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SPECIFICITIES OF ANTISERA DIRECTED AGAINST SOYBEAN LIPOXYGENASES-1 AND -2 AND PURIFICATION OF LIPOXYGENASE-2 BY AFFINITY CHROMATOGRAPHY

MARJAN VERNOOY-GERRITSEN, GERRIT A. VELDINK and JOHANNES F.G. VLIEGENTHART

Department of Bio-organic Chemistry, State University of Utrecht, Croesestraat 79, 3522 AD Utrecht (The Netherlands)

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Antiserum directed against lipoxygenase-1 from soybean (*Glycine Max* (L.) Merr. var. Williams) was developed in rabbits with electrophoretically pure lipoxygenase-1 as an antigen. To remove traces of lipoxygenase-1 from a lipoxygenase-2 preparation the latter was chromatographed over a column of Sepharose 4B to which antibodies directed against lipoxygenase-1 were coupled. During affinity chromatography of lipoxygenase-2 no protein and only a small amount of activity were lost. Affinity-purified enzyme was used for immunization of rabbits to produce lipoxygenase-2 antibodies. Results with double gel immunodiffusion tests with the two soybean lipoxygenases and antisera directed against them demonstrated that there is no immunological relationship between the isoenzymes. Lipoxygenases from different species of leguminosae crossreacted only with antiserum directed against soybean lipoxygenase-2, and not with anti soybean lipoxygenase-1 serum. No crossreaction could be detected between soybean lipoxygenase antisera and lipoxygenases from species of Gramineae, Linaceae and Solanaceae.

Introduction

Lipoxygenase (linoleate : oxygen oxidoreductase EC 1.13.11.12) is a dioxygenase that catalyzes the conversion of polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system into conjugated hydroperoxy fatty acids. Various lipoxygenase isoenzymes have been isolated from soybeans [1–4]. We isolated lipoxygenases-1 and -2, which were apparently pure on polyacrylamide gel electrophoresis.

In a standard polarographic assay with 1.8 mM linoleic acid lipoxygenase-2 shows maximum activity at pH 6.6 and hardly any oxygen consumption at pH 9.0, which is the optimum pH for lipoxygenase-1. However, the reaction rate of dioxygenation of linoleic acid catalyzed by lipoxygenase-2 is much higher at pH 9.0 if the substrate concentration is lowered or Ca^{2+} is added [5].

We searched for new methods of testing the purity of the isoenzyme preparations and localizing the enzymes in soybean tissues. Therefore we investigated the antigenicity of soybean lipoxygenases-1 and -2 and interactions of the antisera with each of the isoenzymes.

Materials and Methods

Assay of lipoxygenase activity. Enzyme activity was measured polarographically in a Gilson Oxygraph equipped with a Clark oxygen electrode in a solution of 1.8 mM linoleic acid in air-saturated 0.1 M sodium borate buffer (pH 9.0) or 1.0 M sodium phosphate buffer (pH 6.6) at 25°C. The specific activity is expressed as μmol O_2 consumed per mg protein per minute.

Purification of soybean lipoxygenases. Soybean (*Glycine max* (L.) Merr. var. Williams) lipoxygenases were isolated according to the method

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of Finazzi-Agrò et al. [6] as modified by Galpin et al. [7]. The specific activity of lipoxygenase-1 at pH 9.0 was 240 μmol $O_2 \cdot min^{-1}$ per mg protein. The lipoxygenase-2 preparation had a specific activity of 4 at pH 6.6 and of 7 μmol $O_2 \cdot min^{-1}$ per mg protein at pH 9.0 in the presence of 1.4 mM $CaCl_2$.

Protein concentrations. Protein concentrations were obtained from the absorbance at 280 nm, using $A_{280}^{0.1\%} = 1.60$ for lipoxygenase-1 [8], 1.43 for lipoxygenase-2 [1] and 1.34 for IgG [9].

Immunization. Rabbits were immunized by monthly intradermal injections of an emulsion of 0.9% (w/v) NaCl containing 500–700 μg lipoxygenase in an equal volume of Freund's complete adjuvant (Miles laboratories Inc.). 10 days after the fourth booster injection the rabbits were bled by heart puncture. Sera were stored at −40°C.

Coupling of IgG to Sepharose 4B. The IgG fraction of antiserum directed against lipoxygenase-1 was isolated by precipitation of the whole serum with 0.15 mM caprylic acid according to Steinbuch et al. [10]. Approx. 10 mg IgG were obtained from 1 ml serum. After centrifugation at 10 000 × *g* the supernatant was dialyzed against bi-distilled water at 4°C with several changes, followed by lyophilization. The IgG preparation was stored at −20°C until use.

CNBr-activated Sepharose 4B (Pharmacia) was swollen and washed with 1 mM HCl. IgG was dissolved in 0.1 M $NaHCO_3$ buffer (pH 8.5) containing 0.5 M NaCl. The IgG solution was gently mixed with the gel suspension for 15 h at 4°C (20 mg IgG per g dry gel). Unreacted active groups of the gel were blocked with 0.2 M glycine in 0.1 M $NaHCO_3$ buffer (pH 8.0) containing 0.5 M NaCl. After five washings alternatively with 0.1 M $NaHCO_3$ buffer (pH 8.3) and 0.1 M sodium acetate buffer (pH 4.0) each containing 0.5 M NaCl, the gel was packed in a column (6 × 1 cm for 2 g dry gel).

Single radial immunodiffusion. For radial immunodiffusion according to the method of Mancini et al. [11] serum was diluted with phosphate-buffered saline (0.05 M sodium phosphate buffer (pH 7.4) containing 5.3 g NaCl per l) and mixed at 50°C with an equal volume of 2.6% (w/v) agar solution containing 0.9% (w/v) NaCl. The antiserum-agar mixture was poured as a 2-mm layer on glass slides. 4-mm-wide holes were punched in the gel and each filled with 10 μl lipoxygenase solution. In each gel a series of known enzyme concentrations was employed as a reference. After incubation in a moist Petri dish for 5 days at room temperature the diameters of the precipitation rings were measured.

Two-dimensional double immunodiffusion. Double gel immunodiffusion according to the method of Ouchterlony [12] was carried out on glass slides (5 × 5 cm) in a gel (1.3% (w/v) agar and 0.9% (w/v) NaCl), thickness 2 mm. The reactants were allowed to diffuse in a moist Petri dish at 4°C. Results were photographed after 12 h to 1 week.

Homogenates of various plant species. Dry seeds of soybean, green peas (*Pisum sativum* L. var. Bliss Abundance and High Germination), maize (*Zea mays* L.) and flax (*Linum usitatissimum* L.) were ground in an overcross-beat mill (Peppink, Amsterdam) together with solid CO_2. Flour of soybean, maize and flax was defatted with cold acetone (−10°C) and vacuum-dried. Flour or acetone-powder was extracted with 0.1 M sodium phosphate buffer (pH 7.0) for 45 min at 0°C.

Fruits of eggplant (*Solanum melongena* L.) and tubers of potato (*Solanum tuberosum* L.) were homogenized in a Sorvall omnimixer. All homogenates were centrifuged at 48 000 × *g* and reactions with the antisera directed against soybean lipoxygenases were determined in a double immunodiffusion test.

Results and Discussion

Antiserum directed against lipoxygenase-1

Lipoxygenase-1 concentrations of 0.05–1.00 mg/ml precipitated in a single sharp line with antiserum in a two-dimensional immunodiffusion test. In the first instance lipoxygenase-2 preparations showed precipitation reactions with sera directed against lipoxygenase-1. At concentrations above 3 mg/ml a lipoxygenase-2 preparation caused, in a double immunodiffusion test with anti-lipoxygenase-1 serum, a precipitation line which fused with the line between the serum and lipoxygenase-1 in the neighboring well (Fig. 1). Because of the need for a high concentration of lipoxygenase-2 the coalescence of the precipitation lines of the two isoenzymes and anti-lipoxygenase-1

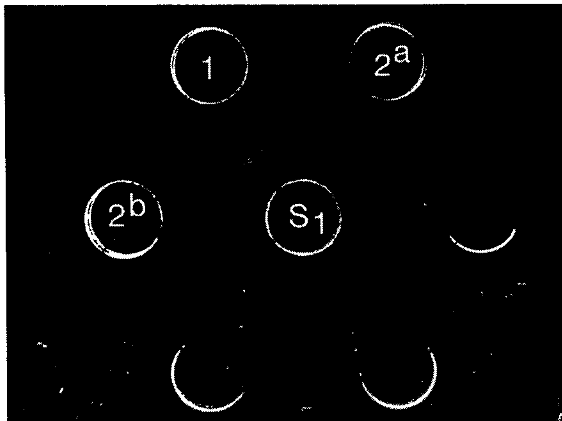

Fig. 1. Double gel immunodiffusion of different concentrations of a lipoxygenase-2 preparation and antiserum directed against lipoxygenase-1: 1, lipoxygenase-1 (1 mg/ml); 2^a, lipoxygenase-2 (10 mg/ml); 2^b, lipoxygenase-2 (1 mg/ml); S_1, antiserum directed against lipoxygenase-1.

serum points to a contamination of lipoxygenase-1 in the sample of lipoxygenase-2, rather than to crossreaction.

Non-immune serum reacted with neither lipoxygenase-1 nor lipoxygenase-2.

Quantification of lipoxygenase-1 in a lipoxygenase-2 preparation

The concentration of lipoxygenase-1 in samples was determined by single radial immunodiffusion in a gel containing antiserum directed against lipoxygenase-1. A solution of electrophoretically pure lipoxygenase-2 (Fig. 2) of 10 mg/ml was analysed on a gel with an 80-fold dilution of antiserum. The lipoxygenase-2 preparation proved to contain 0.65–0.80% (w/w) lipoxygenase-1.

Fig. 2. Polyacrylamide (9%) gel electrophoresis of preparations of soybean lipoxygenases-1 and -2: gel 1, lipoxygenase-1 (15 μg); gel 2, lipoxygenase-2 (15 μg). Gels are stained with Amido black.

TABLE I

EFFECT OF AFFINITY CHROMATOGRAPHIC PURIFICATION ON THE SPECIFIC ACTIVITY OF LIPOXYGENASE-2

pH	Spec. act. (μmol O_2/min per mg protein)		Recovery (%)
	Before treatment	After affinity chromatography	
6.6	4.1	3.7	90.2
9.0	0.5	—	—
9.0 + $CaCl_2$	7.1	6.5	91.6

Purification of lipoxygenase-2 by affinity chromatography

In view of the presence of a small amount of lipoxygenase-1 in a lipoxygenase-2 preparation an additional purification step was needed before the preparation could be used for the immunization of rabbits. Owing to the lability of lipoxygenase-2 a great deal of activity is lost during most purification procedures (cf. Axelrod et al. [13]). In the following procedure the possibility of removing the lipoxygenase-1 contamination by binding to anti-lipoxygenase-1 IgG was used.

A column of anti-lipoxygenase-1 IgG coupled to Sepharose 4B was equilibrated with phosphate-buffered saline at 4°C. After application of the

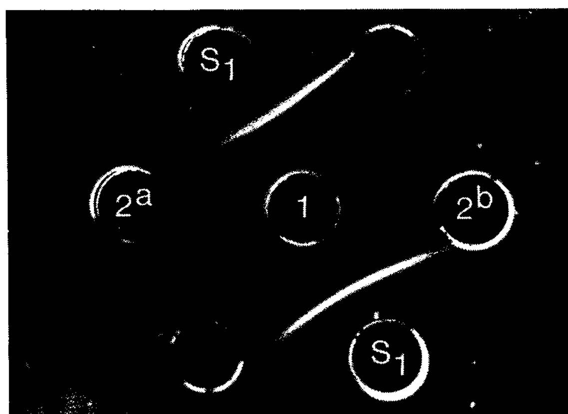

Fig. 3. Effect of affinity purification on the precipitation reaction of lipoxygenase-2 with antiserum directed against lipoxygenase-1: 1, lipoxygenase-1 (0.5 mg/ml); 2^a, lipoxygenase-2 (3.6 mg/ml); 2^b, affinity-purified lipoxygenase-2 (3.6 mg/ml); S_1, antiserum directed against lipoxygenase-1.

lipoxygenase-2 sample in phosphate-buffered saline the flow was stopped for 15 min to allow lipoxygenase-1 to bind to the adsorbent. Then the unbound enzyme fraction was eluted with phosphate-buffered saline and applied once more to the top of the column. After three runs over the column only 0.8% or less of the original activity at pH 9.0 was left (Table I). Virtually all of the protein was recovered and a small amount of activity at pH 6.6 was lost (10%), which can be ascribed to the lability of the enzyme (Table I). In a double immunodiffusion test with the purified lipoxygenase-2, lipoxygenase-1 and antiserum directed against lipoxygenase-1 no fusing precipitation lines could be detected (Fig. 3).

Antiserum directed against lipoxygenase-2

Affinity-purified lipoxygenase-2 was subsequently used for the immunization of rabbits. The titer of the antisera did not reach the same level as for lipoxygenase-2 and hence precipitation lines formed with anti-lipoxygenase-2 sera were less clear. It is unknown why it is so difficult to obtain lipoxygenase-2 antisera with a high titer.

By double immunodiffusion no interaction could be detected between lipoxygenase-1 and anti-lipoxygenase-2 serum (Fig. 4).

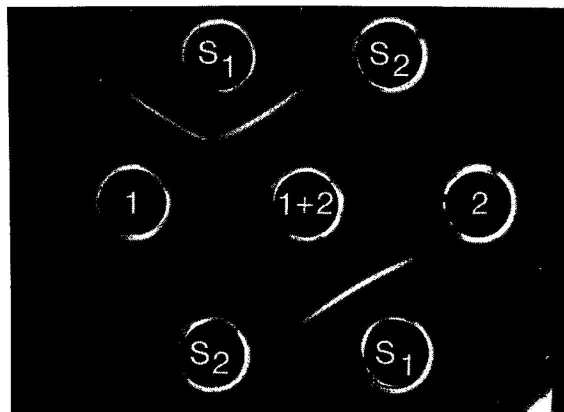

Fig. 4. Interactions of soybean lipoxygenases-1 and -2 and antisera directed against each of the isoenzymes: 1, lipoxygenase-1 (1 mg/ml); 2, lipoxygenase-2 (1 mg/ml); S_1, serum directed against lipoxygenase-1; S_2, serum directed against lipoxygenase-2.

Immunological relationship between various plant lipoxygenases

As can be seen from Table II, only for members of the family Leguminosae is the presence of lipoxygenase-2 activity accompanied by crossreaction with anti-lipoxygenase-2 serum. Trop et al. [14] reported immunological crossreaction between an-

TABLE II

CROSSREACTIONS BETWEEN LIPOXYGENASES FROM VARIOUS PLANTS AND ANTISERA DIRECTED AGAINST SOYBEAN LIPOXYGENASES-1 AND -2

Reactions between the antisera and homogenates of fruits or seeds of plants were investigated in a double immunodiffusion test. Precipitation lines fusing with a reference line were considered as a positive reaction.

Plant source	Precipitation with antiserum directed against		Activity (μmol O_2/ min per ml crude extract)		
	lipoxygenase-1	lipoxygenase-2	pH 6.6	pH 9.0	pH 9.0 + $CaCl_2$
Pure soybean lipoxygenase-1	+	–	109.9	234.7	131.4 [a]
Pure soybean lipoxygenase-2	–	+	3.7	–	6.5 [a]
Glycine max (L.) Merr. (soybean)	+	+	48.0	31.5	29.7
Pisum sativum L. (green pea)	–	+	14.0	–	10.2
Phaseolus vulgaris L. (red kidney-bean)	–	+	11.5	–	4.7
Zea mays L. (maize)	–	–	3.0	0.3	1.4
Triticum aestivum L. (wheat germs)	–	–	26.5	2.2	13.3
Linum usitatissimum L. (flax)	–	–	18.5	9.0	7.5
Solanum melongena L. (eggplant)	–	–	1.7	–	–
Solanum tuberosum L. (potato)	–	–	10.7	0.4	0.3

[a] Activity: μmol O_2/min per ml of a solution of 1 mg enzyme per ml.

tiserum directed against a soybean lipoxygenase preparation and the lipoxygenases from potato tubers and fruits of eggplant. However, the heterogeneity of both enzyme preparations and the antisera makes a direct comparison between the results of their double gel immunodiffusion tests with those presented here virtually impossible.

Concluding remarks

As crossreactions between isoenzymes and antisera directed against them point to the presence of common antigenic determinants, the present results clearly indicate that structural similarities between lipoxygenases occur only within the plant family Leguminosae. Type-2 lipoxygenases are most widespread in the Leguminosae family, while the more stable soybean lipoxygenase-1 takes an exceptional position. Apparently other species known to contain considerable amounts of lipoxygenase(s) have structurally different types of lipoxygenases.

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