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Antibodies with specificities for D-xylose and for D-galacturonic acid residues of flaxseed polysaccharides

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Abstract

Two sets of anti-carbohydrate antibodies, one with specificity for D-xylose residues of flaxseed polysaccharides and the other with specificity for D-galacturonic acid residues, have been isolated by affinity chromatography from the immune serum of rabbits immunized with a vaccine of the polysaccharides and Freunds complete adjuvant. A number of properties of the antibodies are described. Of special note is the finding that, like other anti-carbohydrate antibodies, the new antibodies are biosynthesized in multi-molecular forms.

Keywords: Anti-D-xylose antibodies; Anti-D-galacturonic acid antibodies; Flaxseed polysaccharides

1. Introduction

Polysaccharides from the cell walls of some bacteria and from the seeds of some plants are immunogenic when mixed with Freunds complete adjuvant and injected into experimental animals.[1] Specific residues of these polysaccharides combine with receptor sites on the surface of the immunocytes in the blood stream of animals and this combination initiates the reaction sequence for the biosynthesis of anti-carbohydrate antibodies. In the present study, a mixture of two types of flaxseed polysaccharides, neutral and acidic, have been used to immunize rabbits. Two sets of anti-carbohydrate antibodies were produced and these have been isolated and purified from the immune serum by affinity chromatography. One set of antibodies has specificity for xylose residues of the neutral polymer and the other has specificity for galacturonic acid residues of the acidic polymer. A number of properties of both types of antibodies have been determined by standard immunological methods.[2] Results of density-gradient ultracentrifugation indicate that both antibody preparations are homogeneous and are of molecular weight of 150,000. However, results from coupled isoelectrofocusing–agar diffusion methodology[3] have shown that both sets of antibodies are composed of multi-molecular proteins and each protein possesses antibody activity. Such sets have been designated as isoantibodies, which is consistent with the nomenclature of isoenzymes.[4] The new antibodies may be useful for studies on the elongation of plant cells, for identification of pathogenic bacteria and for the detection of the presence of abnormal glycoproteins in diseased organs.

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Fig. 1. Agar diffusion of anti-flaxseed polysaccharide antibodies and flaxseed polysaccharides. So, S_4 and S_8 = preimmune, 4th, and 8th week serum and P = polysaccharide antigen.

2. Results and discussion

The procedure and the schedule for the immunization of rabbits with a vaccine of the neutral and acidic flaxseed polysaccharides and Freunds complete adjuvant are described in the Experimental section. The serum was obtained from the immunized animals weekly and tested for the presence of antibodies by the Ouchterlony agar diffusion method[5] with the polysaccharide antigens. The agar diffusion results for preimmune, 4th and 8th week serum are presented in the agar plate in Fig. 1. The precipitin bands formed with the 4th and 8th week serum and not with the pre-immune serum. However the 8th week serum yielded two precipitin bands, showing that two types of antibodies were produced on longer periods of immunization.

On the basis of methylation data, the structures of flaxseed polysaccharides (neutral and acidic) have been proposed by others.[6–8] The main feature of the neutral polysaccharide is a main chain of xylose residues joined by (1→4) linkages and with many side chains terminated by xylose residues.[6] The acidic polysaccharide used in this study is composed of a main chain of rhamnose, galactose, and galacturonic acid and with side chains terminated by galactose or galacturonic acid residues.[7,8] Acid hydrolysis of the polysaccharide mixture used in this study, followed by paper-chromatographic analysis showed that rhamnose, xylose, galactose, and galacturonic acid were the monosaccharides liberated.[9] The configuration of galactose from flaxseed polysaccharide is L as shown by the non-reactivity with galactose oxidase.[10] The configuration of rhamnose is L as shown by its reaction with anti-L-rhamnose antibodies.[11,12] The configuration of xylose and galacturonic acid has been previously established to be D by methylation analysis of appropriate derivatives.[8]

The two types of antibodies were isolated by affinity chromatography on columns of Sepharose with appropriate ligands (Fig. 2). One type of antibody was obtained on chromatography on Sepharose adsorbent with D-xylose ligands and eluted with D-xylose. The other type was obtained by affinity chromatography on a Sepharose adsorbent with flaxseed polysaccharide ligands and eluted with D-galacturonic acid. It is pointed out that the anti-xylose and anti-galacturonic acid pure antibodies have been prepared for the first time. An agar diffusion plate of the serum and the purified antibodies is shown in Fig. 3. Whereas the serum yielded two precipitin bands, the purified antibodies yielded single bands.

A number of additional immunological tests were performed. The results of ultracentrifugation in a sucrose gradient[13] are shown in Fig. 4. Calculations from the data and the

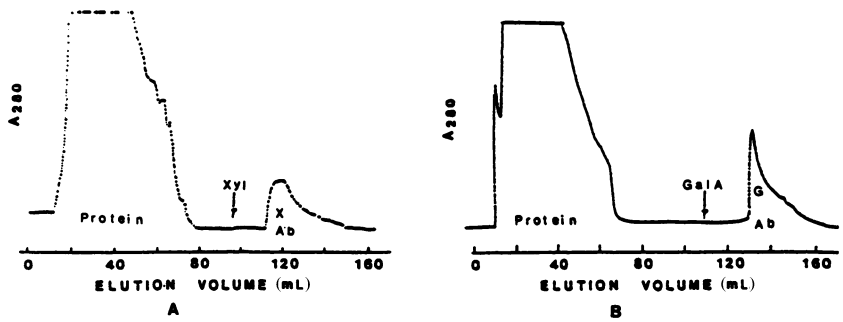

Fig. 2. Affinity chromatography of immune serum of anti-xylose antibodies (A) and anti-galacturonic acid antibodies (B).

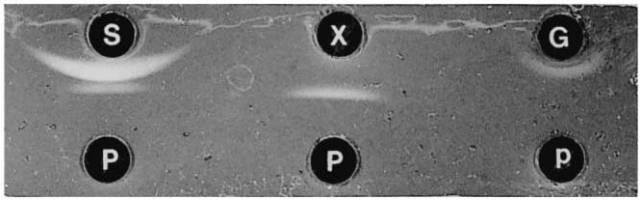

Fig. 3. Agar diffusion of immune serum (S), anti-xylose antibodies (X), and anti-galacturonic acid antibodies (G) against flaxseed polysaccharides (P).

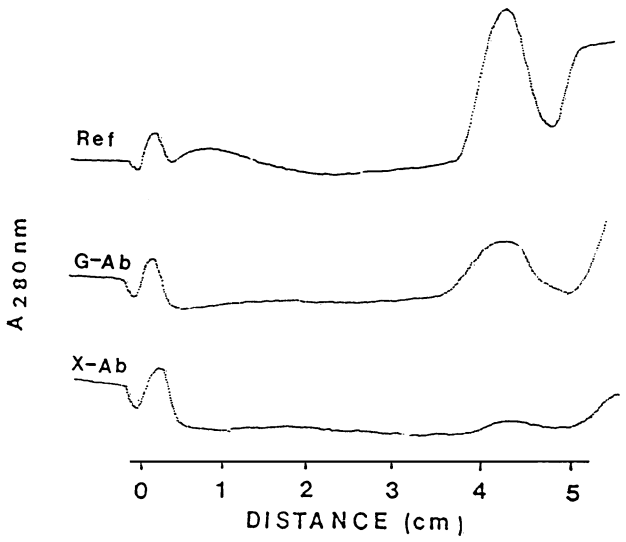

Fig. 4. Density-gradient ultracentrifugation of glucose oxidase (Ref), anti-galacturonic acid antibodies (G), and anti-xylose antibodies (X).

formula developed by Martin and Ames[14] showed both types of antibodies are of molecular weight 150,000. The immunoglobulin type of the antibodies was determined by an agar diffusion method. Goat antibodies specific for rabbit IgA, IgG, or IgM were used in the agar diffusion test. The results in Fig. 5 show that both types of antibodies (anti-xylose and anti-galacturonic acid) reacted with the goat antibodies specific for anti-rabbit IgA. Thus the new antibodies are type A immunoglobulins. In contrast, the preparation of anti-gum arabic antibodies is of the type G immunoglobulins as shown in Fig. 5 on plate C.

The anti-xylose and anti-galacturonic acid isoantibodies were subjected to inhibition tests by incubating the antibodies with the respective sugar and comparing the extent of precipitin formation in the digest with the extent of formation of precipitin in the antibodies devoid of the sugar. The procedure is described in detail in an earlier publication.[15] The inhibition of the anti-xylose antibodies by D-xylose is shown on plate A of Fig. 6. It can be seen that the pure sample of antibody solution devoid of xylose yielded precipitin bands with 20, 10, and 5 μg of the polysaccharide antigen, and that the digest of antibodies incubated with xylose yields only a precipitin band at 20 μg of the antigen. The results of inhibition of anti-galacturonic acid antibodies by galacturonic acid are also shown in Fig. 6 (Plate B). In this test, precipitin bands were obtained with 20, 10, 5, and 2.5 μg of antigen with the antibodies devoid of galacturonic acid, and precipitin bands were obtained only with 20 and 10 μg of antigen with the antibodies incubated with galacturonic acid. Thus the inhibition of the antibodies with xylose and galacturonic acid shows that the determinant units of the polysaccharides for antibody synthesis are xylose or galacturonic acid residues.

Results of gel electrophoresis and isoelectrofocusing experiments with the serum and the purified antibodies are shown in Fig. 7. It is noted in Plate A of the figure that both types of antibodies have been separated from the other proteins in the serum. However Plate B shows that each preparation of the

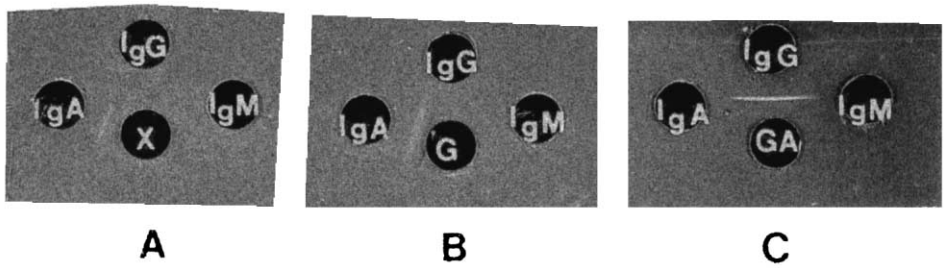

Fig. 5. Agar diffusion of anti-xylose antibodies (X), anti-galacturonic acid antibodies (G), and anti-gum arabic antibodies (GA) against immunoglobulin antibodies A, G, and M.

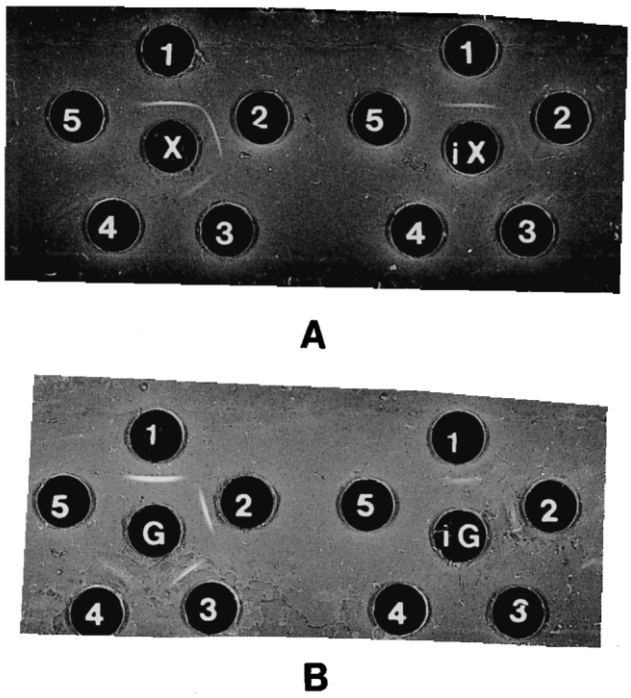

Fig. 6. Inhibition of antibodies by 100 μg of D-xylose and 150 μg of D-galacturonic acid. (A) Anti-xylose antibodies (X), anti-xylose antibodies plus xylose (iX). 20,10, 5, 2.5, 1.25 μg of flaxseed polysaccharides respectively in Wells 1, 2, 3, 4, 5. (B) Anti-galacturonic acid antibodies (G), anti-galacturonic acid antibodies plus galacturonic acid (iG), the numbers as in A.

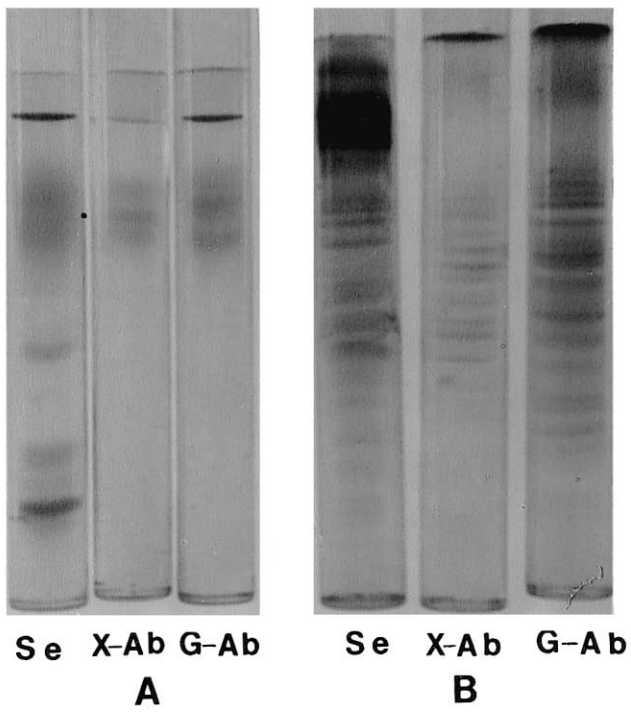

Fig. 7. Gel electrophoresis (A) and isoelectric focusing (B) of serum (Se), anti-xylose antibodies (X-Ab) in anti-galacturonic acid antibodies (G-Ab).

antibodies on isoelectrofocusing was separated into multi-protein components. It should be noted in the figure that there are at least eight proteins with different isoelectric points for the anti-xylose antibodies and at least 11 for the anti-galacturonic acid antibodies. From the coupled isoelectrofocusing–agar diffusion results with the galacturonic acid antibodies in Fig. 8, which were obtained by a published method,[3] it was concluded that all of the protein bands formed a precipitin complex with the antigenic polysaccharide and therefore possessed antibody activity. Like other sets of multi-protein anti-carbohydrate antibodies,[16,17] these new antibodies yielded a continuous precipitin band opposite all the multi-proteins establishing that each protein form possessed antibody activity.

3. Experimental

Immunization.—The immunization was performed by following the directions in a previous article.[16] Solutions of 0.8% of the flaxseed polysaccharides were mixed with equal amounts of Freunds complete adjuvant and 1 mL of the mixture was injected under the skin in the back of the rabbit, weekly for 10 weeks. Blood samples were obtained after the third injection and weekly thereafter, and serum was prepared by a standard procedure. The serum was analyzed for antibody by agar diffusion against a solution of flaxseed polysaccharides. The results of analysis are shown in Fig. 1 for pre-immune, the 4th and the 8th week serum.

Preparation of polysaccharides.—The method for preparing flaxseed polysaccharides was devised by Neville,[18] and was used in this study. Flaxseed (25 g) was soaked in 100 mL of distilled water for 18 h at rt and stirred in a magnetic stirrer. The insoluble material was removed by centrifugation. The supernatant (50 mL) was mixed with 50 mL of EtOH. A white precipitate was formed and precipitation was allowed to proceed for 24 h. The precipitate was collected by centrifugation, redissolved in 50 mL of water and again fractionated by EtOH precipitation. Any undissolved material was removed by centrifu-

gation and the clear solution was lyophilized to dryness, yield 0.5 g. Hydrolysis of a sample of the preparation in 1 M HCl followed by paper-chromatographic analysis showed that the mixture yielded rhamnose, xylose, galactose, and galacturonic acid. The structure of one flaxseed polysaccharide, the neutral polymer, was determined by methylation analysis to be an xylose polymer with many side chains.[6] The structure of an acidic polysaccharide was determined later[8] to be a heteropolysaccharide of rhamnose, galactose, and galacturonic acid. The neutral polysaccharide contains side chains terminated in xylose groups[6] and the acidic polysaccharide has side chains terminated in galacturonic acid groups.[8,19] The terminal units of the polymers activate the immune system to synthesize anti-carbohydrate antibodies.

Affinity adsorbents.—An affinity adsorbent containing D-xylose ligands was prepared from 50 mg of *p*-aminophenyl α-D-xylopyranoside and 3.5 g of cyanogen bromide-activated Sepharose 4B. The procedure is described in Ref. 20. A similar procedure was used to synthesize flaxseed polysaccharide–Sepharose-4B adsorbent from 3.5 g of CNBr-activated Sepharose 4B and 100 mg of the polysaccharide mixture.

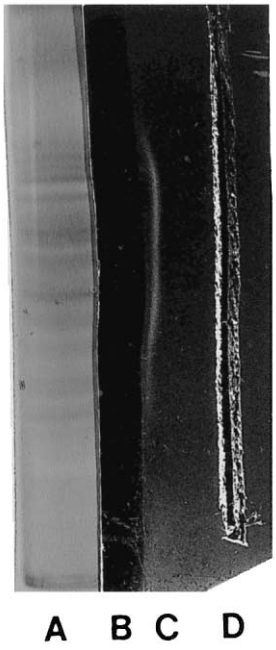

Fig. 8. Coupled isoelectrofocusing and agar diffusion. Gel of galacturonic acid antibodies, isoelectricfocused and stained (A). Gel, not stained but embedded in agar (B). Area of precipitin formation (C). Trough of flaxseed polysaccharides antigen (D).

Affinity chromatography.—Affinity chromatography devised by others for purifying enzymes[21] has been adapted for purifying antibodies.[22] A sample (1 mL) of the 8th week serum was introduced on the xylose–Sepharose column and the antibodies were eluted with 0.5 M D-xylose. Another sample of the 8th week serum was chromatographed on the flaxseed polysaccharide–Sepharose column and the column was eluted with 0.5 M D-galacturonic acid. Prior to elution with the carbohydrate solutions, both columns containing the serum were washed with 0.02 M phosphate of pH 7 containing saline until the unadsorbed protein was removed. The adsorbed protein was then eluted with xylose or galacturonic acid. The eluates from the columns were monitored for UV absorbance at 280 nm and the eluates with UV-absorbing components were collected separately. Each sample was mixed with an equal volume of saturated ammonium sulfate and maintained at 4 °C. The precipitates which formed in 24 h were collected by centrifugation and dissolved in 0.2 mL of 0.02 M phosphate buffer and saline of pH 7. These solutions were used for the antibody tests as described in the following sections. The affinity patterns are shown in Fig. 2. The purity of the isolated antibodies was checked by agar diffusion and the results are shown in Fig. 3.

Density-gradient ultracentrifugation.—A density-gradient ultracentrifugation procedure was used to determine the homogeneity and molecular weight of the antibodies.[13] Samples of 0.2 mL of 0.2% solutions of the antibodies and of reference glucose oxidase (molecular weight 150,000) were placed carefully on top of sucrose density gradients prepared from 5 to 40% sucrose solutions. The samples were centrifuged in an SW-65 rotor at 65,000 rpm for 16 h in a Beckman L-75 ultracentrifuge. At the end of this time, the tubes were removed and the gradient solutions were fractionated by means of an ISCO density gradient fractionator (ISCO Inc., Lincoln, NE). The UV absorbance of the solution from the density-gradient columns was measured continuously at 280 nm during fractionation.

The sedimentation patterns are reproduced in Fig. 4. The sedimentation data for the antibodies and glucose oxidase were used to calculate the molecular weight of the antibodies using the relationship developed by Martin and Ames:[14] $(D_1/D_2) = (M_1/M_2)^{2/3}$. D_1 and M_1 are distance and molecular weight, respectively, for the unknown and D_2 and M_2 for the standard. Glucose oxidase was the standard and molecular weight of the oxidase has been determined by ultracentrifugation in a Spinco centrifuge to be 150,000.[23]

Immunoglobulin type.—The agar-diffusion method and goat antisera were used to determine the immunoglobulin class of the purified antibodies. The goat antisera samples were specific for rabbit immunoglobulin types A, G, and M. The antibody solution was placed in the center well and the goat anti-sera in the outer wells. Diffusion was allowed to proceed for 10 h and the plates were photographed and shown in Fig. 5.

Inhibition of antibodies.—A micro method has been developed previously for measuring inhibition of antigen–antibody complex formation by potential inhibitors.[15] This method was used in the present study as follows. Samples of 1.0 mg of the potential inhibitors (D-xylose or D-galacturonic acid) were dissolved in 50 μL of antibody solution and incubated at rt for 2 h. The incubation mixture (15 μL) and 15 μL of the same antibody solutions but devoid of sugar were placed in separate center wells of an agar plate. The antigen (flaxseed polysaccharide mixture) solutions (15 μL) containing 20, 10, 5, 2.5, 1.25 μg of the antigen were placed in the five outer wells of the gel. The diffusion plates were maintained in a closed petri dish in a moist atmosphere at rt for periods of 6–24 h. The plates were checked periodically for the number and intensity of the precipitin bands and photographed. The photograph is presented in Fig. 6.

Gel electrophoresis.—The purified antibody preparations and the 8th week serum were analyzed by a gel-electrophoretic method.[24] Samples of 10–20 μL of the preparations were used in tube gels of 10% polyacrylamide. Tris–glycine buffer of pH 8.3 was used and electrophoresis was conducted for 3–4 h at a current of 2.5 mA/gel. The finished gels were stained with 0.05% Coomassie Blue to identify protein components and a photograph is shown in Fig. 7.

Gel isoelectric focusing.—Isoelectrofocusing of the purified antibody preparations and the serum was performed in 10% polyacrylamide gels and ampholine–sucrose solution of pH gradient 5–8.[25] The antibody preparation (20 μL) was mixed with an equal volume of 40% ampholine and introduced on the prefocused polyacrylamide gels. The prefocusing was performed at a current of 0.7 mA/gel until a potential of 215 V was reached for 1 h. The sample was introduced into the gel and focusing was maintained constant at 215 V for an additional 6 h. At completion of the isoelectrofocusing, the gels were removed and stained for proteins with Coomassie Blue and destained in 7% AcOH. The photograph of the gels is presented in Fig. 7.

Since both the anti-xylose and the anti-galacturonic acid antibody preparations were found by isoelectrofocusing to consist of multi-proteins (Fig. 7), a coupled method of isoelectrofocusing–agar diffusion described previously[3] was used to detect the antibody activity of the multi-proteins. Duplicate gels of the type of antibody preparation were prepared by isoelectrofocusing as already outlined. One finished gel was stained for proteins with Coomassie Blue and the other gel was not stained but was embedded in fluid agar in a petri dish. When the agar solidified, a trough was cut in the agar about 2 cm from the embedded gel. An 0.8% solution of the antigen (polysaccharides) was placed in the trough and diffusion was allowed to proceed for 24–48 h. Precipitin complex was formed opposite all the protein isomers in the stained gel. The result is shown in Fig. 8 for anti-galacturonic acid antibodies.

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