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Linkage Analysis in a Heptasaccharide from Prosopis juliflora by Methylation and Periodate Oxidation Methods

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Abstract: *Prosopis juliflora* (Sw.) DC. commonly known as mesquite, vilayti babul, vilayti kikar belongs to family leguminosae sub family (mimosoidae). It's a thorny ever-green to semi-evergreen fast growing tree capable of growing in arid and semi-arid regions and attaining heights up to 10m. A polysaccharide was isolated from *Prosopis juliflora* seed and purified. Purified *juliflora* polysaccharide has been partially hydrolysed to get structural oligosaccharides. An oligosaccharide has been isolated from the hydrolysed product of *Prosopis juliflora* seed polysaccharide. On complete hydrolysis it furnished two monosugars, galactose and mannose in the molar ratio of 3:4. On methylation and periodate oxidation study it indicated the presence of (1→4) and (1→6) linkages among structural monosaccharide units.

Keywords: Prosopis, Heptasaccharide, Methylation, Periodate Oxidation, D- Galactose and D- Mannose.

Introduction

P. juliflora commonly known as mesquite, vilayti babul, vilayti kikar belongs to family leguminosae sub family (mimosoidae). *P. juliflora* is often quoted as native to the Caribbean where it is found often in coastal areas, but several authors have suggested that it was introduced possibly with the arrival of the first human settlers from Venezuela (circa 0-1000 AD). It is a large shrub to a small evergreen tree. Usually tree attains a height of 9-12m and a girth of 90cm. Under favorable conditions the tree attains a height of 18m. If repeatedly coppiced from ground level it maintains as a multistemmed shrub of 3-4m in height [1]. Wood is chunked or chipped and sold as a special wood or charcoal for barbeques in the USA and in Australia and found to have strong resistance, considerably stronger than that of teak (*Tectona grandis*), particularly when dry [2]. Bark of the tree exudes gum that forms adhesive mucilage and can be used as an emulsifying agent. From ethno-botanic viewpoint, it shows medicinal properties in particular for treating pharyngeal ailments, dysentery and to strength teeth. Fruit is indehiscent pods that are of high nutritive value as a fodder and food crop. Leaves possess many antibacterial and medicinal properties.

There are number of reports on chemical constituents isolated from the different parts of *Prosopis juliflora* viz. root, stem, leaf, bark, pods and flowers which have been reported in literature [3]. *P. julifora* seed gum solution in water is used as viscosity improver for fracturing geological formations, stable to bacteria, with low residue content and high viscosity was reported [4]. The use of polysaccharide from endosperm of *P.*

juliflora as a thickening agent was reported [5]. Detailed knowledge of the chemical nature of this type of polysaccharide is of interest to the plant chemist for its further utilization.

P.juliflora polysaccharide produces one hepta-, one octa-, and one nonasaccharide on partial hydrolysis. In the present paper only methylation and periodate analysis results of heptasaccharide has been discussed. The present paper is a part of complete structure elucidation of *P.juliflora* polysaccharide.

Experimental and General Methods of analysis

Solutions were concentrated at or below 40^0C in a rotary evaporator under reduced pressure. All melting points are uncorrected. Optical rotation was determined on Autopol-II, automatic polarimeter (Rudolph Research, Flanders, New Jersey) at 589nm, D-lines of sodium. Paper chromatography was carried on Whatmann 1 and 3mm filter paper sheets using the following solvent systems; n-Butanol: Ethanol: Water (4:1:5, S_1) upper layer; n-Butanol: Pyridine: Water (6:4:3, S_2) and Ethyl acetate: acetic acid: n-Butanol: Water (4:3:2:2, S_3). Detection was effected with acetonical silver nitrate (R_1) and Aniline phthalate spray (R_2). Detection with acetonical silver nitrate (solution of $AgNO_3$, water and acetone) was done by treatment with following agents: (a) To silver nitrate solution (12.5 g in 10ml water), one litre acetone was added with continuous shaking. Distilled water was added dropwise with stirring until the white precipitate completely dissolved to form a clear solution. (b) Sodium hydroxide (20g) was dissolved in 400ml of ethanol. (c) Aqueous ammonia solution.

The dried chromatograms were dipped and passed through reagent solution (a) for about 5min, dried at room temperature and passed through reagent (b); when the dark brown spots were visualized the paper was dipped in reagent (c) for some time with shaking (5-10min). Finally the chromatograms were washed with water and dried in air [6].

Gas liquid chromatography of the sugar mixtures was carried out on a Shimadzu Gas Chromatograph GC-9A fitted with flame ionization detector (FID). The samples were analyzed in the form of their alditol acetates on ECNSS-M (3%) on Gas chrom Q (100-200mesh) packed into 5'×1/8" stainless steel column under the following operating conditions. (Column temperature 170^oC, nitrogen flow rate 35-40ml/min (C_1).

Isolation of polysaccharide

The seeds of *Prosopis juliflora* were collected from Jodhpur, Rajasthan. The moisture content of seeds was found to be 11%. The seeds were air dried in shade to moisture content of 0.09%. Endosperm (20 g) was isolated by removing seed coat and germ by impact grinding using high speed domestic grinder. Seed coat was soaked in cold water for 3 hrs and endosperm was isolated from swollen seed coat by separating the seed coat with the help of forceps which was stirred vigorously in distilled water (1000 ml) for 5h at room temperature and centrifuged to remove water insoluble impurities. The supernatant solution was poured into three times its volume of ethanol with constant stirring. The polysaccharide was precipitated out in the form of a fluffy precipitate. The precipitate was again dissolved in water and added to ethanol. Precipitate was treated successively with dry solvent ether and acetone. It was filtered under vacuum and dried in vacuum desiccator at room temperature. The polysaccharide (18.0g) so obtained was deionised by passing the aqueous solution successively through the columns of freshly regenerated cation [Dowex-50W-X8] and anion [Seralite-SRA-400] exchange resins. The columns were washed with distilled water until the washings showed a negative Molisch test for carbohydrates. The combined eluents were concentrated to small volume [1/4th] and subjected to further purification by dialysis. In the process, the concentrated product was transferred into a cellophane bag and dialyzed for 72h in running water. The dialyzed product was concentrated and re-precipitated with a large volume of ethanol to obtain finally the pure polysaccharide. It was kept overnight, alcohol was decanted off, and the precipitated polysaccharide was dried by treating with solvent ether, acetone and absolute ethanol. It was filtered and lyophilised at -40^oC to obtain finally the pure polysaccharide in the form of a white amorphous powder (16.1g).

Partial hydrolysis

Polysaccharide (5.0g) was heated with sulphuric acid (0.05 N, 100 ml) on steam bath at 100^oC for 3 h. The hydrolysate was cooled, neutralized with saturated solution of barium carbonate till neutral pH and filtered. The combined filtrate was concentrated to syrup (4.73g). The mixture of oligosaccharides and monosaccharide was resolved into its components by preparative chromatography on whatmann No.3 mm filter paper sheets

using solvent system S_2. The strips corresponding to individual oligosaccharides were eluted with water, elutes were concentrated separately, to obtain the three oligosaccharides. Homogeneity of the oligosaccharides was checked by paper chromatography in solvent system S_1, S_2, S_3 using R_1 and R_2 as spray reagent.

Methylation of oligosaccharide

Oligosaccharide (0.0283 g) was methylated completely according to the method of Hakomori [7] using sodium hydride-dimethyl sulphoxide followed by Purdie [8]. Each methylated oligosaccharide was recovered by chloroform extraction. After evaporation of the chloroform extracts to dryness, the residues were hydrolyzed with formic acid (90%, 10ml) for 1h on steam bath at 100^{o}C, the solutions evaporated and treated with aqueous sulphuric acid (0.13 M, 15ml) for 18h on a steam bath. The partially methylated compound was subjected to Purdie's methylation by dissolving it in methyl iodide (5 ml) with stirring under inert atmosphere and silver oxide (0.50 g) was added periodically in 4 hr and reaction was allowed for 6 hr. This process was repeated two times on the successive days. The methylated oligosaccharide processed further as above, and obtained in the syrup form with the yield (0.0123 g).

Preparation of alditol acetates

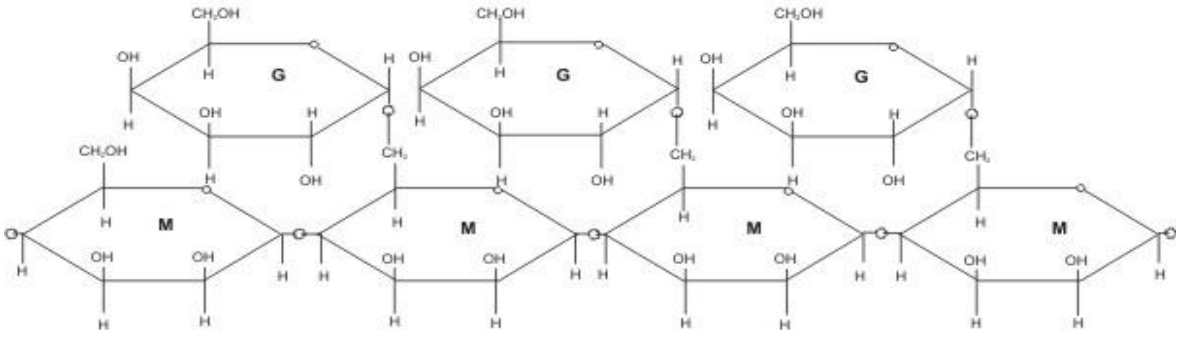
Alditol acetates of the hydrolyzed material were prepared by the method as described [9]. Sodium borohydride (0.020g) was added to hydrolyzates, and the mixture was kept for 18h at room temperature. The mixture was neutralized by slow addition of dilute acetic acid (6ml), and concentrated to dryness in vacuum rotator at 40^{o}C. Sodium was removed by passing it through cation exchange resin (Dowex-50 W-X8). Boric acid was removed by co distillations, in the vacuum rotator with methanol (3 × 5ml). The residue was treated with redistilled acetic anhydride and pyridine, 1:1 (4ml) and refluxed for 6h. Toluene (6ml), which gave an azeotrope with acetic anhydride, was added and the mixture was distilled as above, until the rate of distillation decreases. A new portion of toluene (6ml) was added and the solution was concentrated to dryness. It was dissolved in water (10ml) and the acetylated sugars separated by shaking with dichloromethane (4 × 25ml). Traces of water present in dichloromethane were removed by adding anhydrous sodium sulphate followed by filtration and washing with dichloromethane before concentration.

Periodate oxidation

To a solution of oligosaccharide (0.0510 g in 25 ml for each) in water an aqueous solution of sodium metaperiodate (0.2 g in 50 ml) was added and the volume of the resultant solution was made upto 100ml. A blank solution of sodium metaperiodate (0.2 g in 100 ml) was also prepared. These were kept in dark at room temperature (25^{o}C) for 192 h. To determine periodate consumed, an aliquot (5 ml) of the periodate reaction mixture was added to a solution containing distilled water (20 ml), potassium iodide (20%, 2 ml) and sulphuric acid (0.5N, 3 ml). The liberated iodine was immediately titrated with 0.1N sodium thiosulphate solution using starch as an indicator [10-12].

Liberation of formic acid was determined by the methods reported earlier [11, 13 -15]. To an aliquot (5 ml) of the periodate reaction mixture was added acid free ethylene glycol (0.5 ml), followed by an excess of potassium iodide (20%, 5 ml) after 10min. To the above solution an excess of 0.01 N sodium thiosulphate was back titrated with 0.01 N iodine solution using starch as an indicator. A blank solution was titrated concurrently.

Fig.1: Structure of heptasaccharide showing 1→4 and 1→6 linkages



M: β-D-Mannose; G: α-D-Galactose

Result and discussion

The polysaccharide was isolated from the endosperm of seeds in 81% yield as detailed in experimental section.

Partial hydrolysis

Prosopis juliflora seed endosperm polysaccharide upon hydrolysis with dilute sulphuric acid (0.05N, 3h) furnished a mixture of oligosaccharides along with monosaccharides. Preliminary paper chromatographic examination of the hydrolyzates revealed the presence of three oligosaccharides along with 2 monosaccharides, D-galactose and D-mannose. The Rgal values of oligosaccharides were 0.31, 0.19 and 0.11 respectively in the solvent system S_2. From this mixture, the oligosaccharides were separated by preparative chromatography on whatmann No.3 mm sheets and each oligosaccharide eluted separately and the elutes combined to isolate pure polysaccharide. The homogeneity of the oligosaccharides was checked by paper chromatography using organic solvent systems S_1, S_2 and S_3 and spray reagents R_1 and R_2. The degree of polymerization of three oligosaccharides corresponds to one hepta, one octa and one nonasaccharides. The nature and sequence of glycosidic linkages was determined by methylation analysis, periodate oxidation of the oligosaccharide. Linkage sequences in other two oligosaccharides will be discussed in future communication.

Linkage analysis of heptasaccharide

This heptasaccharide [α] + 1120 (c 0.05%, H2O), m.p. 200-2020C (d), was found homogenous and gave single spot [Rgal 0.31] using organic solvent S_2 and spray reagents R_1 and R_2 upon chromatographic examination. Upon complete acid hydrolysis, it gave D- galactose and D- mannose on paper chromatogram in solvent systems S_1, S_2 and S_3. The alditol acetates of the hydrolyzates of heptasaccharide on GLC analysis under conditions C-1 showed two peaks corresponding to D-galactose and D- mannose in the molar ratio of 3:4.

The heptasaccharide was completely methylated by Hakomori method [7] followed by Purdie [8]. Complete methylation was confirmed by IR spectrum of the methylated heptasaccharide which showed complete absence of –OH band (3590-3225cm-1). It was hydrolyzed and transformed into its alditol acetates according to the method of Jansson et al. [9]. GLC of the resulting alditol acetate under conditions C1, furnished 2, 3, 4, 6- tetra-O-methyl-D-galactose, 2, 3-di-O-methyl-D-mannose, 2, 3, 4, 6-tetra-O-methyl-D-mannose in the molar ratio of 3:3:1, respectively.

On the basis of methylation study it was found that 2, 3, 4, 6 - tetra-O-methyl mannose (1mole) and 2, 3, 4, 6 - tetra-O-methyl- galactose (3moles) showed four non reducing end chain groups. Occurrence of 2, 3- di-O-methyl-mannose (3moles) indicated that these mannose residues are joined through O-4 and O-6 and branched structure of oligosaccharide in which non-reducing galactose units is attached by $1\rightarrow6$ linkages to $1\rightarrow4$ linked mannose units of the backbone.

Evidence supporting the presence of $1\rightarrow4$ and $1\rightarrow6$ linkages in the framework of heptasaccharide has been obtained from the results of periodate oxidation [10-15]. The heptasaccharide when subjected to oxidation with sodium metaperiodate, consumed 1.69 mol of periodates per anhydrohexose unit and released 0.69 mol of formic acid per anhydrohexose unit. As the heptasaccharide structure consists of 7 sugar residues, the above results can be interpreted in terms of the consumption of 11.83 mol of periodate with simultaneous liberation of 4.83 mol of formic acid by the repeating unit of oligosaccharide. Thus, periodate oxidation data of oligosaccharide as calculated on the basis of proposed structure and obtained experimentally are found to be in close agreement with each other.

Conclusion

From the results of methylation and periodate oxidation study of the oligosaccharide obtained from *P. juliflora* seed polysaccharide, it can be concluded that it contains two type of linkage i.e. $1\rightarrow4$ and $1\rightarrow6$ in its framework. The linkage sequence also indicated that in the heptasaccharide, non reducing galactose units is attached by $(1\rightarrow6)$ linkages to $(1\rightarrow4)$ linked mannose units of the backbone. On the basis of the results of methylation and periodate oxidation study, structure represented in fig.1 justify the linkage position in *Prosopis* polysaccharide.

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