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An immunological method for the determination of the D and L configurations of monosaccharides

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An immunological method is described by which the D and L configuration of monosaccharides can be determined. The method utilizes agar diffusion, specificity of anti-carbohydrate antibodies and inhibition of the antigen–antibody reaction by the D or L forms of monosaccharides The method has been applied to the differentiation of configuration of isomers of glucose, fucose, and galactose. The configuration of galactose in two different polysaccharides has also been determined.

INTRODUCTION

Monosaccharides may occur in nature in free form or as structural units of many types of carbohydrate containing polymers. The configuration of the monosaccharide units may be D or L, and some monosaccharides may be present in one form only, while others may be present in both forms. In the past the configuration of monosaccharides has been assigned on the basis of optical rotation values, circular dichroism data or susceptibility to enzymes of known D or L specificity (see review by Pazur, 1986). In order to determine the configuration of monosaccharide residues of carbohydrate polymers, it is first necessary to liberate the monosaccharides by acid or enzymic hydrolysis followed by isolation of the monosaccharide and application of one of the above methods A chemical method which is based on the identification of diastereomeric products formed from the monosaccharide and chiral alcohols has been described for the determination of the configuration of certain monosaccharides (Gerwig *et al.*, 1978; Leontein *et al.*, 1978). This method has been described in a recent Methods volume (Leontein & Lonngren, 1993) but has some limitations.

In our laboratory an immunological method has been utilized for the differentiation of the configuration of monosaccharides. This method is based on the inhibitory property of monosaccharides in antibody–antigen reactions. The method has been tested with glucose, fucose, and galactose and two polysaccharide preparations, one from the cell wall of *Streptococcus faecalis* (Pazur *et al.*, 1973) and the other from flaxseed (Neville, 1913). Results of the immunological tests show that the configurations of monosaccharides can be assigned by the immunological method and verify that a streptococcal polysaccharide contains D-galactose (Pazur *et al.*, 1971) and a flaxseed polysaccharide contains L-galactose (Anderson, 1933). The method is generally applicable to the analysis of configurations of other monosaccharides and monomeric units of carbohydrate containing polymers.

The immunological procedure requires the use of purified antibodies having specificity for the monosaccharide under study. The inhibition of the antibodies specific for the D or L configuration of carbohydrates is measured by a macro agar diffusion assay (Pazur & Kelley, 1984). One or the other configuration of the monosaccharide will cause inhibition and from the inhibition or lack of inhibition the absolute configuration of the monosaccharide can be deduced. Antibodies with specificity for other monosaccharides (mannose, rhamnose, *N*-acetylglucosamine and glucuronic acid) in addition to those listed above are available (Pazur, 1981; Pazur *et al.*, 1992) and can be used for determination of the configuration. Details of the immunological method are presented in this report.

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MATERIALS AND METHODS

Preparation of glycoconjugates and polysaccharides

The glycoconjugates of D-glucose, L-fucose and D-galactose were prepared from the appropriate *p*-aminophenyl glycosides and bovine serum albumin by the carbodiimide coupling reaction as outlined in a recent publication (Pazur *et al.*, 1992). In a typical preparation, samples of 0·05 g of the glycoside and 2 g of bovine serum albumin were used and the protocol recommended by the supplier was followed (Pharmacia, 1986). The final product was dialyzed for 48 h and then taken to dryness by lyophilization. The yields of D-Glc-BSA, L-Fuc-BSA and D-Gal-BSA were approximately 0·2 g.

The polysaccharide in the cell wall of *Streptococcus faecalis* strain N was isolated as previously described (Pazur *et al.*, 1971). The structure of the glycan has been determined by methylation analysis and enzymic methods to consist of a chain of eighteen units of D-glucose-D-glucose-D-galactose with lactosyl units attached to the central unit (Pazur *et al.*, 1973). Flaxseed mucilage was isolated by the original method (Neville, 1913). On acid hydrolysis and paper chromatographic analysis, rhamnose, xylose, arabinose, galactose and galacturonic acid were confirmed as constituents of the mucilage (Tipson *et al.*, 1939; Anderson & Lowe, 1947). The mucilage fraction of flaxseed is probably a mixture of a neutral and an acidic polysaccharide as shown by fractionation and methylation studies (Easterby & Jones, 1950; Hunt & Jones, 1962).

Preparation of affinity adsorbents

The affinity adsorbents were prepared from *p*-aminophenyl glycosides and cyanogen bromide-activated Sepharose-4B (Cuatrecasas, 1970) by the protocol outlined in a recent bulletin (Pharmacia, 1986). A sample of 4 g of CNBr-activated Sepharose-4B and 0·05 g of the appropriate *p*-aminophenyl glycoside was suspended in 25 ml of 0·1 M $NaHCO_3$ of pH 8·3 containing 0·5 M NaCl. The mixture was shaken for 2·5 h at room temperature. The derivatized Sepharose was then isolated by filtration and washed with dilute acetic acid followed by dilute sodium bicarbonate. Unreacted imidocarbonate groups were blocked with 1 M ethanolamine and the Sepharose derivative was washed again with the acid and base solutions. The product was placed in a column (1 cm × 20 cm) and equilibrated with 0·02 M phosphate buffer of pH 7. The β-D-Glc-, α-L-Fuc- and β-D-Gal-Sepharose-4B were synthesized by this method and used for isolating the anti-β-D-glucose, anti-α-L-fucose and the anti-β-D-galactose antibodies from immune sera of rabbits immunized with appropriate glycoconjugates.

Immunization of rabbits

Two types of immunization procedures are used for producing antisera with antibodies having specificity for the carbohydrate units of glycoconjugates or polysaccharides. In the first procedure (Pazur *et al.*, 1992) 100 mg of the glycoconjugate was dissolved in 20 ml of sterile saline and for each immunization 1 ml of this solution was mixed with an equal volume of Freunds complete adjuvant. Portions (0·2 ml) of the suspension of adjuvant and antigen were injected in multisites in the back of the rabbit each week for 6 consecutive weeks. After a rest period of 3 weeks, a secondary response was evoked by additional immunizations. Antisera were prepared from the blood samples collected from the animals after the sixth week of immunization and thereafter by a standard procedure. Agar diffusion tests showed that all samples of serum in the sixth and subsequent immunization periods possessed anti-carbohydrate antibodies.

In the second procedure (Pazur *et al.*, 1973) a non-viable cell suspension was prepared from *S. faecalis* cells collected from a 500 ml culture. The cells were suspended in 30 ml of phosphate buffer (pH 7) containing 0·2% formaldehyde for 6 h at room temperature. At the end of this time the suspension was diluted several-fold with the buffer solution. This suspension was used for immunizing two rabbits by intravenous injection of 0·3 ml of the suspension daily for 4 days followed by a rest period of 3 days. The schedule was repeated for 6 additional weeks. After a rest period of 5 weeks, a second immunization was performed following the above schedule. Blood samples were collected from the rabbits weekly and the serum was tested for potency against the *S. faecalis* polysaccharide as the antigen. Serum samples from the 9th and subsequent weeks exhibited high titers against the antigen. The sample with the highest titer was employed for the immunological experiments reported in this paper.

Affinity chromatography of immune sera

A sample of 2 ml of immune serum was used for affinity chromatography on appropriate adsorbents by the method previously described (Pazur *et al.*, 1978). In brief, the method involved introduction of the serum sample onto the proper adsorbent and washing with 0·02 M sodium phosphate buffer of pH 7 in saline until all the unadsorbed protein was removed as determined by UV measurements on the eluate. The column was then washed with 10 ml of 1 M glucose, fucose, galactose, or ammonium thiocyanate dissolved in the 0·02 M phosphate buffer of pH 7 depending on the immune serum being used. The UV absorbance of the eluate was measured in an ISCO analyzer during the affinity chromatographic step. Fractions containing the substance which eluted with the carbohydrate solutions or ammo-

nium thiocyanate were collected separately and mixed with an equal volume of saturated ammonium sulfate solution. After refrigeration for 24 h a white precipitate was obtained and collected by centrifugation. The sample was redissolved in 0·2 ml of 0·02 M phosphate buffer of pH 7 and this solution contained the desired antibodies.

Carbohydrate analysis of polysaccharides

Samples of 6 mg of *S. faecalis* polysaccharide and 10 mg of the flaxseed mucilage were dissolved in 0·2 ml of 2 N hydrochloric acid in a small test tube, the tubes were stoppered tightly and heated in a boiling-water bath for 2 h. Initially at time 0, a 10 μl sample of each solution was taken for analysis for monosaccharides by paper chromatography (French *et al.*, 1950). At the end of the heating period, 10 μl samples were again taken for carbohydrate analysis. The hydrolysates and reference carbohydrates were analyzed by paper chromatography (two ascents) in a solvent of *n*-butyl alcohol, pyridine, and water (6:4:3 by volume). The carbohydrates were detected by staining a chromatogram with silver nitrate and sodium hydroxide reagents (Meyer & Larner, 1959). The stained chromatogram was photographed.

The inhibition assay

In a typical assay, 10–20 μM of the D- or L-monosaccharides were dissolved in 20 μl of antibody solution with specificity for β-D-glucose, α-L-fucose or β-D-galactose units of antigens. Each solution was incubated for 2 h at room temperature, in which time a complex was formed between the antibody and the carbohydrate having the complimentary structure. Ten μl of the reaction mixture and 10 μl of antibody solution devoid of carbohydrate were then used in agar diffusion tests against decreasing amounts of antigen. In the glucose tests the amounts of antigen (β-D-Glc-BSA) were 20, 10, 5, 2·5, 1·25, and 0·625 μg in the outer wells, in the fucose tests the amounts of antigen (α-L-Fuc-BSA) were 80, 40, 20, 10, 5, and 2·5 μg in the outer wells, and in the galactose test the amounts of antigen (*S. faecalis* polysaccharide) in the outer wells were as listed for the glucose tests. The center wells of the glucose experiment (Plate A) contained 10 μl of untreated antibody solution in Well A_1, 10 μl of the mixture of Ab and D-Glc in Well A_2, and 10 μl of the mixture of Ab and L-Glc in Well A_3. The wells of the fucose experiment (Plate B) contained 10 μl of untreated antibody in Well B_1, 10 μl of Ab and D-Fuc in Well B_2, and 10 μl of Ab and L-Fuc in Well B_3. In the galactose test (Plate C), the center wells contained 10 μl of Ab in Well C_1, 10 μl of Ab and D-Gal in Well C_2, and 10 μl of Ab and L-Gal in Well C_3. The β-D-galactose antibodies were used for configurational analyses of galactose units in the bacterial and flaxseed polysaccharides. The polysaccharides were hydrolyzed as described in a previous section. The hydrolyzates were neutralized with solid $NaHCO_3$. Samples (10 μl) of the hydrolyzates were used for incubation with β-D-Gal antibodies by the above protocol. In the results shown in Fig. 3, Well 2 and Well 3 contained hydrozylates of *S. faecalis* and flaxseed polysaccharides which had been incubated with β-D-galactose antibodies. All plates in the tests were maintained in a closed petri dish and in a moist atmosphere at room temperature for periods up to 24 h. The plates were checked periodically for precipitin band formation. The numbers and the intensities of the precipitin bands were noted and the plates were photographed for a permanent record.

RESULTS AND DISCUSSION

The differentation of the D or L configurations of glucose and fucose by the immunological method is based on inhibition with the monosaccharides during formation of the antibody–antigen complex. The results in Fig. 1, Plates A and B, are typical. It is seen in Plate A that the precipitin pattern of β-D-glucose antibodies (Well A_1) with decreasing amounts of antigen (β-D-Glc-BSA in the outer wells) consists of five precipitin bands of decreasing intensity. The antibodies which were incubated with L-glucose (Well A_3) also yield five precipitin bands. The precipitin pattern around Well A_2, which contains antibodies incubated with D-glucose, is quite different from the patterns around Wells A_1 and A_3. Only at the two highest concentrations of the antigen (β-D-Glc-BSA) was a precipitin complex formed. Calculation of the amount of antigen that did not combine with the antibody showed that 80% inhibition had occured in this test. The D-glucose is therefore an inhibitor of the precipitin reaction and prevents the formation of precipitin bands at the lower concentration of antigen. Since the L-glucose is not an inhibitor, the configuration of glucose is distinguished by these results.

Plate B of Fig. 1 shows the results with D- and L-fucose. The α-L-fucose is an inhibitor of the precipitin reaction of the α-L-Fuc Ab with the α-L-Fuc-BSA, but the D-fucose is not. Therefore, the assignment of the configuration of fucose is also possible by the immunological test. The L-fucose is a strong inhibitor since a precipitin complex was obtained only with the highest concentration of antigen. In this experient 90% inhibition was obtained.

Inhibition data were obtained for β-D-galactose antibodies with D- and L-galactose and hydrolyzates of polysaccharides from *S. faecalis* and flaxseed. That the polysaccharides contain galactose was shown in the following manner. Samples of each polysaccharide were hydrolyzed and the monosaccharides thus liberated were identified by paper chromatography. A photo-

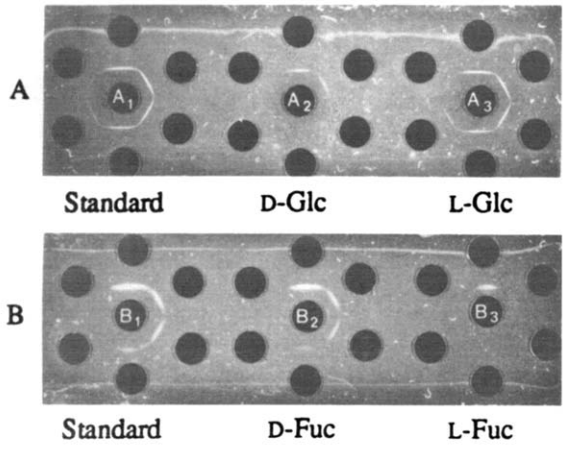

Fig. 1. Agar diffusion plates of β-D-Glc antibodies and glucose (Plate A) and α-L-Fuc antibodies and fucose (Plate B). Well A_1 contained untreated antibodies. Wells A_2 and A_3 contained antibodies incubated with D- or L-glucose, respectively. The outer wells contained 20, 10, 5, 2·5, and 0·625 μg of β-D-Glc-BSA clockwise, beginning with the top well. Well B_1 contained untreated antibodies, and Wells B_2 and B_3 contained antibodies incubated with D- or L-fucose. The outer wells contained 80, 40, 20, 10, 5, and 2·5 μg of α-L-Fuc-BSA, respectively.

graph of the chromatogram of the products of hydrolysis and reference D- and L-galactose is reproduced in Fig. 2. It is noted that both polysaccharides yield galactose as one of the hydrolysis products. The mobilities of the galactose from the polysaccharides on paper chromatography and the mobilities of D-galactose and L-galactose on the same chromatogram are identical (*Rf*, 0·47). The flaxseed preparation yielded rhamnose (*Rf*, 0·80), xylose (*Rf*, 0·69), arabinose (*Rf*, 0·60), and galacturonic acid (*Rf*, 0·10) in addition to galactose. The bacterial polysaccharide yielded glucose (*Rf*, 0·55) in addition to the galactose. It has been shown that polysaccharide preparations from flaxseed consist of neutral and acidic components with the latter containing galactose (Hunt & Jones, 1962). A separation of these polymers was not attempted since the objective of the present study was to test the immunological method for determination of the configuration of the galactose.

The configurations of galactoses from the polysaccharides were determined with β-D-Gal antibodies by the immunological method. Results with galactose and the products from the polysaccharides are shown in Fig. 3, Plate C and Plate D. The β-D-galactose antibodies did not diffuse as rapidly in agar as the other antibodies that were studied. However, it can be seen in Plate C that the standard antibody solution (Well C_1) and the antibody incubated with L-galactose (Well C_3) yield precipitin bands at five concentrations of the antigen (*S. faecalis* polysaccharide). Therefore the L-galactose is not an inhibitor of the antibody–antigen reaction. By contrast, antibodies incubated with D-galactose yield precipitin bands only at the two highest concentrations of antigen and the immunological method differentiates the two configurations of the galactose.

Plate D of Fig. 3 shows an experiment carried out with the hydrolyzates of the polysaccharides and the β-D-galactose antibodies. Well D_1 contained the β-D-galactose antibodies, Well D_2 contained the hydrolyzate of bacterial polysaccharide incubated with the antibodies and well D_3 contained the hydrolyzate of flax-

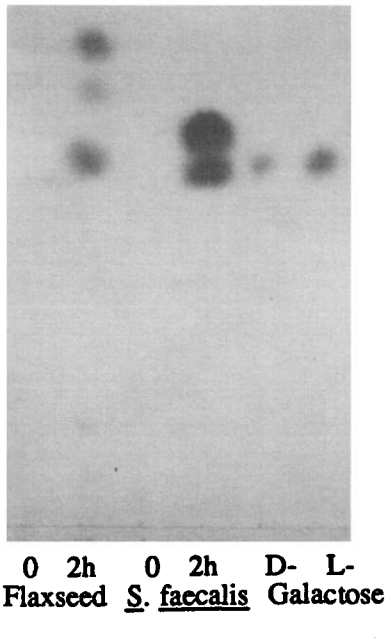

Fig. 2. Photograph of a paper chromatogram of D-galactose, L-galactose and 0 and 2 h acid hydrolyzates of flaxseed polysaccharide and *S. faecalis* polysaccharide.

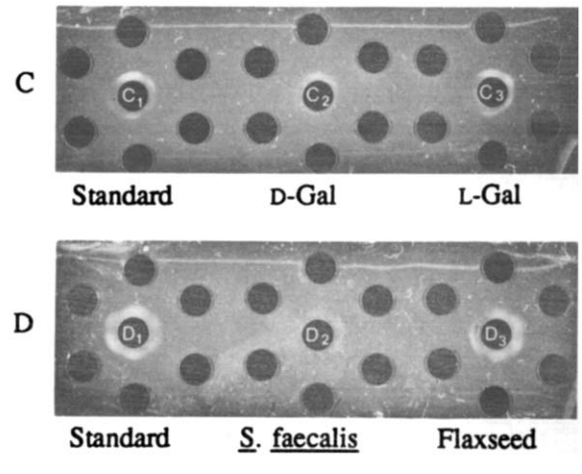

Fig. 3. Agar diffusion plates of purified D-gal antibodies and antibodies incubated with D-galactose or L-galactose (Plate C) and with hydrolyzates of *S. faecalis* and flaxseed polysaccharides (Plate D). Wells C_1 and D_1 contained untreated antibodies. Wells C_2, C_3, D_2, and D_3 contained antibodies incubated with the carbohydrates indicated in the photograph. The outer wells contained 20, 10, 5, 2·5, 1·25, and 0·625 μg of *S. faecalis* polysaccharide in both Plate C and Plate D, respectively.

seed polysaccharide incubated with the antibodies. The outer wells contained decreasing amounts of the antigen (β-D-Gal-BSA). It is noted that around Wells D_1 and D_3 the same type of precipitin patterns were obtained and bands were formed at all concentrations of antigen. However, the hydrolyzate of bacterial polysaccharide yielded precipitin bands (Well D_2) only at the three highest concentrations of antigen. That galactose in the hydrolyzate of the bacterial polymer inhibited the precipitin reaction and therefore it is of the D configuration. The flaxseed polysaccharide contains galactose of the L configuration since this galactose had no effect on the antibody antigen reaction (Well D_3). These results show conclusively that the D or L configurations can be determined by the immunological method and verify previous reports that the bacterial polysaccharide contains D-galactose (Pazur *et al.*, 1971) and flaxseed mucilage contains L-galactose (Anderson, 1933). The configuration was determined in the earlier reports by specific rotation +80 for the D isomer (Isbell & Pigman, 1937) and −79 for the L isomer (Anderson, 1933).

Antibodies specific for α-D-mannose, β-D-rhamnose, *N*-acetyl β-D-glucosamine and β-D-glucuronic acid are available and can be used to determine configuration of these monosaccharides in carbohydrate polymers containing such units. For other monosaccharides, the methods described in this report are readily adaptable for producing the required antibodies and applying immunological analyses. The method is of general applicability for the analyses of polymers containing carbohydrate units and further it is not necessary to purify the monosaccharides under investigation.

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