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IMMUNOCHEMICAL STUDIES ON A POLYSACCHARIDE FROM *MICROCOCCUS LYSODEIKTICUS* CONTAINING GLUCOSE AND *N*-ACETYL-MANNOSAMINURONIC ACID

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SUMMARY Rabbits immunized with *M. lysodeikticus* cells produced antibodies which reacted with a polysaccharide of the organism containing glucose and *N*-acetyl-mannosaminuronic acid.

The specificity of the antigen-antibody reaction was examined by the quantitative precipitation inhibition assay. Phenyl α-D-glucopyranoside was a better inhibitor than the β-anomer, and methyl 2-acetamido-2-deoxy-β-D-mannopyranosiduronate was a better inhibitor than the α-anomer. These results suggest that the antigenic determinants of the polysaccharide contained α-linked D-glucopyranoside and β-linked 2-acetamido-2-deoxy-D-mannopyranosiduronic acid structures.

INTRODUCTION

Polysaccharide containing glucose and *N*-acetyl-mannosaminuronic acid (2-acetamido-2-deoxy-mannuronic acid) was extracted with formamide or trichloroacetic acid from cell walls of *M. lysodeikticus* by Perkins (1963). It was shown to be composed of alternate residues of the two sugars, the glucose molecules being linked at their 6-position (Perkins, 1963a; Jeanloz, 1967). Hase and Matsushima (1970, 1971, and 1972) extracted the polysaccharide from digests of a cell wall preparation of the organisms with lysozyme or lysozyme and L-11 enzyme and purified it by ECTEOLA-cellulose chromatography. Extensive studies with the purified preparations showed that *N*-acetyl-mannosaminuronic acid residues in the polysaccharide were linked at their 4-positions and had the D-configuration, and that the anomeric structures of glucose and *N*-acetyl-mannosaminuronic acid were α- and β- linkages, respectively.

In the present study rabbit antisera capable for precipitating with the polysaccharide were prepared and the structure of its antigenic

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determinant was studied by quantitative precipitation inhibition assay.

MATERIALS AND METHODS

1. *Antigen*

The preparation of polysaccharide containing glucose and *N*-acetyl-mannosaminuronic acid used was the same as that described previously (Hase and Matsushima, 1972).

2. *Antisera*

Two rabbits (219L and 220L) were immunized with formalin-killed cells of *M. lysodeikticus* strain P, and two rabbits (222L and 224L) with heat-killed cells. Suspensions of the cells (4 mg dry cells/ml saline) were injected into an ear vein twice a week in volumes of 0.5, 0.5, 1.0, 1.0, 2.0, 2.0, 3.0, 3.0, 4.0 and 4.0 ml, respectively. Seven days after the last injection, blood was taken by cardiac puncture and sera were prepared by the usual method. The lyophilized cells of *M. lysodeikticus* strain P were generously supplied by Dr. J. T. Park, Tufts University School of Medicine, Boston, Massachusetts, U.S.A.

3. *Serological test*

Quantitative precipitation and quantitative precipitation inhibition were performed using the usual methods (Kabat, 1961). Precipitated protein was determined by the method of Goodman et al. (1968) with the slight modification that the final volume was 2.0 ml. The precipitin reaction in agar was carried out by Ouchterlony's double diffusion technique (Ouchterlony, 1949) with Bacto Special Agar Noble.

4. *Inhibitors*

D-Glucose, kojibiose (*O*-*α*-D-glucopyranosyl-(1→2)-D-glucose), nigerose (*O*-*α*-D-glucopyranosyl-(1→3)-D-glucose), maltose (*O*-*α*-D-glucopyranosyl-(1→4)-D-glucose) and isomaltose (*O*-*α*-D-glucopyranosyl-(1→6)-D-glucose) were the same samples as those described previously (Sakakibara et al., 1972a). Phenyl *α*-D-glucopyranoside, phenyl *β*-D-glucopyranoside, *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), methyl *N*-acetyl-*α*-D-glucosaminide and methyl *N*-acetyl-*β*-D-glucosaminide were obtained from Nakarai Chemicals, Ltd., Kyoto, cellobiose (*O*-*β*-D-glucopyranosyl-(1→4)-D-glucose) from Wako Pure Chemical Industries, Ltd., Osaka and gentiobiose (*O*-*β*-D-glucopyranosyl-(1→6)-D-glucose) from Tokyo Chemical Industry Co. Ltd., Tokyo.

Sodium methyl 2-acetamido-2-deoxy-*α*-D-mannopyranosiduronate was synthesized as follows: methyl 2-acetamido-2-deoxy-*α*-D-mannopyranoside (217 mg) (Beychok et al., 1971) was oxidized in water (40 ml) containing sodium bicarbonate (77.5 mg) by oxygen gas at 75 C for 4 hr in the presence of platinum catalyst (99 mg) (Heyns and Beck, 1957). When the reaction mixture became about pH 4.5, the catalyst was removed by filtration or centrifugation. The clear solution was concentrated to about 0.5 ml and then ethanol (5 ml) was added. The precipitate was dissolved in a small volume of water and reprecipitated with ethanol. The colorless precipitate was washed with ethanol and dried *in vacuo* over P_2O_5 and NaOH. The supernatants were combined, concentrated and subjected to alcohol precipitation. The total yield was 202 mg. The material was very hygroscopic and was not crystallized, but it was shown to be a single component by thin layer chromatography using Silica gel G (Merck) with chloroform-methanol (5 : 10, v/v) as solvent. M.p. 220 C (Sint.); $[\alpha]_D^{20}$ +42.9° (c 0.34, water); i.r. data: ν_{max}^{KBr} 1560 and 1410 cm^{-1} (ionized carboxyl). Anal. Calc. for $C_9H_{14}O_7$ NNa·$1\frac{1}{2}H_2O$: C, 36.24; H, 5.75; N, 4.70. Found: C, 36.05; H, 5.69; N, 4.34.

Sodium methyl 2-acetamido-2-deoxy-*β*-D-mannopyranosiduronate (82.0 mg) was synthesized from methyl 2-acetamido-2-deoxy-*β*-D-mannopyranoside (98.5 mg) (Beychok et al., 1971) in the same way. It was also hygroscopic and was not crystallized, but was shown to be one component by thin layer chromatography. M.p. 235–240 C (decomp.); $[\alpha]_D^{20}$ −70.7° (c 0.48, water); i.r. data: ν_{max}^{KBr} 1560 and 1410 cm^{-1} (ionized carboxyl). Anal. Calc. for $C_9H_{14}O_7NNa\cdot H_2O$: C, 37.37; H, 5.58; N, 4.83. Found: C, 37.71; H, 5.65; N, 4.73.

Hydrolysis of these compounds by treatment with 1 N HCl at 100 C for 30 minutes gave mannosaminuronic acid which was identified with authentic material by paper electrophoresis (Mayer and Westphal, 1968) and paper chromatography using Toyo filter paper, No. 51A and ethyl acetate-pyridine-water-acetic acid (5 : 5 : 3 : 1, v/v) as solvent. Mannosaminuronic acid, used as a standard, was prepared from a polysaccharide of *M. lysodeikticus* containing mannosaminuronic acid, as described by Biely and

Jeanloz (1969).

RESULTS

1. *Precipitin reaction*

Four antisera were tested with the polysaccharide by the gel diffusion technique, as shown in Fig. 1. Serum 219L gave less precipitate than sera 220L, 222L and 224L, but the precipitin lines were completely fused. Preimmune sera did not give any precipitin line. Similar preparations from strains NRLL 384 and B361 of *M. lysodeikticus* which was kindly supplied by Toyo Jyozo Co. also gave precipitin lines which fused with the line of the polysaccharide.

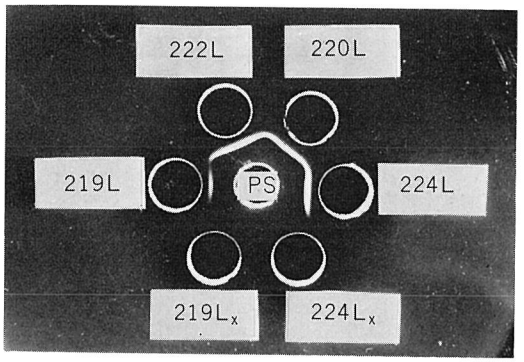

FIGURE 1. *Precipitin reaction in agar of the polysaccharide with rabbit antisera. PS, glucose and N-acetyl-mannosaminuronic acid containing polysaccharide; 219Lx and 224Lx, preimmune sera; 219L, 220L, 222L and 224L, antisera to M. lysodeikticus.*

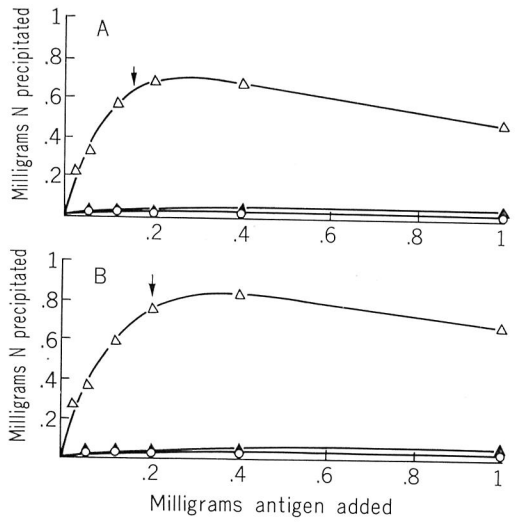

FIGURE 2. *Quantitative precipitin curves of polysaccharides with antisera. A, with antiserum 220L (1 ml); B, with antiserum 222L (1 ml); △, polysaccharide containing glucose and N-acetyl-mannosaminuronic acid; ○, dextran B1397; ▲, K15-antigen of Vibrio parahaemolyticus.*

2. *Quantitative precipitin reaction*

The quantitative precipitin curves obtained with sera 220L and 222L are given in Fig. 2. The arrows in the figure indicate the equivalent points at which subsequent inhibition analyses were carried out. Quantities of antisera which gave 6–8 μg of precipitable antibody nitrogen were used for the inhibition assay. Sera 219L and 224L also gave similar precipitin curves.

3. *Quantitative precipitation inhibition*

The major components of the polysaccharide were found to be glucose and *N*-acetyl-mannosaminuronic acid, so the inhibitory effects of various compounds related to these two sugars were tested. The results obtained with sera 220L and 222L are given in Fig. 3. With both antisera phenyl α-D-glucopyranoside was a stronger inhibitor than the β-anomer and sodium methyl 2-acetamido-2-deoxy-β-D-mannopyranosiduronate was a stronger inhibitor than the α-anomer. Two α-linked glucose disaccharides, maltose and nigerose, were stronger inhibitors than the β-linked disaccharides, cellobiose and gentiobiose. Other α-linked glucose disaccharides, kojibiose and isomaltose, showed the same level of inhibition as the β-linked disaccharides. *N*-acetyl-D-glucosamine and its methyl α- and β- glycosides were also tested and shown to be less inhibitory than D-glucose.

4. *Examination of the cross-reactions with dextran and K-15 antigen of Vibrio parahaemolyticus*

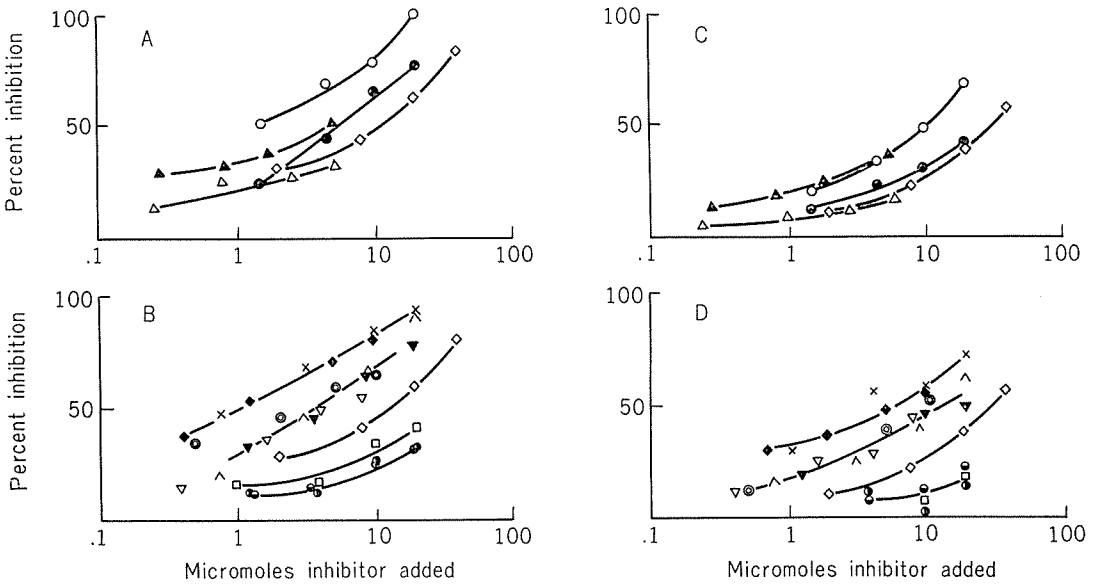

FIGURE 3. *Quantitative inhibition by sugars and sugar-derivatives of the precipitin reaction. A and B, with serum 220L and M. lysodeikticus polysaccharide; C and D, with serum 222L and M. lysodeikticus polysaccharide;* ◇, *D-glucose;* ○, *phenyl α-D-glucopyranoside;* ●, *pheny β-D-glucopyranoside;* △, *sodium methyl 2-acetamido-2-deoxy-α-D-mannopyranosiduronate;* ▲, *sodium methyl 2-acetamido-2-deoxy-β-D-mannopyranosiduronate;* ◎, *kojibiose;* ◆, *nigerose;* ×, *maltose;* ∧, *isomaltose;* ▽, *gentiobiose;* ▼, *cellobiose;* □, *N-acetyl-D-glucosamine;* ◑, *methyl 2-acetamido-2deoxy-α-D-glucopyranoside;* ◒, *methyl 2-acetamido-2-deoxy-β-D-glucopyranoside.*

The polysaccharide contains α-linked glucose and β-linked *N*-acetyl-mannosaminuronic acid, so its cross-reactions were examined with dextran NRRL B1397 (Miyaji et al., 1969) and K-15 antigen of *V. parahaemolyticus*, which contains mannosaminuronic acid (Torii and Kuroda, 1971; Sakakibara et al., 1972). As seen in Fig. 2, dextran and K-15 antigen did not cross-react with anti *M. lysodeikticus* serum.

DISCUSSION

Rolica and Park (1969) showed that an antigen prepared by digestion of *M. lysodeikticus* cell walls with lysozyme precipitated with heterologous rabbit antiserum to *Streptococcus pyogenes* A variant as well as homologous rabbit antiserum to *M. lysodeikticus*. In the heterologous system, the mucopeptide portion was the main antigen, but in the homologous system it was not a significant part of the antigen. The antigenic specificity of the antigen in the homologous system was not identified, as *Staphyloccocus aureus* UDP-muramyl-pentapeptide and mucopeptide were poor inhibitors and glucose was not inhibitory.

In the present study the polysaccharide was prepared by a similar method and shown to consist of mainly glucose and *N*-acetyl-mannosaminuronic acid. It reacted with anti *M. lysodeikticus* serum and the precipitin reaction was strongly inhibited by D-glucose and its derivatives. The precipitin reaction was also strongly inhibited by methyl 2-acetamido-2-deoxy-β-D-mannopyranosiduronate, but *N*-acetyl-D-glucosamine and its methyl α- and β-glycosides were poor inhibitors. These results suggest that D-glucose and *N*-acetyl-D-mannosaminuronic acid, rather than mucopeptide, are responsible for the specificity of the precipitin reaction.

The facts that α-D-glucopyranosyl com-

pounds were stronger inhibitors than the corresponding β-anomeric compounds and that sodium methyl 2-acetamido-2-deoxy-β-D-mannopyranosiduronate was a stronger inhibitor than the α-anomer, suggest that the antigenic determinant of the polysaccharide includes α-linked D-glucopyranose and β-linked 2-acetamido-2-deoxy-D-mannopyranosiduronate residues. This fact is in accordance with the results of structural studies, which showed that the glucose and *N*-acetyl-mannosaminuronic acid residues were bound with α- and β-linkages, respectively (Hase and Matsushima, 1972).

Maltose and nigerose were the strongest inhibitors among those tested, but it is uncertain whether the antigenic structure contains α-linked D-glucose disaccharide residues or not.

The lack of a cross-reaction with dextran and K-15 antigen may be because the common consituent, glucose or *N*-acetyl-mannosaminuronic acid, is linked to different constituents of the antigens.

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