity. Tests with ^{3}H-labeled acetic anhydride showed that this loss of activity is probably due to denaturation, as only traces of ^{3}H-acetic anhydride were bound to the enzyme.

Modification with 2-Hydroxy-5-nitrobenzylbromide (HNB)

The inhibition increased with increase in the HNB:enzyme ratio. When the ratio was 2500:1 the inhibition was 97%. When the pH of the HNB enzyme reaction was varied, the inhibition as measured by oxygen consumption was virtually complete at 3·5 and decreased with increase in pH (by 20% up to pH 5·5). Above this pH, the inhibition increased up to 100% inhibition at pH 7·5. Calculation of the E_{410}/E_{280} ratio for the enzyme which was modified by comparison with the unmodified enzyme shows that in the absence of urea, 7·6 units of tryptophan reacted, while in the presence of urea 9·3 units reacted. These results were obtained when the HNB enzyme ratio was 2500:1.

Modification with N-bromosuccinimide (NBS)

At a ratio of 22:1 of NBS to enzyme, the inhibition was 93%. Similar results were obtained in the presence of 2 M urea. A decrease in absorption at 280 nm was observed in the presence of urea. Calculation showed that 2·5 tryptophan residues were modified in the absence of urea and 5 in its presence.

DISCUSSION

Although the potato lipoxygenase may be extracted without the use of Triton X100, this detergent was used here because the Triton-extracted enzyme was much more stable. A more active preparation was obtained by extraction without Triton X100[2] but this preparation was unstable, losing its activity after a few days. The preparation extracted with Triton on the other hand was stable for several weeks at 4°. It was shown by Pinsky

et al.[2] that Triton inhibits the enzyme, but this presents no difficulty since the detergent is removed during the column chromatography.

The two isoenzymes can be separated by chromatography on Ecteola cellulose. Although the ammonium sulphate precipitation caused a considerable loss in activity, from 87 to 65% yield, this step was considered necessary in order to obtain a concentrate for further purification since the volume of the Ecteola cellulose eluate was generally large, being about 250 ml for even a small column (10 cm). Molecular filtration of fraction-*A* results in a relatively homogeneous protein with traces of impurities, as shown by acrylamide gel electrophoresis (one main and one minor band). However, a determination of the isoenzymes by activity measurement on cellulose acetate strips[5] showed 4 active fractions after Ecteola cellulose chromatography of fraction-*A*. Two of these remained after molecular filtration. Fraction-*B* on the other hand contained only one active fraction after Ecteola cellulose chromatography.

Fraction-*A* still shows 2 active fractions after molecular filtration on Sephadex G200. The minor fraction may be fraction-*B* from the migration distances of both fractions on cellulose acetate gel.

A comparison of both potato isoenzymes shows slight differences in specificity of isoenzymes-*A* and -*B* with the *B* enzyme being more specific for esters and triglycerides. In contrast to the soybean isoenzymes, neither of the potato lipoxygenases was affected by Ca^{2+}. The enzymes from both sources on the other hand were inhibited by cysteine.[6]

The nitration of isoenzyme-*A* showed quite clearly that one or more tyrosine residues were essential for the enzyme action. When the TNM caused 90% inhibition, 22·8 of the 40 tyrosine residues in the molecule had been nitrated; at 40% inhibition, 14·6 residues had been nitrated. Riordan *et al.*[7] have indicated that tyrosine is nitrated at alkaline pH values (above 8) with cysteine reacting in acid conditions. Since the main inhibition occurs when the nitration is at pH 8 and the inhibition decreases to 30% when the nitration is effected at pH 5–6, cysteine is not in the active center, and the results obtained with *p*-chloromercuribenzoate confirm this finding. In addition, the acetylation experiments indicate that lysine is not involved since the acetylated enzyme retained full activity.

The tryptophan reagents appeared to show differences in their effect, the HNB being active at a relatively high concentration and the NBS, at a low concentration. This discrepancy can be explained by the fact that HNB is rapidly hydrolysed in H_2O and the active concentration is therefore much less than the amount originally introduced. The pH effect of the HNB (increase in inhibition in the alkaline range) may be explained by the observation of Horton and Koshland[8] that in this range the tyrosine reacts as well. The high inhibitory effect of these reagents shows that tryptophan is essential for the lipoxygenase activity. Furthermore, the results with NBS (spectra) indicate that two tryptophan residues are involved.

EXPERIMENTAL

Materials. New potatoes were bought from the Tel Aviv market.

Preparation of extracts. Extracts were prepared at 4°. 150 g potatoes were homogenized for 2–3 min in a Waring blendor in 300 ml 5 mM cold phosphate buffer pH 6·5 to which 3·75 ml Triton X100 had been

[6] MITSUDA, H., YASUMOTO, K. and YAMAMOTO, A. (1967) *Agric. Biol. Chem.* **31**, 853.
[7] RIORDAN, I. F. and VALLEE, B. L. (1967) in *Methods in Enzymology* (HIRS, C. H. W., ed.), Vol. 11, p. 565, Academic Press, New York.
[8] HORTON, H. R. and KOSHLAND, JR., D. E. (1965) *J. Am. Chem. Soc.* **87**, 1126.

added. The homogenate was filtered through gauze and the filtrate was centrifuged for 10 min at 20 000 *g*. The clear supernatant was then dialysed for 12 hr against 5 mM phosphate buffer (pH 6·5). The clear dialysate (no ppt. appeared) was the crude extract later purified by column chromatography.

Chromatography on Ecteola (epichlorohydrin triethanolamine) cellulose. The crude extract was pipetted onto an Ecteola cellulose column (10 × 2 cm) which had been equilibrated with 5 mM phosphate buffer (pH 6·5). The first eluent, the equilibration buffer, removed the detergent and inactive proteins. When the buffer concentration was raised to 0·05 M, fraction-*A* containing most of the activity was eluted. At 0·1 M buffer concentration, fraction-*B*, less active, was eluted.

Determination of protein. Protein was estimated from the E_{280} readings by assuming that a reading of 1·0 corresponds to a protein concentration of 1 mg/ml. The protein values thus obtained were used for the calculation of specific enzyme activities.

Assay of enzyme activity. Lipoxygenase activity of the fractions obtained with the various substrates as listed in Table 2 was determined by either a polarographic method[9] or a spectrophotometric method.[10]

Electrophoresis. Cellulose acetate gel electrophoresis[5] in 0·05 M sodium phosphate buffer (pH 6·5) or in 0·05 M acetate buffer (pH 4·6) at 250 V and 5 mA for 20–45 min was used to determine the purity of the active fractions. The lipoxygenase fractions were identified with ferrous ammonium sulphate and ammonium thyocyanate after incubating the electrophoresis strip on linoleate. Polyacrylamide disc gel electrophoresis of the lipoxygenase was carried out according to Orenstein and Davis[11] with 7% gel and riboflavin catalyst.

Nitration. The enzyme was nitrated by the method described by Riordan *et al.*[7] Varying quantities (1–100 μl) of tetranitromethane in EtOH were added to the lipoxygenase, 4 mg/ml 0·05 M buffer at room temp. The reaction was terminated by passing the mixture through a Sephadex G25 column (40 × 2 cm). The enzyme was eluted with 0·05 M Tris buffer pH 8·0, before the reagent. This nitrated enzyme was tested for activity and nitrated phenol groups were estimated from its absorption at 428 nm. The blank contained 1–100 μl EtOH and the enzyme (4 mg/ml) as above.

Acetylation. Enzyme (2 ml) in half satd. NaOAc (1 mg/ml) was mixed in the cold room. Up to 30 μl Ac_2O was added (an enzyme reagent ratio of 1:20 000).[12] The blank contained double the amount of HOAc, with respect to the Ac_2O used. To determine the extent of acetylation, labeled ^3H-Ac_2O (Amersham) was used. The same procedure as above was adopted, adding 30 μl Ac_2O having 130 000 cpm.

Modification with HNB was by the method of Horton and Koshland.[13] 10–100 μl of 0·2 M acetone solution of HNB were added to enzyme solution (0·8 mg/ml) in 0·2 M NaOAc (pH 4·0) buffer. The blank contained equivalent amounts of acetone. Linoleate oxidizing activity was tested polarographically after 10 min incubation at 25°. To determine the absorption of modified enzyme it was filtered through a column of Sephadex G25 (40 × 2 cm). The enzyme was eluted with 0·2 M acetate buffer (pH 4·0) and its absorption at 410 nm was determined, after adding a few drops of conc. NaOH to raise the pH to 10·0. The same procedure was adopted with the blank solution and its absorption at 280 nm was read.

Modification with NBS was by the method of Spande and Witkop.[14] An aq. soln of 0·01 M NBS was prepared and 2–15 μl quantities of this were added to the enzyme soln (0·6 mg/ml) in 0·2 M acetate buffer (pH 4·0). After 30 min incubation at 25°, the enzyme was tested for activity and its spectrum was determined. Similar experiments with NBS and HNB were conducted with the enzyme in 2 M urea, all other conditions being identical.

[9] Grossman, S., Ben-Aziz, A., Budowski, P., Ascarelli, I., Gertler, A., Birk, Y. and Bondi, A. (1969 *Phytochem.* **8,** 2287.

[10] Ben-Aziz, A., Grossman, S., Ascarelli I. and Budowski, P. (1970) *Anal. Biochem.* **34,** 88.

[11] Orenstein, L. and Davis, B. J. (1961) *Disc. Electrophoresis,* Distillation Products Industries.

[12] Riordan, I. F., Sokolowski, M. and Vallee, B. L. (1966) *J. Am. Chem. Soc.* **88,** 4104.

[13] Horton, H. R. and de Koshland, Jr. (1967) in *Methods in Enzymology* (Hirs, C. H. W., ed.), Vol. 11, p. 556, Academic Press, New York.

[14] Spande T. F. and Witkop, B. (1967) in *Methods in Enzymology* (Hirs , C.H. W., ed.), Vol. 11,p. 565, Academic Press, New York.