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Purification of soybean lipoxygenase isoenzyme-1 and characterization of its inhibition by 13-hydroperoxides

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RESUMEN

Purificación de la isoenzima-1 de la lipoxigenasa de soja y caracterización de su inhibición por 13-hidroperóxidos.

La purificación de la isoenzima-1 de la lipoxigenasa de soja fue reinvestigada mediante un nuevo procedimiento. Diferentes parámetros que influyen en la producción de hidroperóxidos fueron estudiados. Nuestros estudios se centraron particularmente en la inhibición de la actividad enzimática por los productos de reacción. Las representaciones de Dixon mostraron inhibición competitiva con valor de K_i de 0.45 y 1.5 mM respectivamente para los 13-hidroperóxidos de los ácidos linoleico y linolénico.

PALABRAS-CLAVE: Ácido linoleico – Ácido linolénico – Hidroperóxido – Lipoxigenasa – Soja.

SUMMARY

Purification of soybean lipoxygenase isoenzyme-1 and characterization of its inhibition by 13-hydroperoxides.

Purification of soybean lipoxygenase isoenzyme-1 was reinvestigated furnishing a new procedure. Different parameters influencing the hydroperoxide production were studied. Our study focused particularly on the enzyme activity inhibition by the reaction products. Dixon plots revealed competitive inhibition with K_i value of 0.45 and 1.5 mM respectively for 13-linoleic and 13-linolenic acid hydroperoxides.

KEY-WORDS: Hydroperoxide – Linoleic acid – Linolenic acid – Lipoxygenase – Soybean.

1. INTRODUCTION

Lipoxygenase (E. C. 1.13.11.12) catalyses the addition of molecular oxygen on polyunsaturated fatty acids containing a *cis, cis* 1-4 pentadiene structure (linoleic or linolenic acid). It leads to the production of conjugated hydroperoxides which can be further degraded into volatile aldehydes by hydroperoxide lyases. Depending on the origin of the enzyme and on the reaction conditions, variable amounts of 9-and 13-hydroperoxides are formed (figure 1). Linolenic acid 13-hydroperoxide (13-LnOOH) leads to the formation of high value aroma compounds: cis-3– hexenol and *trans*-2-hexenal, so called leaf alcohol and leaf aldehyde (Fukushima, 1994; Hatanaka, 1993; Hildebrand, 1989). In soybean, three isoenzymes are described. Isoenzyme-1 (Lox-1) can be easily distinguished from isoenzyme 2 (Lox-2) and 3 (Lox-3) on the PH optimum basis (9 and 6.5 respectively). Lox-1 produces large amounts of 13-hydroperoxides while Lox-2 and Lox-3 gives equal amounts of the 9 and 13 isomers. (Siedow, 1991; Chism, 1985; Axelrod et al., 1981; Christopher et al., 1972; Christopher et al., 1970). Different methods have been described for the purification of soybean lipoxygenase. Most of them result in very long procedures or in impure enzyme extracts (Nishiyama et al., 1993; Kuninori et al., 1992; Funk et al., 1985; Axelrod, 1981; Finazzi-Agro et al., 1973; Christopher et al., 1972). We describe here a new method for purifying Lox-1. The purity was checked by SDS-PAGE electrophoresis; the lack of Lox-2 and Lox-3 was confirmed by specific tests. Different parameters affecting the production of hydroperoxides by the enzyme (temperature, pH, ionic strength of the reaction medium) were studied. Dixon plots were established using linoleic (LaOOH) and linolenic (LnOOH) acid hydroperoxides in order to evaluate the enzyme inhibition by the reaction products.

2. EXPERIMENTAL

2.1. Enzyme assay

Enzyme assay was based on the increase of absorbance at 234 nm due to appearance of the conjugated diene in fatty acid hydroperoxides (Surrey, 1964). Three different tests were used to determine lipoxygenase activity of each isoenzyme in the extracts (Axelrod, 1981). The tests are based on the specificity of the different isoenzymes for a particular substrate and on their optimal pH activity. For the Lox-1, the test was realized with linoleic acid as substrate at pH 9. At this pH, Lox-2 and Lox-3 are not active. The activity of

Lox-2 and Lox-3 (together) can be measured using linoleic acid as substrate at pH 6.5. At this pH, Lox-1 has a negligible activity. The measurement of Lox-2 activity (alone) was achieved using arachidonic acid as substrate at pH 6.1. This test is specific for isoenzyme 2 because the Lox-1 is not working at this pH and because Lox-3 doesn't transform the arachidonic acid.

One unit of activity is defined as the amount of enzyme that generates an increase of absorbance of 0.001 in one minute at 20°C. When specific activity is mentioned, V_0 is always considered. V_0 is the initial rate of substrate transformation.

2.2. Enzyme purification

Lipoxidase from soybean, type I-S (Sigma ref: L8383) was used for the purification. The enzyme was solubilized in distilled water and dialysed at 4°C against water for two days. The lipoxygenase precipitate was separated by centrifugation (1000 g, 15 minutes). The residue was solubilized in a small volume of phosphate buffer 50mM, 100mM NaCl, pH 7. The resulting enzyme solution was loaded on a 30 mm X 500 mm column of Sephadex G-100 (Pharmacia) and eluted with the same buffer solution.

The lipoxygenase was eluted rapidly. Lox-1 was easily separated from Lox-2 and 3 by ion exchange chromatography on DEAE sepharose CL 6B column (internal diameter: 15 mm, length: 400 mm). The dialysed and lyophilized sample was solubilized in 20 mM, pH 6.8 phosphate buffer (buffer A). Buffer B was phosphate buffer 220mM pH 6.8. The flow rate was 3ml/min and the elution profile was:

$$0\%\ B \xrightarrow{5\ min} 0\%\ B \xrightarrow{15\ min} 45\%\ B \xrightarrow{4\ min} 45\%\ B \xrightarrow{15\ min} 85\%\ B \xrightarrow{7\ min} 85\%\ B \xrightarrow{5\ min} 100\%\ B$$

The purity of the recovered enzyme was checked by SDS-PAGE electrophoresis with EXCEL gel SDS, gradient 8-18 for EPH (Pharmacia). The proteins were measured using the method of Folin.

2.3. Preparation and analysis of 13-LnOOH and 13-LaOOH

a) Synthesis and purification: 10 mg of linoleic or linolenic acid and 4 mg of commercial soybean lipoxygenase were added to 100 ml of oxygenated borate 0.2 M pH 9 buffer. The reaction was carried out at 2°C for 15 minutes under a constant flow of oxygen (saturation of oxygen). The pH was than adjusted to 3 with HCl 6 N and the reaction products were purified on C-18 cartridges (C-18 chromabond of 500 mg from Macherey-Nagel) according to Sanz *et al.* (1993). The columns were eluted with 3 ml of n-hexane and 2 ml of methanol. The products of interest were recovered in the methanol fraction.

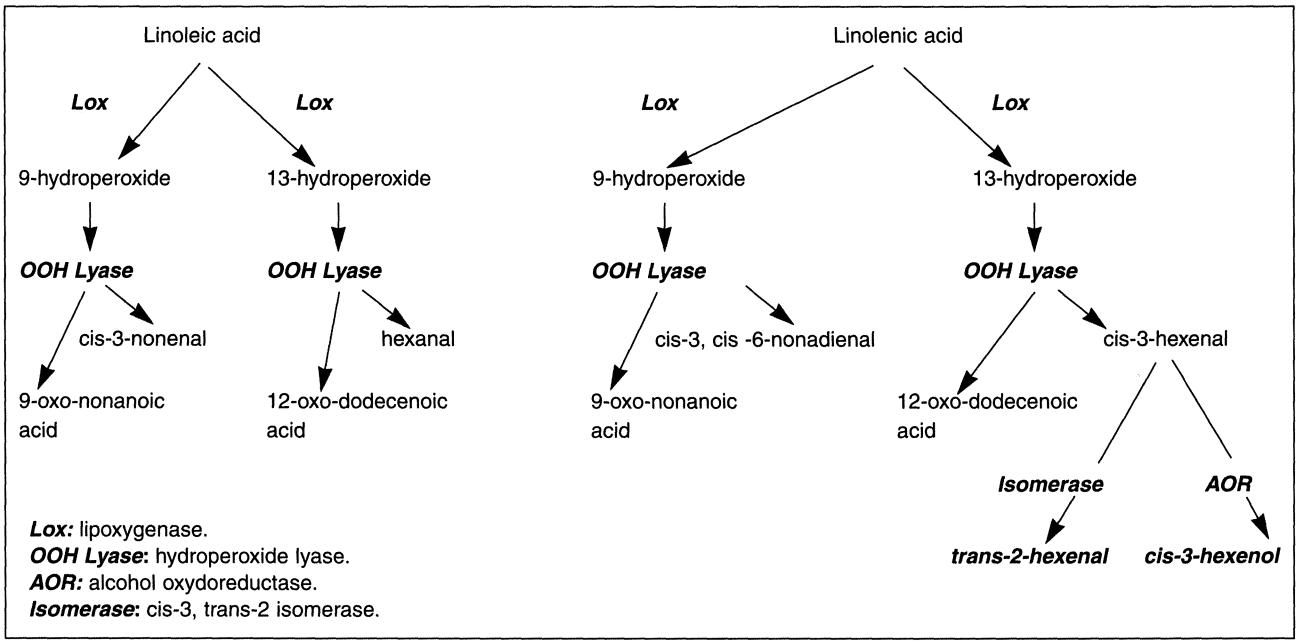

Figure 1
Enzymatic degradation of polyunsaturated fatty acids

b) Analysis (Sanz *et al.,* 1993): the positional and geometrical isomers of hydroperoxides were separated by HPLC after reduction with $NaBH_4$ and methylation with diazomethane. The HPLC products were collected and analyzed by GCMS after double bonds saturation and trimethylsilylation. The purity of the synthesized 13-LnOOH and 13-LaOOH was>98%.

2.4. Influence of temperature, pH and ionic strength of the buffer on the hydroperoxide production

The tests were performed in triplicate. Typical assays consisted of (2950-x) ml of buffer, 50 ml of linoleic or linolenic acid (final concentration of 0.0166 mM) and 2 units of enzyme (x ml).

– t° from 2°C to 60°C were checked in borate buffer (0.2 M; pH 9) with both substrates.

– pH range from 7 to 11 were assayed with linoleic and linolenic acid at 20°C.

For pH 7, phosphate buffer 0.2 M was used and for pH from 8 to 11, borate buffer 0.2 M. The pH was always checked after adding the enzyme extract.

– Ionic strength of the buffer: water adjusted to pH 9 with NaOH; borate buffer 0.05 M, 0.1 M and 0.2 M were used at 20°C. The pH of the medium was controlled after the addition of the enzyme solution.

The proportion of 13- and 9-hydroperoxides produced with linoleic and linolenic acid at 2°C and at 25°C was determined by HPLC. The productions were realized in 10 ml vials in the same conditions than the activity tests.

2.5. Inhibition tests

Inhibition tests were performed in triplicate with borate buffer 0,2 M pH 9 at 20°C.

– For linoleic acid: three concentrations of substrate were used (0,0166 mM, 0,05 mM, 0,166 mM). For each concentration of substrate, increasing concentrations of 13-LaOOH were added to the reaction medium (from 0 to 3,8 mM). For linolenic acid: comparable procedure was used with 13-LnOOH at concentrations ranging from 0 to 2,3 mM.

The evolution of V_0 versus 13-OOH concentration for different substrate concentrations allowed the elaboration of Dixon plots.

3. RESULTS AND DISCUSSION

3.1. Purification of soybean lipoxygenase isoenzyme-1

The data shown in table I clearly demonstrate the purification of Lox-1 during the procedure. The Sephadex G-100 purification increased specific activity but did not allow isoenzyme separation. During the DEAE Sepharose step (figure 2), two fractions contained lipoxygenase activity (fraction 2 and 3). Fraction 2 (45% of buffer B) exhibited high specific activity at pH 6.5 and almost no activity at pH 9 when linoleic acid was used as substrate; this is typical for Lox-2 and Lox-3. On the other hand, specific tests demonstrated that fraction 3 (85% of buffer B) contained Lox-1. Indeed, the specific test for Lox-3 revealed that the activity in this fraction was only 0.5% of the initial activity in the commercial extract; the low activity observed at pH 6.5 can be due to the Lox-1 itself because this isoenzyme still exhibits little activity at this pH. The purity of fraction 3 was checked by SDS-PAGE electrophoresis, revealing a single band at 94000 daltons.

The purification procedure described here allows the isolation of pure Lox-1 with a high massic recoverage (40% for fraction 3).

Table I
Evolution of specific activity (U/mg of protein) during purification. / : test not realized

Isoenzyme activity Substrate	Lox-1 Linoleic acid pH 9	Lox-2 and Lox-3 Linoleic acid pH 6.5	Lox-2 Arachidonic acid pH 6.1
Commercial Lox	61300	36000	18300
After dialysis	71200	55000	/
After Sephadex G-100	206600	106900	/
DEAE Sepharose Fraction 2	800	97600	/
DEAE Sepharose Fraction 3	291400	2300	100

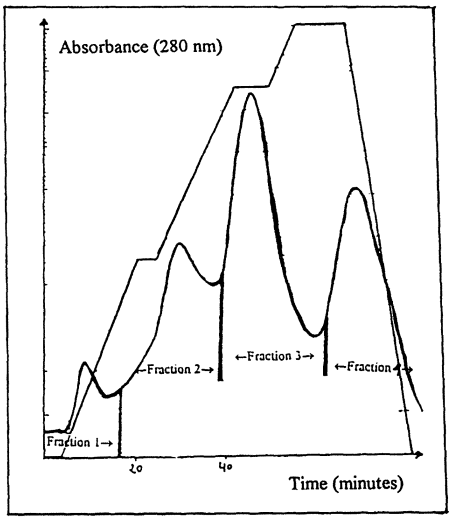

Figure 2
Chromatographic profile of the DEAE purification step

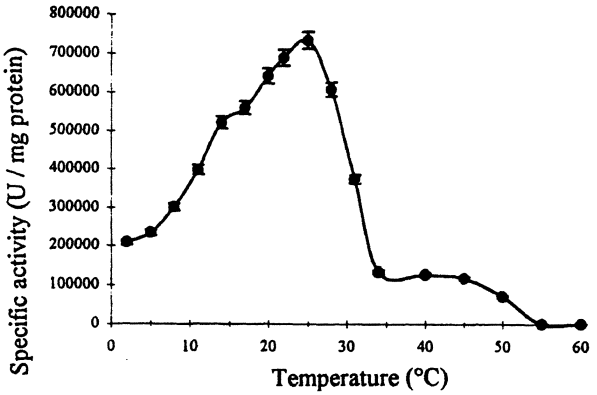

Figure 3
Influence of the temperature on the production of linolenic acid hydroperoxides by Lox-1

3.2. Influence of temperature, pH and ionic strength of the medium on the production of hydroperoxides

The enzyme exhibits and optimum of t° at 25°C (linoleic or linolenic acid as substrate). The specific activity for linolenic acid is 1.7 times higher than for linoleic acid. The enzyme keeps a good specific activity at 2°C (25% of the maximum specific activity), whereas the enzyme is rapidly denatured over 40°C (figure 3).

The enzyme has an optimal pH at 9.5 but is still active at pH 11 (40% of maximum activity). The ionic strength of the reaction medium is linearly related to the specific activity: when water adjusted at pH 9 is used instead of 0.2 M borate buffer the specific activity is only 40% of the maximum activity.

HPLC analysis of the hydroperoxides produced at 2°C and 25°C with both linoleic and linolenic acid revealed always high 13-OOH to 9-OOH ratios (96%). These last results are in agreement with specific tests because Lox-1 forms almost only 13-OOH at pH 9 (Chism, 1985).

3.3. Inhibition of Lox-1 by 13-OOH

Lox-1 is clearly inhibited by the hydroperoxides formed during polyunsaturated fatty acid biotransformation. Indeed, the Dixon plots established with linoleic acid as substrate (inhibition with 13-LaOOH) or with linolenic acid (inhibition with 13-LnOOH) demonstrate competitive inhibition (figure 4). The k_i (constant of inhibition) values are respectively 1,5 mM and 0,45 mM (for 13-LaOOH and for 13-LnOOH).

Small amounts of product (fatty acid hydroperoxides) activate Lox-1 because Fe (II) lipoxygenase must be oxidized to the Fe (III) lipoxygenase form by its product (P) before it can again take part in the oxygenation cycle (Schilstra et al., 1992). In this case, as the product accumulates during the reaction, regeneration occurs more rapidly and the fraction of active enzyme as well as the oxygenation rate increases. When the concentration of P is higher (in our case), P and S (substrate) compete for the same binding site on both enzyme forms and the reaction is competitively inhibited as shown in Dixon plots.

4. CONCLUSIONS

When 13-OOH are produced with commercial lipoxygenase, the reaction must take place at low temperature where Lox-1 is the only isoenzyme active but the reaction conditions are far from the optimal ones (Leu, 1974). With Lox-1 purified like described herein, the reaction can be conducted at 25°C furnishing high yield of very pure 13-OOH especially at pH 9.5 in a high ionic strength buffer. The results obtained with the inhibition tests demonstrate that the enzyme is rapidly inhibited by its products. This last feature must be kept in mind if a continuous process is envisaged for the production of hydroperoxides, for example with immobilized Lox.

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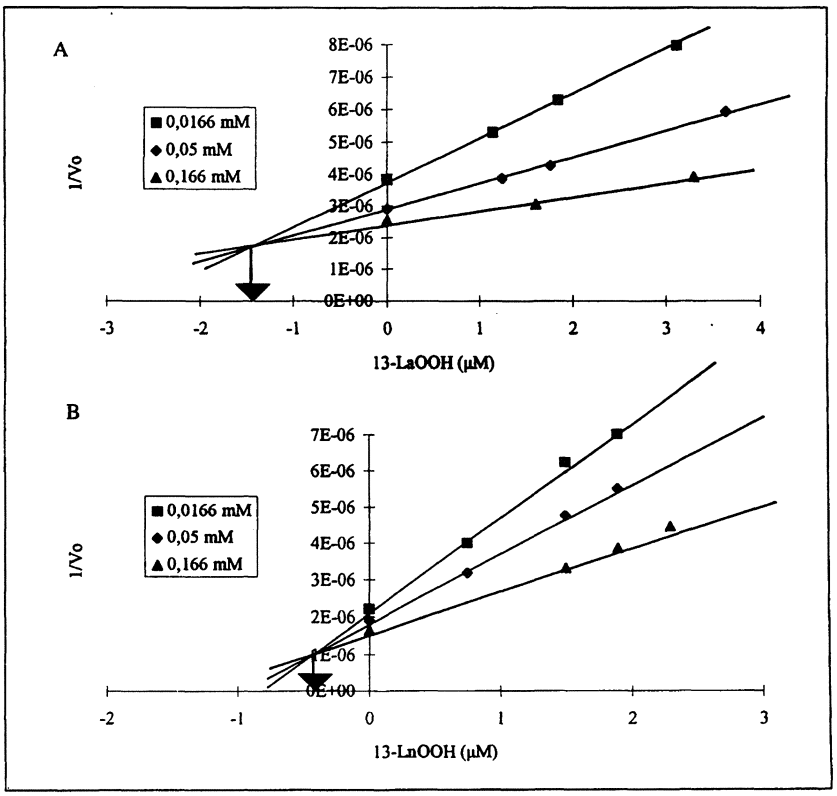

Figure 4
Dixon's plots. A: $1/V_0$ versus concentration of 13-La00H for different concentrations of linoleic acid. V_0: U/mg of protein. B: idem With 13-Ln00H.

BIBLIOGRAPHY

Axelrod, B., Cheesbrough, T. M. and Laaksa, S. (1981). –«Lipoxygenase from soybean».– Methods in enzymology, **71**, 441-446.

Chism, G. W. (1985). –«Soy lipoxygenase».– in «Flavor Chemistry of Fats and Oil» p. 175- D. B. Min and T. H. Smouse (Ed.).– American Oil Chemists Society, Columbus and St Louis.

Christopher, J., Pistorius, T. and Axelrod, B. (1970). –«Isolation of an isoenzyme of soybean lipoxygenase».– Biochimica and biophysica acta, **198**, 12-18.

Christopher, J., Pistorius, T. and Axelrod, B. (1972). –«Isolation of a third isoenzyme of soybean lipoxygenase». Biochimica and biophysica acta, **284**, 54-62.

Finazzi-Agro, A., Avigliano, L., Veldink, G. A., Vliegenthart, J. F. G. and Bolding, J. (1973). –«The influence of oxygen on the fluorescence of lipoxygenase».– Biochimica and Biophysica Acta, **326**, 462-470.

Fukushina, D. (1994). –«Recent progress on biotechnology of soybean proteins and soybean protein food products».– Food Biotechnology, **8**, (2 and 3), 83-135.

Funk, M. O., Whitney, M. A., Hausknecht, E. C. and O'Brien, E. M. (1985). –«Resolution of the isoenzymes of soybean lipoxygenase using isoelectric focusing and chromatofocusing».– Analytical Biochemistry, **146** 246-251.

Hildebrand, D. (1989). –«Lipoxygenases».– Physiologia Plantarum, **76**, 249-253.

Hatanaka, A. (1993). –«The biogeneration of green odour by green leaves».– Phytochemistry, **34** (5), 1201-1218.

Kuninori, T., Nishiyama, J., Shirakawa, M. and Shimoyama, A. (1992). –«Inhibition of soybean lipoxygenase-1 by n-alcohols and n-alkylthiols». Biochemica and biophysica acta, **1125**, 49-55.

Leu, K. (1974). –«Formation of isomeric hydroperoxides from linoleic acid by lipoxygenase».– Lebensm. Wiss. u. **7** (2), 82-85.

Nishiyama, J., Shizu, Y. and Kuninori, T. (1993). –«Inhibition of soybean lipoxygenase-1 by sucrose esters of fatty acids».– Biosc. Biothech. Biochem, **57**, (4), 557-560.

Sanz, L. C., Pérez, A. G., Ríos, J. J. and Olias, J. M. (1993). –«Positional specificity of ketodienes from linoleic acid aerobically formed by lipoxygenase isoenzymes from kidney bean and pea».– J. of Agricultural and Food Chemistry, **41** (5), 696-699.

Schilstra, M. J., Veldink, G. A., Verhagen, J. and Vliegenthart, J. F. G. (1992). –«Effect of lipid hydroperoxide on lipoxygenase kinetics»–. Biochemistry, **31**, 7692-7699.

Siedow, J. N. (1991). –«Plant lipoxygenase: structure and function»– Rev. Plant Physiol. Plant Mol. Biol., **42**, 145-158.

Surrey, K. (1964). –«Spectrophotometric method for determination of lipoxidase activity»–. Plant Physiol., **39,** 65-70.

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