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AFFINITY CHROMATOGRAPHY OF ANTIBODIES DIRECTED AGAINST SOYBEAN LIPOXYGENASE-1 AND -2 AND AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANTIBODIES AND LIPOXYGENASES

MARJAN VERNOOY-GERRITSEN, ADRI L.M. BOS, GERRIT A. VELDINK and JOHANNES F.G. VLIEGENTHART

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

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Crude immunoglobulin G (IgG) fractions of antisera directed against soybean lipoxygenase-1 and -2 were purified by being passed through an immunoadsorbent column containing lipoxygenase coupled to CNBr-activated Sepharose 4B. Bound immunoglobulin was desorbed with pulses of 2 M or 3 M ammonium thiocyanate or 0.1 M glycine-HCl buffer (pH 2.5). The total column recoveries of anti-lipoxygenase-1 IgG and anti-lipoxygenase-2 IgG were 45% and 58%, respectively. The affinity for lipoxygenase of immunospecific antibodies was determined in an enzyme-linked immunosorbent assay (ELISA). In a reaction with lipoxygenase-1, anti-lipoxygenase-1 IgG, which was eluted with glycine-HCl buffer (pH 2.5) with recovery of 24%, had a 6.5-times higher affinity than the whole IgG fraction of antiserum. The affinity of anti-lipoxygenase-2 IgG for lipoxygenase-2 increased 2.2-times after chromatography of IgG over an immunoadsorbent column using 2 M ammonium thiocyanate as eluent (recovery 21%).

The enzyme lipoxygenase (EC 1.13.11.12) oxidizes unsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system to hydroperoxides. Although significant amounts of lipoxygenase occur in a wide variety of plant seeds, little is known of the function and the localization of the enzyme in seed tissues. To gain a better insight into these features we decided to study first the localization of the isoenzymes lipoxygenase-1 and -2 in tissues of germinating soybean seeds (*Glycine max* (L.) Merr. var. Williams). To this end we introduced immunocytochemical techniques that are based on the application of specific antibodies directed against the enzymes [1,2]. We report here the purification of whole IgG fractions of sera directed against soybean lipoxygenase-1 and -2 by affinity chromatography using columns of the isoenzymes coupled to CNBr-activated Sepharose 4B. The effect of various eluents on the immunochemical reactivity of anti-lipoxygenase IgG is tested by enzyme-linked immunosorbent assay (ELISA).

Soybean (*Glycine max* (L.) Merr. var. Williams) lipoxygenases were isolated according to Finazzi-Agrò et al. [3] as modified by Galpin et al. [4]. The isoenzymes were homogeneous on 9% polyacrylamide gel electrophoresis [1].

Protein concentrations were obtained from the absorbance at 280 nm, using $A_{280}^{0.1\%} = 1.60$ for lipoxygenase-1 [5], 1.43 for lipoxygenase-2 [6] and 1.34 for IgG [7].

Rabbits were immunized by five monthly intradermal injections of an emulsin of 0.9% (w/v) NaCl containing 500–700 μg lipoxygenase in an equal volume of Freund's complete adjuvant (Miles Laboratories Inc.). Before immunization with lipoxygenase-2 the enzyme was affinity-purified over a column of Sepharose 4B (Pharmacia Fine Chemicals) to which antibodies directed against lipoxygenase-1 were coupled [1]. 10 days after the last booster injection the rabbits were bled by heart

puncture. Sera were stored at −40°C.

CNBr-activated Sepharose 4B was swollen and washed with 1 mM HCl. Per g dry gel (7–8 ml wet wt.) 20 mg lipoxygenase-1 was dissolved in 0.1 M $NaHCO_3$ (pH 8.5) and gently mixed with the gel suspension for 15 h at 4°C. Affinity-purified enzyme was used for the column with lipoxygenase-2. Because of the sensitivity of lipoxygenase-2 towards high pH a 0.1 M sodium phosphate buffer (pH 7.8) was used for coupling the enzyme to the gel (20 mg/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) and the gel was washed five times alternately with 0.1 M $NaHCO_3$ buffer (pH 8.3) and 0.1 M sodium acetate buffer (pH 4.0). Each buffer contained 0.5 M NaCl.

The Ig fraction of antiserum (minimum 90% IgG) was isolated by precipitation of whole serum with 0.15 mM caprylic acid according to Steinbuch and Audran [8]. After centrifugation at $10000 \times g$ the supernatant was dialyzed at 4°C against buffer 1 (0.05 M sodium phosphate buffer (pH 7.4) containing 0.09 M NaCl).

Whole IgG fractions of sera directed against lipoxygenase-1 and -2 were chromatographed over a column filled with the corresponding lipoxygenase-Sepharose 4B and Sephadex G-50 (coarse). Therefore a layer of 25 mm lipoxygenase-Sepharose 4B was applied onto the top of a column packed with Sephadex G-50 (160 × 18 mm) which was equilibrated with buffer 1. The column was washed with buffer 1 with a flow rate of 18 ml per h until the absorbance at 280 nm of the effluent was zero. Buffer 1 (15 ml) containing 40 mg IgG was applied to the column with ten intervals of 15 min. Unbound IgG was removed by washing the column with buffer 1 until absorbance at 280 nm in the effluent was minimal. Bound IgG was eluted with pulses of solutions of 2 M NH_4SCN (pH 7.2) and 3 M NH_4SCN (pH 7.2). Each pulse of 3 ml was followed by buffer 1 until A_{280} of the effluent was negligible. Eluted antibody was separated from thiocyanate by the Sephadex G-50 part of the column (Fig. 1).

IgG remaining on the column after several pulses of the NH_4SCN solutions was eluted with 5-ml pulses of 0.1 M glycine-HCl buffer (pH 2.5) each followed by buffer 1. The pH of the effluent was 7.4 due to the desalting capacity of Sephadex G-50. Results of the affinity purification of anti-lipoxygenase-1 IgG generally were similar to those of the purification of anti-lipoxygenase-2 IgG. During the second elution with 3 M NH_4SCN a smaller amount of bound anti-lipoxygenase-1 IgG was desorbed. Absorbance at 280 nm of the effluent during the elution of anti-lipoxygenase-2 IgG from the immunoadsorbent column is shown in Fig. 1. The recoveries of anti-lipoxygenase-1 IgG and anti-lipoxygenase-2 IgG after two pulses of each eluent are given in Table I.

The immunochemical reactivity of the obtained IgG fractions was determined by an ELISA. Flat-bottom wells in a microtiter plate of clear polystyrene (Nutacon) were coated with 0.2 ml of 1 μg/ml solution of lipoxygenase in 0.1 M $NaHCO_3$ buffer (pH 9.6). After incubation for 3 h at 37°C

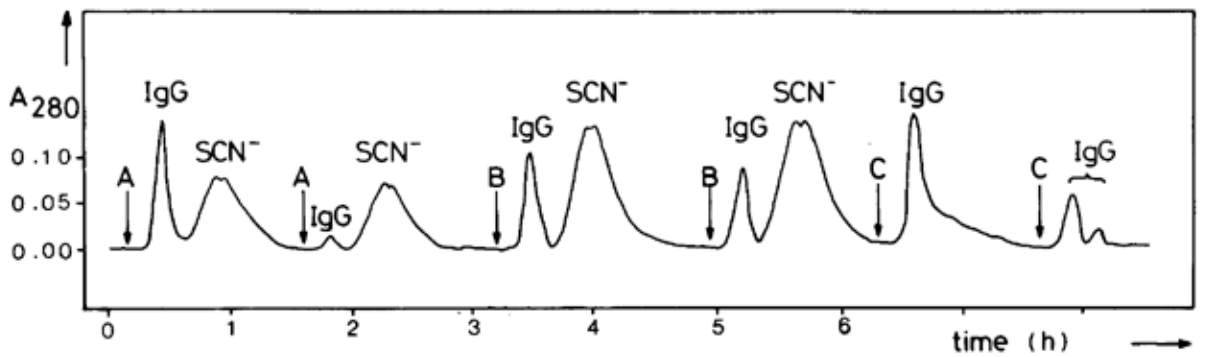

Fig. 1. Absorbance at 280 nm of the effluent of an immunoadsorbent column with lipoxygenase-2 coupled to Sepharose 4B during the elution of bound anti-lipoxygenase-2 IgG. A, 3 ml 2 M NH_4SCN (pH 7.2); B, 3 ml 3 M NH_4SCN (pH 7.2); C, 5 ml 0.1 M glycine-HCl buffer (pH 2.5).

TABLE I

RECOVERY OF IMMUNOGLOBULIN G AFTER CHROMATOGRAPHY OVER AN IMMUNOADSORBENT COLUMN OF LIPOXYGENASE COUPLED TO SEPHAROSE 4B

IgG fraction	IgG (mg)	% of total IgG	% of bound IgG
Anti-lipoxygenase-1			
Whole IgG	40.0	100.0	
Unbound IgG	29.1	72.8	
Bound IgG	10.9	27.3	100.0
Total eluted IgG	4.9	12.3	45.0
2 M NH_4SCN fractions	0.8	2.0	7.3
3 M NH_4SCN fractions	1.5	3.8	13.8
pH 2.5 fractions	2.6	6.5	23.9
Anti-lipoxygenase-2			
Whole IgG	40.0	100.0	
Unbound IgG	31.9	79.8	
Bound IgG	8.1	20.3	100.0
Total eluted IgG	4.7	11.8	58.0
2 M NH_4SCN fractions	1.7	4.3	21.0
3 M NH_4SCN fractions	2.0	5.0	24.7
pH 2.5 fractions	1.0	2.5	12.4

the plate was washed with buffer 2 (buffer 1 containing 0.05% Tween 20). After the third washing the wells were filled with 0.2 ml anti-lipoxygenase IgG solutions in buffer 2. Stepwise 5-fold dilutions were made on the plate. The plate was incubated for 1 h at 37°C followed by 14 h at 4°C. After three washings with buffer 2 the wells were filled with affinity-purified goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma, 1:250 dilution in buffer 2) and incubated for 3 h at 37°C. The plate was then washed three times with buffer 2 and the amount of bound enzyme was determined by adding 0.2 ml substrate solution to each well (*p*-nitrophenyl-phosphate (0.1 mg/ml) in 0.1 M glycine-HCl buffer (pH 10.3) containing 1 mM $MgCl_2$). The reaction was stopped after 25 min at room temperature by adding 50 µl 2 M NaOH and the extinction at 405 nm was measured in a rapid microplate reader (Titertek Multiskan).

As can be seen from Fig. 2, the activities of all fractions of anti-lipoxygenase-1 IgG were higher than the activity of untreated whole IgG. Unbound IgG showed no activity at all. The affinity of IgG for lipoxygenase can be expressed as the

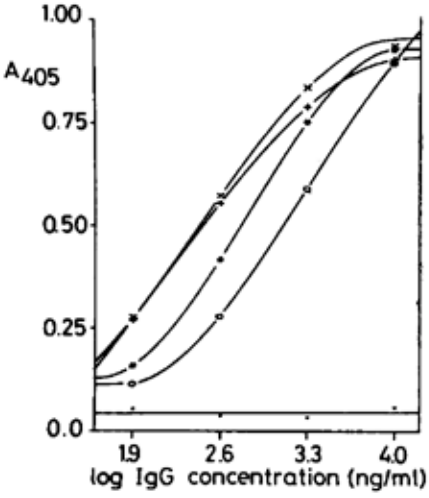

Fig. 2. ELISA of affinity-purified immunoglobulin G directed against lipoxygenase-1. Coating: lipoxygenase-1 (1 µg/ml); ○, whole IgG; ·–·, unbound IgG; ★, 2 M NH_4SCN fraction; +, 3 M NH_4SCN fraction; ×, pH 2.5 fraction.

equilibrium constant K in the following equation:

$$K = \frac{k_a}{k_d} = \frac{[\text{IgG}\cdot\text{lipoxygenase}]}{[\text{IgG}][\text{lipoxygenase}]}$$

where k_a and k_d represent the association and dissociation rate constants, respectively. In the

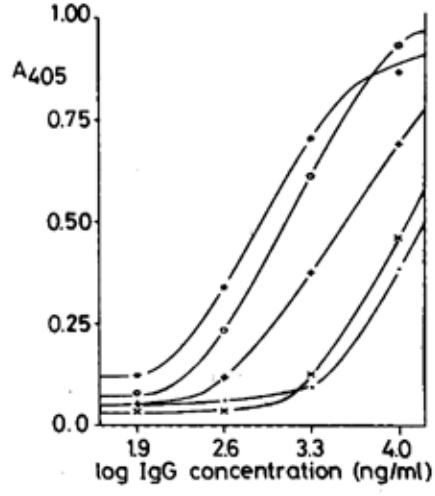

Fig. 3. ELISA of affinity-purified immunoglobulin G directed against lipoxygenase-2. Coating: lipoxygenase-2 (1 µg/ml); ○, whole IgG; ·–·, unbound IgG; ★, 2 M NH_4SCN fraction; +, 3 M NH_4SCN fraction; ×, pH 2.5 fraction.

ELISA the concentration of lipoxygenase is constant because of the amount bound to the wells during coating of the microtiterplate. The absorbance at 405 nm is directly proportional to the concentration of the IgG-lipoxygenase complex. The IgG concentration causing an absorbance of 0.45 was taken as a measure of the affinity of the reaction between IgG and lipoxygenase, because minimal absorbance at 405 nm is 0.05 and maximal absorbance is 0.95 (Figs. 2 and 3). Relative affinity (K_r) of the reaction is given by the equation:

$$K_r = K\frac{[\text{lipoxygenase}]}{[\text{IgG}\cdot\text{lipoxygenase}]} = \frac{1}{[\text{IgG}]}$$

In Table II the relative affinities of the monospecific IgG fractions of the immunoadsorbent columns with the two soybean lipoxygenases are given.

Apparently most of the anti-lipoxygenase-2 IgG fractions did not withstand the chromatography conditions. Only the IgG fraction eluted with 2 M NH_4SCN (pH 7.2) was more active than untreated whole IgG. The IgG fraction eluted with 0.1 M glycine-HCl buffer (pH 2.5) precipitated after storage at 4°C for 48 h. The presence of activity in the unbound IgG fraction showed that the immunoadsorbent column had been overloaded.

Eluents used in immunoadsorbent chromatography should have adequate desorbing power but should have no negative effects on the immunochemical reactivity of the eluted antibodies. In the chromatographic procedure as described above, the period of interaction of the eluent with the immobilized lipoxygenase and desorbed antibodies was minimized by using small eluent volumes and immediate separation of the eluent and the antibodies by the lower part of the column packed with Sephadex G-50. Recovery dropped to approx. 50% of the maximum due to the short time of contact and the small eluent volumes used [9]. Even strongly chaotropic ions such as SCN^- are much more effective in preventing the formation of immunocomplexes than in breaking those already formed, because of the inaccessibility of the binding sites [10]. After elution with 2 M and 3 M NH_4SCN a glycine-HCl buffer (pH 2.5) was still able to desorb another portion of antibodies from the immunoadsorbent column (24% anti-lipoxygenase-1 IgG and 12% anti-lipoxygenase-2 IgG).

The ELISA turned out to be very useful in testing the affinity of the purified IgG fractions for lipoxygenase. The pH 2.5 fraction of anti-lipoxygenase-1 IgG had an affinity for lipoxygenase-1 which had increased by a factor of 6.5 when compared to whole IgG. Affinity for lipoxygenase-2 of the anti-lipoxygenase-2 IgG fraction eluted with 3 M NH_4SCN or pH 2.5 buffer was decreased to 30% and 6%, respectively, with respect to untreated IgG (Table II). Anti-lipoxygenase-2 IgG eluted with 2 M NH_4SCN has a 2.2-times higher affinity for lipoxygenase-2 than whole IgG. The decrease of affinity of the IgG fractions which were eluted with 3 M NH_4SCN or pH 2.5 buffer

TABLE II

RELATIVE AFFINITY OF PURIFIED IMMUNOGLOBULIN G SAMPLES DIRECTED AGAINST SOYBEAN LIPOXYGENASE-1 AND -2

Anti-lipoxygenase-1 IgG (anti-1) was tested in an ELISA using a microtiter plate coated with lipoxygenase-1 (1 μg/ml) and anti-lipoxygenase-2 IgG (anti-2) in an ELISA with a coating of lipoxygenase-2 (1 μg/ml).

IgG sample	IgG concentration causing A_{405} = 0.45 (μg/l)		Relative affinity (K_r) (l/ng)	
	anti-1	anti-2	anti-1	anti-2
Whole IgG	489	602	2.05	1.66
2 M NH_4SCN fraction	202	276	4.95	3.62
3 M NH_4SCN fraction	79	2099	12.66	0.48
pH 2.5 fraction	75	9493	13.33	0.11
Unbound IgG	> 200000	15163	< 0.01	0.07

may be caused by denaturation of the antibodies. Another cause may be the leakage of (denatured) lipoxygenase-2 from the column. In that case the total recovery of anti-lipoxygenase-2 is lower than 58% (Table I). Precipitation of the pH 2.5 fraction after 48 h at 4°C points to the latter cause. Kennedy and Barnes [11] showed that both glycine-HCl and KSCN had a more drastic effect on antigen proteins than on several antibodies.

The results of the application of monospecific pH 2.5 fractions of anti-lipoxygenase-1 IgG and 2 M NH_4SCN fractions of anti-lipoxygenase-2 IgG for the immunocytochemical localization in tissues of germinating soybean seeds will be published elsewhere.

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