ELSEVIER

0144-8617(95)00048-8

Carbohydrate Polymers 27 (1995) 85–91
Elsevier Science Limited.
Printed in Great Britain.
0144-8617/95/$09.50+.00

Properties and applications of anti-xanthan antibodies

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(Received 28 November 1994; revised version received 5 April 1995; accepted 10 April 1995)

Xanthan, an exocellular bacterial polysaccharide, was used to immunize rabbits to induce the synthesis of anti-xanthan antibodies. The antibodies were isolated by affinity chromatography on an appropriate adsorbent. The antibodies are of the IgG type and possess a molecular weight of $1{\cdot}55 \times 10^5$. On immunization with xanthan, eight isomers of anti-xanthan antibodies are produced in the immune response to the polysaccharide and all isomers exhibit anti-xanthan activity. Several lines of evidence from inhibition tests and antibody reactivity with chemically modified xanthan indicate that the immunodominant group of the antigen is 4,6-pyr-β-D-Man-(1 → 4)-β-D-GlcA. The antibodies were used to identify xanthan in gum blends of commercial origin and in several food items.

INTRODUCTION

Xanthan is an exocellular polysaccharide produced by the bacterium *Xanthomonas campestris* (Jeanes *et al.*, 1961) and in the present study, it has been used to produce antibodies by immunization of experimental rabbits. In structure, the polysaccharide is composed of a pentasaccharide repeating unit with a main chain of →4)-β-DGlc-(1 → 4)-β-D-Glc-(1→ and side chains of 4,6-Pyr-β-D-Man-(1 → 4)-β-D-GlcA-(1 → 2)-6-Ac-α-D-Man-(1→ units linked to the 3 position of alternating glucose residues of the main chain (Jansson *et al.*, 1975; Melton *et al.*, 1976; Stankowski *et al.*, 1993). The polysaccharide is a large polymer with a molecular weight of several millions (Dintzis *et al.*, 1970). As a result of the unique structure and the high molecular weight, xanthan has some remarkable rheological properties and can function as a thickening, stabilizing, and emulsifying agent in aqueous solutions (Pettit, 1982). Based on these properties many uses for xanthan have been developed in the formulation of food items, adhesives, pharmaceuticals, and personal care products (Sanderson, 1981; Sandford *et al.*, 1984). The detection and determination of xanthan in these products is at times difficult to achieve. Anti-xanthan antibodies should be suitable for this purpose and the preparation of monoclonal (Haaheim *et al.*, 1989) and polyclonal (Pazur *et al.*, 1993) anti-xanthan antibodies has been reported. Anti-xanthan antibodies of the polyclonal type have now been isolated in pure state by affinity chromatography of immune serum from rabbits immunized with xanthan and Freund's adjuvant. Experiments on the properties of the antibodies, identification of the immunodominant group of the antigen, and analytical applications of the antibodies are being reported.

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MATERIALS AND METHODS

Xanthan is produced by *Xanthomonas campestris* by an aerobic submerged fermentation process (Pettit, 1982). The production of xanthan involves the use of a multistep inoculum fermentation process, heat treatment of the final broth to kill the organism and recovery of the xanthan from the broth by alcohol precipitation. The alcohol is removed and the precipitate is collected and dried. The dried product is used for the characterization studies being reported. Food items (salad dressing, mayonnaise, cottage cheese, and ice cream) containing xanthan were purchased from local grocery stores. Similar products which were made with other gums were obtained at The Pennsylvania State University Creamery. Samples of commercial preparations of gums were provided by Kelco, San Diego, CA 92123, USA.

Chemical modification of xanthan

Reduction of uronic acid residues

The glucuronic acid residues of xanthan gum were subjected to reduction of the carboxyl groups by using water-soluble carbodiimide and sodium borohydride according to a published procedure (Taylor & Conrad, 1972). For reduction of the uronic acid residues, 1 g of xanthan was dissolved in 40 ml of distilled water and the pH was adjusted to 4·5 using 0·1 M NaOH. The solution was then reacted with 0·8 g of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) for 2 h at room temperature. The pH of the reaction mixture was checked periodically and adjusted to pH 4·5 with 0·1 N HCl. At the end of the reaction period, 0·8 g of sodium borohydride in 10 ml of distilled water was added dropwise to reduce the glucuronic acid moieties of the gum. The pH of this reduction mixture was allowed to rise to 7 and maintained at this pH by addition of 2 N HCl. After the pH of the reaction mixture stabilized, the mixture was cooled to a temperature of 4°C and allowed to react for 16–24 h, after which time the borohydride was removed by dialysis. The reduced gum was recovered by lyophilisation and analyzed for uronic acid content by a carbazole method. The analysis showed that approximately 60% of the glucuronic acid moieties had been reduced. The reduction procedure was repeated twice as outlined above. Analysis of the product after the second and third reductions by the carbazole method showed that 87% and 94% of the glucuronic acid in xanthan had been reduced, respectively. Pyruvate joined to terminal mannose units of xanthan by acetal linkage is not likely to be susceptible to reduction.

Deacetylation

A sample of 5 g of xanthan was *O*-deacetylated using a modification of a published procedure (Tako & Nakamura, 1984). A 5% solution of polymer in 0·1% KCl was treated with 0·2 N KOH for 1 h at 60°C with constant mixing. The solution was cooled to room temperature, neutralized with 0·05 N hydrochloric acid, and the product was recovered by precipitation with isopropanol. The recovered product was rehydrated to yield a 0·25% solution in 0·1% KCl, dialysed against deionized water, passed through ion exchange resins, and reprecipitated. The dried, milled product was used for investigation.

Depyruvylation

Depyruvylation of the xanthan polymer involved treatment of a 1% solution of xanthan gum in deionized water with 5 mM trifluoroacetic acid at 100°C for 1·5 h (Bradshaw *et al.*, 1983). The solution was dialysed against deionized water, passed through ion-exchange resins, and precipitated with isopropanol. The recovered product was rehydrated in 0·1% KCl and reprecipitated with isopropanol. Several samples of depyruvylated xanthan were prepared by varying the time of acid treatment and analysis of five different samples for pyruvate. The samples were hydrolysed in 1 N HCl and reacted with 2,4-dinitrophenyl hydrazene and the absorbance was measured at 375 mu (Sloneker & Orentas, 1962). The analyses showed that the samples contained 6·0, 4·0, 2·8, 0·5, and 0·2% pyruvate.

Periodate oxidation

A sample of 50 mg of xanthan was subjected to periodate oxidation (Forsberg & Pazur, 1979). The sample was dissolved in 10 ml of 0·02 M sodium meta-periodate at pH 5. The oxidation was allowed to proceed at room temperature and in the dark for 24 h. At this point the excess periodate was destroyed with ethylene glycol and the oxidation mixture was dialysed for 24 h. The oxidized xanthan was removed from the dialysis tubing and taken to dryness by lyophilisation. The native and oxidized xanthan were tested for reactivity with the antibodies by agar diffusion.

Treatment of xanthan with α-mannosidase

α-Mannosidase was prepared from jack bean meal by extraction with phosphate buffer of pH 7·5 and recovered by alcohol precipitation. The enzyme solution hydrolysed methyl α-D-mannoside at a high rate, liberating free mannose identified by Rf value on paper chromatography. Although the enzyme is an exo-enzyme it seemed possible that the enzyme was capable of hydrolysing the α-glycosidic linkage near the terminus of the side chains. However, treatment of a solution of xanthan with the α-mannosidase preparation had no effect on the xanthan as no hydrolytic products were produced as shown by paper chromatography, and no loss of antigenicity occurred as shown by agar diffusion.

Detection of xanthan with antibodies

Three commercial gum blends of xanthan were supplied by Kelco, San Diego, CA, USA. Solutions (0·5%) of these samples and standard xanthan were subjected to agar diffusion with anti-xanthan antibodies. The diffusion assay method is described in a subsequent section. The agar plate was photographed and the photograph of the results is reproduced in the Results section.

A number of food items containing xanthan and similar samples containing other gums were examined by the agar diffusion method. The items included salad dressing, mayonnaise, ice cream, and cottage cheese. Samples of 5–10 g of the items were extracted with 5 ml of water and centrifuged to remove insolubles and the lipids. The supernatant was lyophilised to reduce the volume to one-quarter of its original volume. Samples of solutions were subjected to agar diffusion tests using anti-xanthan antibodies. The results for the salad dres-

sing and ice cream tests are recorded in the Results section.

Immunological methods

A 1 ml sample of a 1% xanthan solution in 0·02 M phosphate buffer of pH 7·2 was used in suspension with an equal volume of Freund's complete adjuvant to immunize rabbits. The immunization was performed interdermally at multi-sites on the back and repeated weekly for 6 weeks. Two milliliters of the suspension was used for each injection. Blood samples were drawn weekly and the samples after the sixth week were used to prepare serum by a standard method. Agar diffusion tests showed that antibodies against xanthan were present in a high level in these serum samples.

Affinity chromatography

An affinity adsorbent of AH-Sepharose 4B with ligands of xanthan was prepared for use in the purification of the antibodies. Three grams of AH-Sepharose 4B, 100 mg of the xanthan and 250 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) from Aldrich Chemical (Milwaukee, WI) were used for the synthesis. The preparation of the adsorbent followed the protocol outlined in a Pharmacia bulletin (Pharmacia, 1986).

In the affinity chromatography method (Pazur *et al.*, 1978) samples of the immune serum were applied to a column of the adsorbent and, after the adsorption of the sample, the column was washed with 0·02 M phosphate buffer pH 7·2 containing saline until the unadsorbed proteins were removed. The antibodies were then eluted with 1 M ammonium thiocyanate and the eluate was monitored with a UV analyzer. The components which eluted as a single peak were collected, combined and mixed with an equal volume of saturated ammonium sulfate. The precipitate which formed on refrigeration overnight was then collected by centrifugation and dissolved in a small volume of phosphate buffer. Agar diffusion tests to detect antibodies were performed on these solutions.

Immunodiffusion assays

The Ouchterlony agar diffusion method (Ouchterlony, 1949) was used for detecting precipitin formation in the antibody–antigen reactions. Immune serum (10 μl) or purified antibody solution (10 μl) was placed in the center well of an agar diffusion plate. Antigen (10 or 20 μl) was placed in the outer wells of the agar plate. The diffusion plates were placed in a Petri dish on moist filter paper, covered, and maintained in a moist atmosphere at room temperature for periods of 24–48 h. The plates were checked periodically for precipitin formation and the intensities of the precipitin bands which formed were noted. The plates were also photographed for a permanent record of the results.

A micro method combining inhibition and agar diffusion has been developed in this laboratory for detecting potential inhibitors of the antibody–antigen reactions (Pazur & Kelly, 1984). This method was used to test glucose, glucuronic acid and mannose as potential inhibitors of the precipitin reaction. In the inhibition-agar diffusion method, samples of 0·1–5 mg of the potential inhibitor were dissolved in 20 μl of the appropriate antibody solution or antiserum and incubated for 4 h. At the end of this time, a 10-μl sample of the incubation mixture was placed in the center well of a diffusion plate. Antigen solutions (10 μl) containing decreasing amounts of the antigen (1%, 0·5%, 0·25%, 0·125%, 0·625%, and 0·03125%) were placed in the outer wells of the agar plate. The plates were placed in a moist chamber and allowed to develop for 24–48 h. The extent of inhibition was determined by comparing the intensity of the precipitin bands at the different concentrations of antigen on the inhibition plates with the intensity of bands on a plate with the antibody solution and antigen but no inhibitor.

Isoelectrofocusing

In order to examine the antibody preparation for isomeric forms of the antibody, gel isoelectric focusing was performed. In the method a 10% polyacrylamide gel and ampholine–sucrose solution of pH gradient 5–8 was used following a procedure described in the literature (Doerr & Chrambach, 1971). A coupled isoelectric focusing and agar diffusion method developed in this laboratory (Pazur *et al.*, 1990) was also used to detect antibody activity of the components in the purified antibody preparations. In this procedure, duplicate samples of antibody preparations were subjected to identical conditions of gel isoelectric focusing. One finished gel was stained for protein with Coomassie blue, and the other gel was embedded in liquid agarose. After the agarose had solidified around the polyacrylamide gel, a trough was cut about 2 cm from the gel and a 0·5% solution of xanthan was introduced into the trough. Diffusion was allowed to proceed for a period of 24–48 h in a closed, moist chamber at room temperature. The plates which developed precipitin arcs were photographed.

Density gradient ultracentrifugation

A density gradient ultracentrifugation procedure was used to determine the homogeneity and molecular weight of the antibodies (Pazur *et al.*, 1962). Samples of 0·2 ml of 0·2% solutions of the antibodies and of reference glucose oxidase were placed carefully on top of sucrose density gradients prepared from 5 to 40% sucrose solutions. The samples were placed in a swing-

ing bucket SW-65 rotor and centrifuged at 65,000 r.p.m. for 16 h in a Beckman L-65 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. The UV absorbance of the solutions from the density gradient columns was determined continuously at 280 nm during fractionation. The sedimentation data for the antibodies and glucose oxidase were used to calculate the molecular weight of the antibodies using an empirical relationship (Martin & Ames, 1961).

Immunoglobulin type

The immunoglobulin class of the antibodies was determined by the double immunodiffusion method. A 10 μl sample containing purified xanthan antibodies was placed in the center well of an agar plate, and goat-anti-rabbit IgA, goat-anti-rabbit IgG, and goat-anti-rabbit IgM antisera (Cappel Labs, Organon Corp., West Chester, PA, USA) were placed in separate outer wells. Diffusion of the samples was allowed to proceed in a moist chamber at room temperature for 24 h, after which time the type of anti serum reacting with the gum antibodies was noted.

RESULTS

In Fig. 1, a diagrammatic structure for the pentasaccharide repeating unit of a xanthan molecule is drawn on the basis of literature data (Jansson *et al.*, 1975; Melton *et al.*, 1976). The xanthan molecule is made up of approximately 10,000 such units with the main chain of glucose units linked by β-(1 $\rightarrow$ 4) glucosidic linkages. The side chain of the xanthan molecule consists of a terminal mannose unit with a ketal linkage of pyruvate to positions 4 and 6 of the mannose, glucuronic acid and an internal mannose residue with acetyl groups at position 6. Such structural features and the high molecular weight impart unique properties to the xanthan molecule.

Several serum samples were obtained from rabbits immunized with xanthan and anti-xanthan antibodies have been isolated from the immune serum by affinity

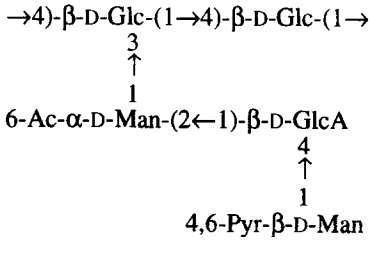

Fig. 1. Structure of the repeating unit of xanthan: Ac, acetate; Pyr, pyruvate; Man, mannose.

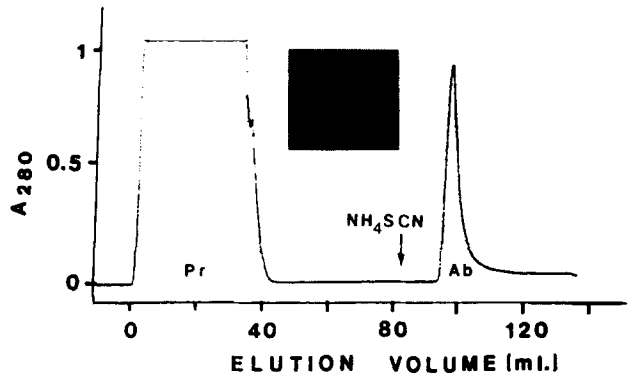

Fig. 2. An affinity chromatography pattern and agar diffusion of the fractions of the immune serum: Pr, protein; Ab, antibody; Xa, xanthan.

chromatography. The elution pattern from the adsorbent of Sepharose–xanthan is shown in Fig. 2. Also shown are agar diffusion results which demonstrate that anti-xanthan antibodies have been obtained.

The molecular weight and homogeneity of the antibodies has been determined by the density gradient centrifugation method (Pazur *et al.*, 1962). The pattern is shown in Fig. 3.

As shown by the results of the isoelectric focusing and agar diffusion experiment in Fig. 4, the anti-xanthan antibody preparation contains several isomeric forms of the antibody. The isoelectrofocusing gel (A) shows that eight major isomers and some minor isomers constitute the purified antibody preparation. The agar diffusion results in the figure show that the isomers possess anti-xanthan activity.

The results in Fig. 5 establish the immunoglobulin type of the antibodies and the effect of chemical modification of xanthan on the precipitin reaction of the gum with the antibodies. The modifications include removal of the pyruvate group, reduction of the carboxy group of glucuronic acid, oxidation with periodate, removal of the acetyl group and α-mannosidase treatment of xanthan. The lower set of plates shows results of inhibition of the precipitin reaction by glucuronic acid and mannose.

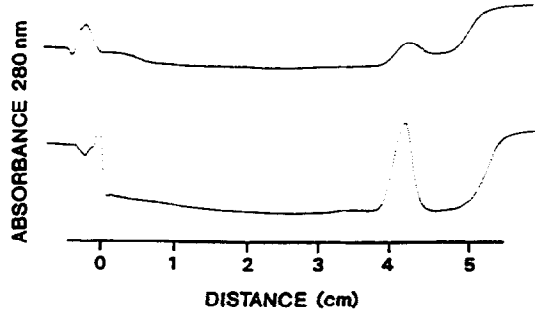

Fig. 3. Ultracentrifugation of anti-xanthan antibodies (above) and glucose oxidase (below) in sucrose gradient.

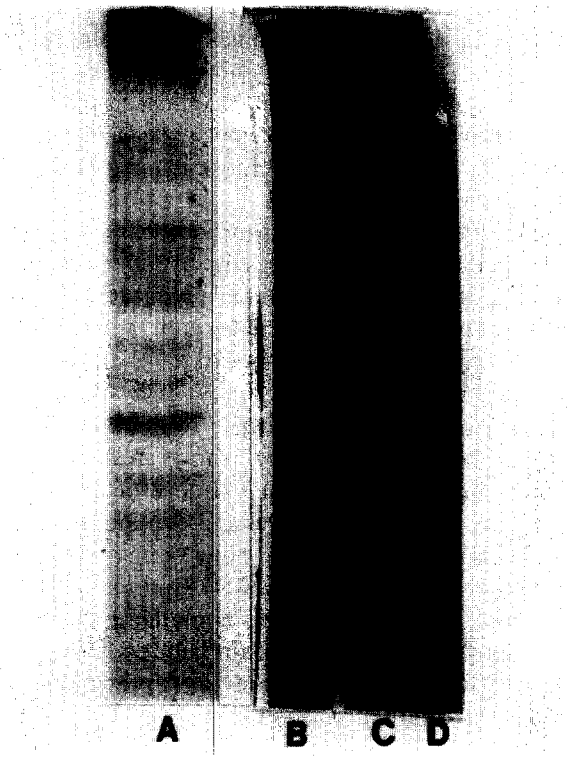

Fig. 4. Isoelectrofocusing and agar diffusion of isomeric anti-xanthan antibodies: A, gel stained for protein; B, gel embedded in agarose; C, precipitin area; D, trough of antigen.

Figure 6 contains results showing the utility of the antibodies in the detection of xanthan in products. Commercial gum blends have been tested and the results are shown in (A). (B) contains results of tests on ice cream and salad dressing samples which contain xanthan and similar products that contain other gums.

DISCUSSION

The structure of the repeating unit of xanthan is shown diagrammatically in the formula in Fig. 1 and is in accord with published findings (Jansson *et al.*, 1975; Melton *et al.*, 1976). The polysaccharide, when injected into animals, can activate the immune system to produce anti-xanthan antibodies (Haaheim *et al.*, 1989; Pazur *et al.*, 1993). Antibodies directed at xanthan have now been isolated by affinity chromatography from immune sera of rabbits immunized with the gum. As detailed in the following, the antibodies are shown to be polyclonal consisting of eight major isomeric forms. The antigen, xanthan, has an immunodominant group of 4,6-Pyr-β-D-Man-(1 $\rightarrow$ 4)-β-D-GlcA moiety.

The anti-xanthan antibodies were isolated by affinity chromatography from the serum of rabbits which were immunized with the suspension of xanthan and Freund's adjuvant. Sepharose–xanthan was used as the adsorbent and the antibodies were eluted with 0·5 M

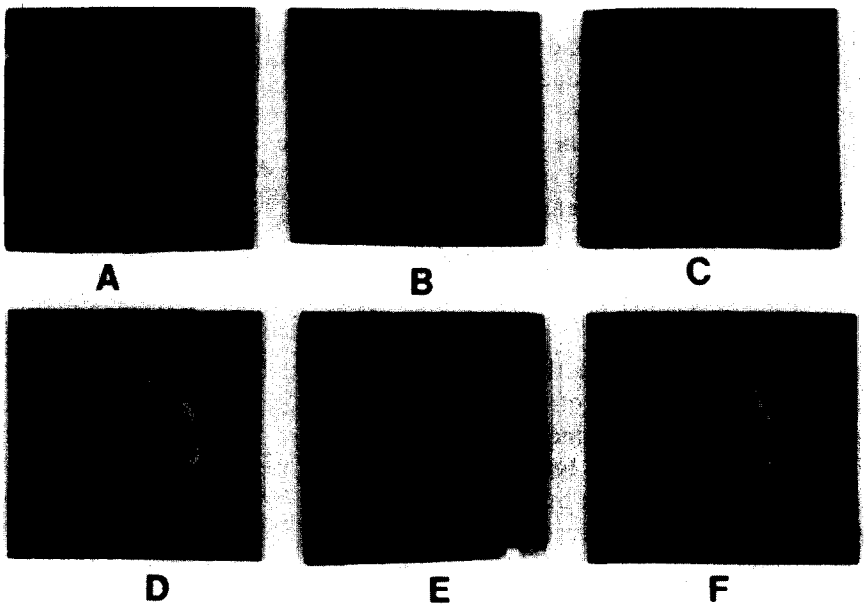

Fig. 5. Agar diffusion patterns of antibodies (Ab) and of xanthan and chemically modified xanthan. (A) 1, goat anti-IgA; 2, goat anti-IgG; 3, goat anti-IgM. (B) 1, xanthan with 6% pyruvate; 2, xanthan with 4% pyruvate; 3, xanthan with 2·8% pyruvate; 4, xanthan with 0·5% pyruvate; and 5, xanthan with 0·2% pyruvate. (C) 1, xanthan; 2, xanthan reduced with borohydride; 3, xanthan oxidized with periodate; 4, deacetylated xanthan; 5, enzymically modified xanthan. (D), (E) and (F) 1–6 contains 1%, 0·5%, 0·25%, 0·125%, 0·0625%, and 0·03125% solutions of xanthan; In_1, antibody + glucuronic acid; In_2, antibody + mannose.

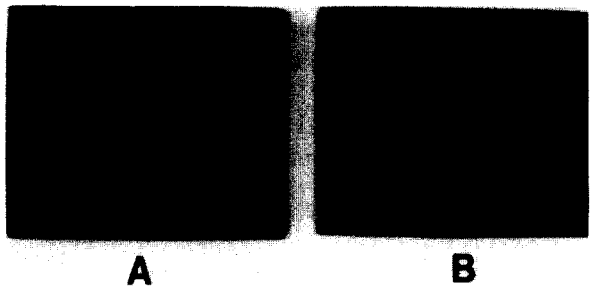

Fig. 6. Detection of xanthan in gum preparations and in food items by anti-xanthan antibodies and agar diffusion. (A) 1, blank; 2, xanthan; 3, sample A; 4, sample B; 5, sample C. (B) 1, xanthan; 2, salad dressing; 3, salad dressing with other gum; 4, ice cream; 5, ice cream with other gums.

ammonium cyanate. It can be seen in Fig. 2 that the antibodies eluted from the adsorbent in a uniform manner. The inset in the figure shows that this eluate contained the anti-xanthan antibodies.

On ultracentrifugation by the density gradient method (Pazur *et al.*, 1962), the antibodies yielded a single symmetrical peak (upper pattern) shown in Fig. 3. On comparison of the antibody pattern to that of glucose oxidase (lower pattern) and applying an empirical formula (Martin & Ames, 1961) the molecular weight of the antibodies was calculated to be $1{\cdot}55 \times 10^5$.

In order to check for the existence of isomeric forms in the purified antibody preparation it was subjected to an analysis by the coupled isoelectric focusing and agar diffusion method (Pazur *et al.*, 1990). A composite figure of the results was prepared and is shown in Fig. 4. Eight major bands of protein components and some minor components can be seen in the stained gel (Fig. 4A). The precipitin arc (Fig. 4C) shows that a precipitin complex was obtained opposite all the major components and these components form a set of isomeric anti-xanthan antibodies. Sets of anti-carbohydrate antibodies are induced by immunization with other glycans as well as glycoproteins and glycoconjugates (Pazur *et al.*, 1978, 1990, 1994; Miskiel & Pazur, 1991).

The agar diffusion pattern of Fig. 5A shows that the anti-xanthan antibodies are of the IgG class of immunoglobulins, since a precipitin band was obtained with antisera against IgG but not against IgA or IgM. (B) shows that decreasing pyruvate in the xanthan by treatment with trifluoroacetic acid yielded decreasing amounts of precipitin. Precipitin complex did not form when the pyruvate content was less than 0·5%. Thus pyruvate groups of xanthan participate in the formation of the antibody–antigen complex. No hydrolytic products were noted during treatment of xanthan with trifluoroacetic acid. (C) shows that reduction of the carboxy group of glucoronic acid (well 2) or oxidation with periodate (well 3) of xanthan destroyed the antigenicity of the gum. However, deacetylation (well 4) or treatment with a mannosidase (well 5) of the xanthan did not destroy antigenicity. (D), (E), and (F) show results of an inhibition experiment. (D) is the standard devoid of carbohydrate, (E) shows GlcA is a potent inhibitor and (F) shows that mannose is not. Other considerations showed that pyruvated mannose is required but deacetylated mannose is not necessary for precipitin complex formation.

The utility of anti-xanthan antibodies as an analytical reagent is illustrated by the results in Fig. 6. (A) shows agar diffusion results with three preparations of gum blends supplied by Kelco. The gum solutions were placed in wells 3, 4 and 5, standard xanthan solution in well 2 and a blank (H_2O) in well 1. It is apparent that sample C (well 5) contains xanthan but samples A and B do not contain xanthan. (B) shows results of agar diffusion of antibodies with extracts of food items. These items include salad dressing containing xanthan (well 2), salad dressing with other gums (well 3), ice cream containing xanthan (well 4), and ice cream with other gums (well 5). The items containing xanthan are readily identified by the precipitin bands while the items containing other gums did not yield precipitin bands with the anti-xanthan antibodies.

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