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Studies on plant gums. Role of calcium in polysaccharide-protein interaction in the neem (*Azadirachta indica*) gum

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Abstract. The partial removal of tightly bound Ca^{2+} from dialysed neem (*Azadirachta indica*) gum, resulted in the release of a basic protein from a highly anionic polysaccharide-protein complex as evidenced by chromatographic studies on TEAE-cellulose. Complete removal of Ca^{2+} caused, in addition, the release of a minor heteropolysaccharide which was found in association with the basic protein. These processes were reversed on the addition of Ca^{2+}. The gum, in addition, contained a protein-rich component accounting for 35% protein and 7·5% total carbohydrate. This component behaved as a distinct entity during ion-exchange chromatography of the native gum solutions, or which were either partially or completely depleted of bound Ca^{2+}.

Keywords. Neem; *Azadirachta indica* gum; calcium; basic protein; polysaccharide-protein interactions

Introduction

Neem (*Azadirachta indica*) exudate gum which belongs to the family of galactan gums (Aspinal, 1969) has unusual structural features in that, it contains appreciable amounts of D-glucosamine and proteins (Ushalakshmi and Pattabiraman, 1967) unlike other plant gums. The present study is aimed at providing some insight into the nature of interaction between proteins and heteropolysaccharides in neem gum. Evidence is provided to show that Ca^{2+} plays a role in such an interaction.

Materials and methods

Materials

Neem gum samples were hand-picked and stored at room temperature. The powdered gum was dissolved in water, clarified by charcoal treatment and centrifuged at 12000 × g for 20 min. The clear supernatant was extensively dialysed against water and stored at – 5o C until use. A typical preparation contained

40 mg of carbohydrate and 22 mg of protein per ml. This is designated as native gum solution. TEAE-cellulose was purchased from Bio-Rad Laboratories, Richmond, CA, USA. Sephadex G–15 and G–100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were of the analytical grade.

Methods

General methods: Total carbohydrate was estimated by the method of Dubois *et al.* (1956) with galactose as standard. Protein was estimated by the method of Lowry *et al·* (1951) using bovine serum albumin as standard. Uronic acid was determined by carbazole method (Bitter and Muir, 1962) using glucuronic acid as standard· Calcium was estimated by the method of Trinder (1960). For the quantitative estimation of basic amino acids, the property of binding of these amino acids to Sephadex (Gelotte, 1960) was used. The neutralised hydrolysate (600 μg total amino acid) was applied on a column of Sephadex G–15 (bed volume 18 ml, 0·9 × 28 cm) and washed with 36 ml of water. The basic amino acids were eluted with 0·1 M NaCl. Fractions of 1·5 ml were collected. Fractions 8–10 were pooled and assayed by the ninhydrin method (Moore and Stein, 1948) with leucine as standard.

Removal from and reconstitution of Ca^{2+} into the gum: Weakly bound calcium was precipitated by mixing equal volumes of the gum solution and 0·1 M phosphate buffer, pH 7·0. After standing for 15 min, the solution was centrifuged and the supernatant solution was dialysed against 50 vol. of water for 16 h with a change of water after 8 h. This solution is designated as phosphate treated (stage I) gum. Tightly bound calcium was partially removed by heating the above solution with equal volume of phosphate buffer at 95°C for 10 min and by processing as described above. This sample is designated as phosphate treated (stage II) gum· Complete removal of bound calcium was effected by dialysing twice, the stage II gum solution, against 50 vol. of 0·01 M EDTA for 6 h, followed by dialysis against water. This sample is designated as metal-free (stage III) gum. Stage II and stage III gums were also prepared from native gum solution directly. Irrespective of the method of preparation, the modified gum solutions behaved similarly during chromatographic studies. Reconstitution with calcium was performed by mixing EDTA-treated gum solution with 9 mg of $CaCl_2 . 2H_2O$. After 2 h, the solution was dialysed against 600 vol. of water for 18 h with a change of water after 9 h.

Results

A typical sample of native gum solution contained 12·4 mg of calcium per g dry wt. At stage I, 54% of the metal ion was removed. An additional 21% of calcium was removed at stage II and the residual calcium was completely removed by EDTA treatment. There was no loss of carbohydrate or protein during the different stages of calcium removal.

The chromatographic pattern of the native gum solution on TEAE-cellulose is Shown in figure l. A small fraction (peak A) which was not retained on the

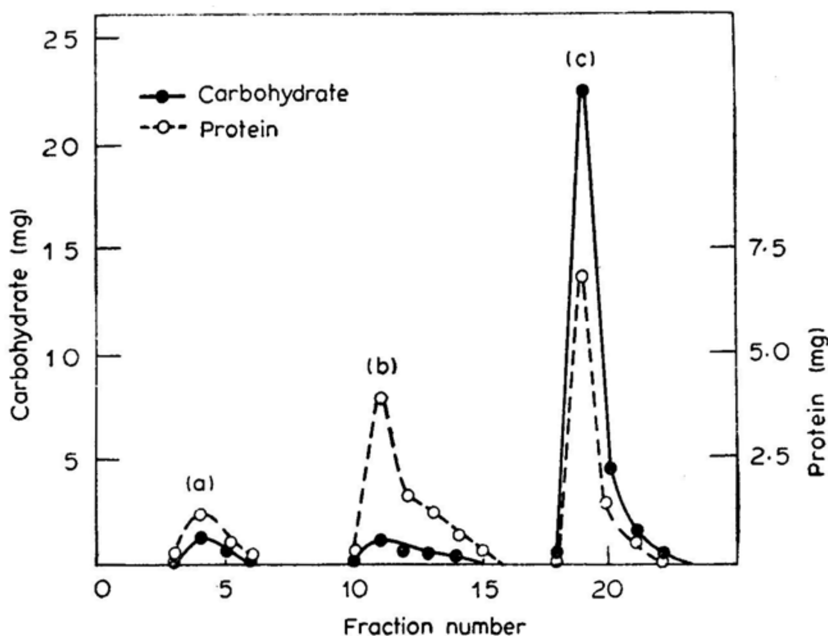

Figure 1. Chromatography of the native gum on TEAE-cellulose.
One ml gum solution (40 mg carbohydrate and 22 mg protein) was processed Column size 0·9 × 39 cm. Bed. volume 25 ml. Flow rate 20ml/h. Fraction volume 5·0 ml. Successive elution with 50 ml of 0·01 M phosphate buffer pH 7·0, 0·1 M phosphate buffer pH 7·0 and 50 ml of 0·1 M phosphate pH 5·0 containing 1 M NaCl.

column and which was eluted with the equilibration buffer accounted for 4·3% of carbohydrate and 7·5% of protein in the gum. A protein-rich fraction (protein: carbohydrate ratio, 2·7) was eluted with 0·1 M phosphate buffer pH 7·0 (peak B) It accounted for 7·6% of carbohydrate and 36% of protein of the native gum. The bulk of the carbohydrate (74%) and the remaining protein, which was tightly bound to the column, was eluted with 0·1 M phosphate pH 5·0 containing 1 M NaCl (peak C). The stage I gum and also gum solution, heat-treated at 95° C for 10 min showed similar elution profiles on chromatography (data not given).

The chromatographic pattern of the metal ion-free gum on TEAE-cellulose is shown in figure 2. The middle fraction was similar in composition to the corresponding fraction separated from native gum. A major difference between the elution profiles of the native and metal ion-free gums is with respect to fraction A. In the case of the metal ion-free gum, a considerable amount of protein (31%) and carbohydrate (20%) came out with the equilibration buffer. There was a corresponding decrease in the protein and carbohydrate content of fraction C as compared to native gum (compare figure 2 with figure 1). The data suggest that complete removal of calcium results in the release of a fraction from the highly anionic complex (peak C, figure 1). This released fraction does not bind to TEAE-cellulose and hence comes in the ‘position of peak A upon TEAE-cellulose chromatography (peak A, figure 2). This fraction was made up of more than one component as indicated by chromatographic studies of stage II treated on TEAE-cellulose. The results are shown in figure 3. It can be surmised that partial

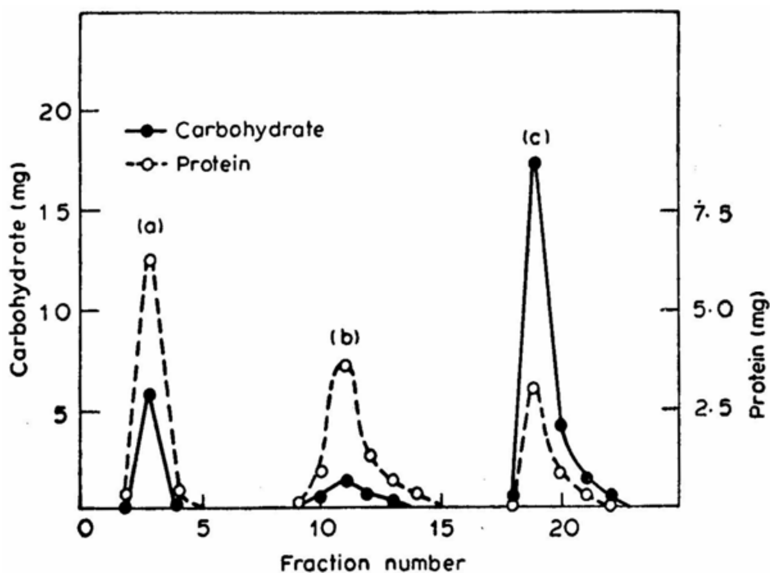

Figure 2. Chromatography of the metal ion-free gum on TEAE-cellulose. Two ml gum solution (40mg carbohydrate and 22·5 mg protein) was processed. Details are as described in figure 1.

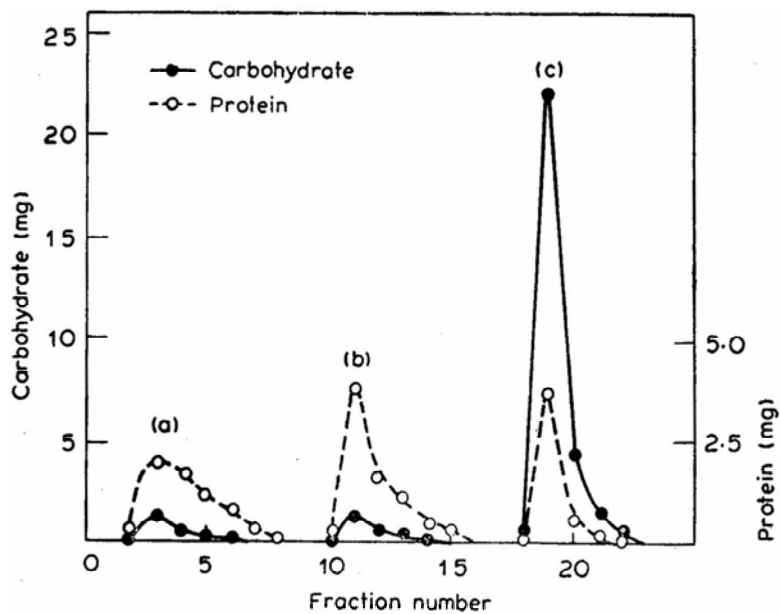

Figure 3. Chromatography of the stage II gum on TEAE-cellulose.
Two ml gum solution (39·5mg carbohydrate and 21·9 mg protein) was processed. Details are as described in figure 1.

removal of tightly bound calcium released a protein-rich component from the anionic complex (peak C, figure 1), whereas complete removal of metal, resulted in the further release of a heteropolysaccharide unit that associated with the released protein and was eluted with the equilibration buffer. The middle fraction was similar in composition to corresponding fractions obtained from native and metal ion-free gum solutions. The carbohydrate and protein contents of the different gum fractions are shown in table 1.

Table 1. Carbohydrate and protein composition of neem gum fractions obtained by TEAE-cellulose chromatography.

Fractions	Carbo-hydrate (mg)	% of total	Protein (mg)	% of total
Native gum	40·0	100	22·0	100
Stage II gum	40·0	100	22·5	100
Stage III gum	39·5	100	21·9	100
Fraction A—native gum	1·7	4	1·6	7
Fraction A—stage II gum	2·3	6	6·7	30
Fraction A—stage III gum	6·2	16	6·9	31
Fraction B—native gum	3·0	8	8·0	36
Fraction B—stage II gum	2·9	7	7·6	35
Fraction B—stage III gum	2·8	7	7·5	34
Fraction C—native gum	29·6	74	9·2	42
Fraction C—stage II gum	29·1	73	4·8	22
Fraction C—stage III gum	23·8	60	4·5	21

Stage II—Native gum treated with 0·1 M phosphate buffer pH 7·0 at 95° for 10 min.
Stage III—Native gum treated with 0·01 M EDTA for 6 h, followed by dialysis.
Fractions A, B, C represent material within peaks A, B, C from TEAE-cellulose chromatography of the native gum, stage II gum and stage III gum.

The released protein fraction was found to be basic in nature. The total basic amino acids in this fraction was found to be 52% compared to 39% in the native gum based on sephadex sorption data (quantitative estimation). The elution profile of fraction A of stage II gum on Sephadex G–100, with water as eluant, is shown in figure 4. Retardation of the bulk of the protein is evident. It is well known that basic proteins show anomalous elution behaviour on cross-linked dextrans due to ionic interactions (Glazer and Wellner 1962). Further studies to isolate and characterise this basic protein fraction are in progress. Glucuronic acid contents

of fractions A, B and C from the metal ion-free gum and the corresponding fractions from the native gum were estimated. It was found that glucuronic acid content in the individual fractions were not significantly different from that of the native gum (20% of total carbohydrate). This would suggest that the failure of the minor heteropolysaccharide released, on removal of the metal to bind to TEAE-cellulose, is not due to the absence of glucuronic acid. Presumably this heteropolysaccharide complexes with the basic protein resulting in the neutralisation of its negative charge.

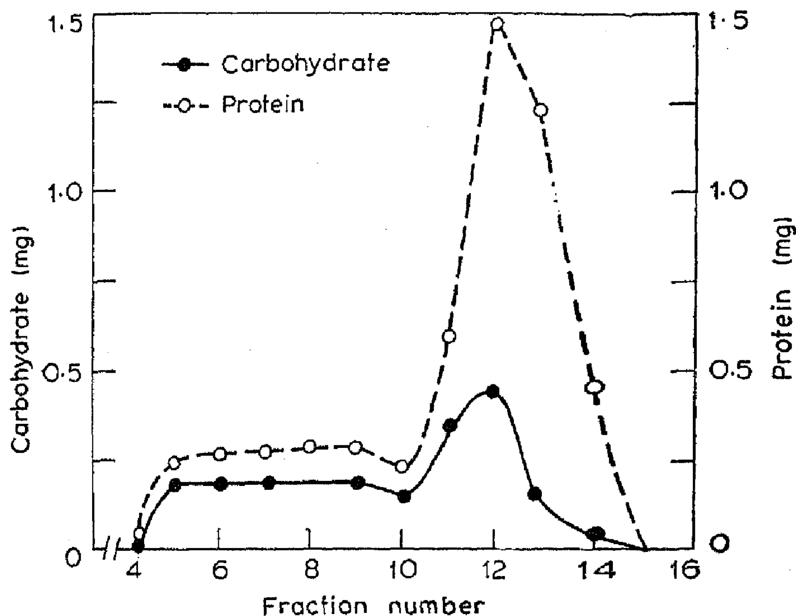

Figure 4. Chromatography of TEAE-cellulose 'fraction A' of stage II gum on Sephadex G–100

One ml solution (2·3 mg carbohydrate and 6·7 mg protein) was processed Column size 0·9 × 59 cm. Bed volume 38 ml. The column was eluted with water.

Further evidence for these observations are provided by gel chromatographic studies on Sephadex G–100. The elution profile of the stage II treated gum with water as eluant is shown in figure 5. A protein fraction (fractions 14, 15), eluted. after the void volume (13 ml), approximately corresponds to the protein peak in the elution profile of fraction A of the stage II gum on Sephadex G–100 (figure 4). The elution profile indicates the heterogeneity of the gum. The elution patterns of stage II gum and stage III gum on Sephadex G–100 with 0·05 M NaCl as eluant is shown in figure 6. With the stage II gum, suppression of ionic interaction between the basic protein and cross-linked dextran is not complete as indicated by the shoulder. However, in the case of the metal ion-free gum, no trailing of protein can be noticed. This could be explained due to the decrease in the basicity of the protein due to complexing with the uronic acid containing minor heteropolysaccharide. The native gum also showed a similar profile with 0·05 M Nacl as eluant.

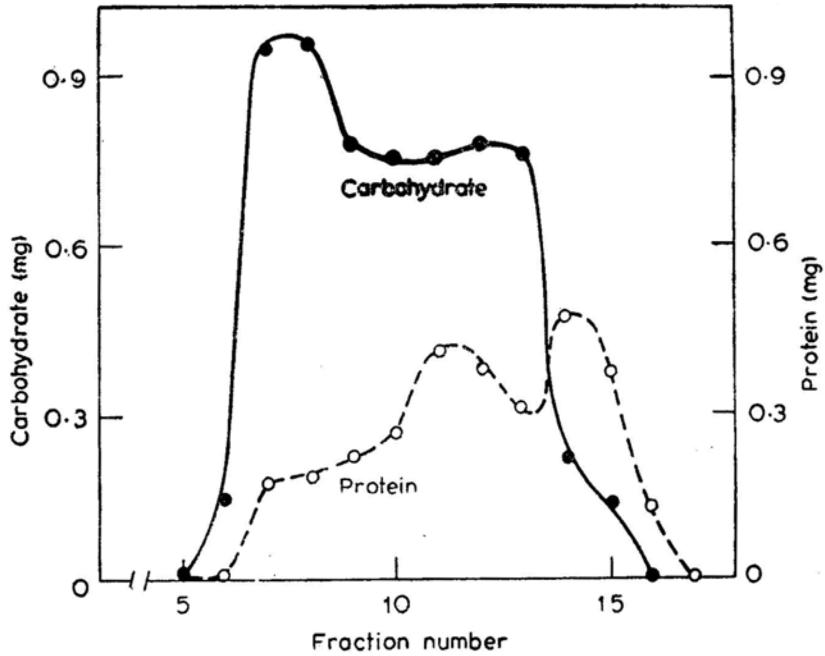

Figure 5. Chromatography of stage II gum on Sephadex G-100. The Gum solution (0·2 ml) was processed. Other details are as described in figure 4.

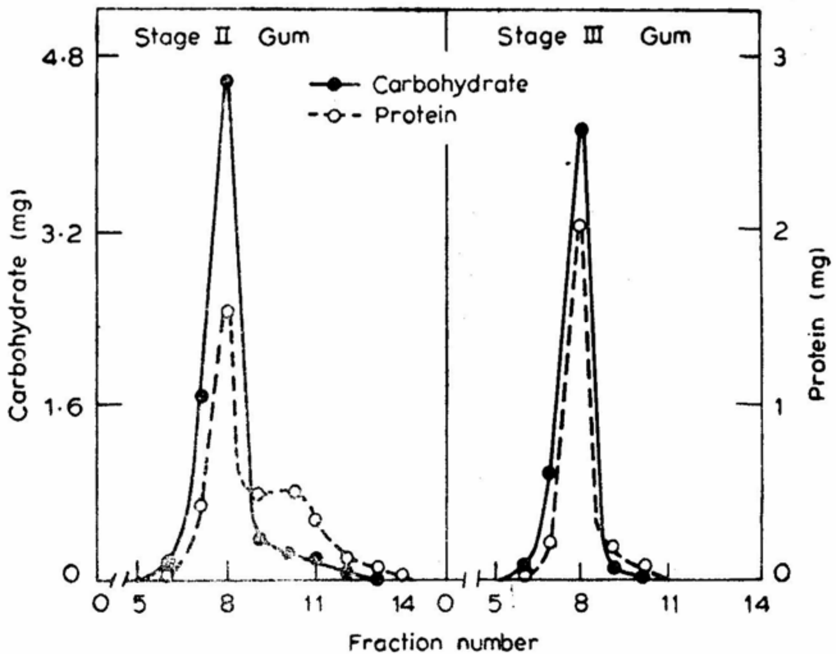

Figure 6. Chromatography of stage II gum and stage III gum on Sephadex G-100 The gum solutions (0·2 ml) each were processed. NaCl (0·05 M) was used for elution. Other details are as described in figure 4.

The metal ion-free gum was reconstituted with calcium and was subjected to chromatography on TEAE-cellulose. The elution profile obtained was similar to that of the native gum (figure 1).

Discussion

Satya Narayan and Pattabiraman (1971) provided evidence for the heterogeneity of neem gum and for the presence of a protein-rich component in this gum· The present studies confirm this finding. It was observed that this protein-rich fraction with a protein carbohydrate ratio of 2·6:1 was bound to TEAE-cellulose and was eluted with 0·1 M phosphate buffer pH 7·0 during chromatography of the native gum and after partial and complete removal of the metal (peak B) The data show that Ca^{2+} does not play a role in the interaction of this fraction with the remaining components of the gum. However, Ca^{2+} appears to play a decisive role in polysaccharide protein interaction with respect to the other components (peaks A and C) in neem gum. Partial removal of tightly bound Ca^{2+} resulted in the release of a basic protein as evidenced by chromatographic studies on TEAE-cellulose and Sephadex G–100 and by the amino acid composition of the released fraction. Complete removal of Ca^{2+} caused further release of a minor heteropolysaccharide unit which tends to associate with the basic protein. The dissociation and reassociation reactions due to step-wise Ca^{2+} removal were found to be reversible as indicated by chromatographic studies after addition of Ca^{2+}. The present studies, thus, provide evidence for the presence of at least two protein-rich components and two polysaccharide components in neem gum. Further studies are needed to isolate and characterise the individual components in neem gum.

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