CHAPTER - III

ISOLATION AND PURIFICATION OF THE POLYSACCHARIDE OCCURING IN THE SEEDS OF SESBANIA GRANDIFLORA

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The matured Sesbania grandiflora seeds were powdered in a low speed grinder. Then the endosperm and the seed coat broken off from the cotyledons. The husk was removed and fine powder of dried seeds was obtained (100 g). This seed powder was soxhlet extracted with petroleum ether ($80^{o}C$) for 15 hours on a boiling water bath. The oil content was removed (10%). The defatted material was soaked in water (1.5 litre) and left overnight. The swollen material was then heated at $50^{o}C$ for 12 hours with constant stirring and mixed with additional quantity of water. It was then filtered through a muslin cloth. The filtrate was centrifuged at 3000 r.p.m. for 30 minutes and the supernatent solution was decanted. It was then acidified with glacial acetic acid. The turbid solution so obtained was again centrifuged at 3000 r.p.m. for 30 minutes and finally filtered through Kiesulghur bed to furnish a clear solution. This solution was concentrated to 1 litre under reduced pressure at $50^{o}C$, then was poured in a thin stream into large excess of distilled ethanol (4 litre) with constant stirring and crude polysaccharide in the form of creamy white precipitate was furnished. After decantation of aqueous ethanol, the precipitate was treated with acetone alcohol until the creamy white precipitate became granular in nature (yield 10.8 g).

PURIFICATION OF CRUDE POLYSACCHARIDE :

1) Cation Exchange Treatment :

In this process, the aqueous solution of the compound (10 g in 800 ml. water) was passed through the column of freshy regenerated cation exchange resin [Amberlite IR - 120 (H^+)]. The column was washed with water until the effluent showed a negative test for carbohydrate (phenol sulphuric acid test). The total 2.5 litre solution was obtained was concentrated to 500 ml under reduced pressure at 50°C. This solution was then dialysed.

2) Dialysis :

500 ml of above solution was dialysed through a cellophane paper against running distilled water at room temperature. The dialysed solution was again concentrated to 250 ml.

3) Copper Complex Formation :

Preparation of Fehling's A Solution :

6.928 g. of $CuSO_4$ was dissolved in minimum quantity of distilled water and diluted to 100 ml with distilled water.

Preparation of Fehling's B Solution :

12 g. of NaOH and 34.6 g. Rochelle salt was dissolved in minimum quantity of water and diluted to 100 ml distilled water.

25 ml Fehling's solution A and 25 ml Fehling's solution B were mixed. The dialysed 250 ml solution was taken in a 1000 ml beaker and kept stirring on magnetic stirrer. The Fehling's solution was added through burette drop by drop to the aqueous solution of polysaccharide. As the addition continued, the viscocity of the solution increased considerably and then decreased as soon as copper complex was precipitated. The precipitate was then separated by centrifugation and the filtrate was again treated with Fehling's solution until there was no precipitate. 40 ml Fehling's solution was required for this process. The blue ppt. of copper complex was washed with distilled water till upper solution of the ppt became colourless. The blue copper complex was taken in 250 ml distilled water and 10 ml cold 1N HCl was added with constant stirring. The colourless syrup was formed, which was slowly added to 1 litre distilled ethanol. The white precipitate of polysaccharide separated out was filtered, washed with ethanol and dried under vacuum to get polysaccharide (3.5 g).

4) Purification by Ion Exchange Resin :

The dried polysaccharide obtained from copper complex process was dissolved in the minimum quantity of water and deionised by passing through the columns of freshly regenerated cation exchange resin [Amberlite IR - 120 (H^+)] and anion exchange resin [Dowex 3(OH^-)] successively.

Regeneration of Cation Exchange Resin [Amberlite IR - 120(H^+)] :

40 g of cation exchange resin was taken in a beaker and washed with distilled water 4-5 times by decantation. Then about 100 ml 2 N HCl was added and the same solution was kept overnight. Then the column was packed and resin was washed in the column with distilled water till the effluent showed pH = 4.5.

Regeneration of Anion Exchange Resin [Dowex 3(OH^-)] :

40 g of anion exchange resin was taken in a beaker and washed with distilled water 4.5 times by decantation. Then about 100 ml 2N NaOH was added and the same solution was kept overnight. Then the column was packed with the resin and same was washed in the column with distilled water till the effluent showed pH = 7.

After passing the polysaccharide solution through cation exchange resin the pH of the effluent was reduced to 1.9. This solution was further passed through anion exchange resin when the pH was increased to 6.2. Both columns were washed with about 1 litre distilled water till the total effluent was about 3 litres in volume. It was then concentrated to 250 ml under reduced pressure at 50°C. This solution was then added to 1 litre ethyl alcohol to get a white precipitate of polysaccharide. It was filtered on suction pump and dewatered by solvent exchange with acetone followed by absolute ethanol

and finally dried in a vacuum descicator at room temperature to furnish white fibrous powder of the purified and fractionated polysaccharide (3.1 g).

Preliminary Analysis of Sesbania grandiflora Seed Polysaccharide :

Pure polysaccharide $[\alpha]_D^{28}$ + 50° (C 0.5% water) was free from starch as indicated by the absence of blue colour with iodine solution and reduces Fehling's solution or Tollen's reagent. The usual tests showed the absence of nitrogen, sulphur, halogens and methoxy groups. The polysaccharide dissolves slowly in distilled water to form clear solution almost neutral in character (pH = 6.7). The homogeneity of Sesbania grandiflora seed polysaccharide has been established by paper electrophoresis.

Paper Electrophoresis :

Electrophoresis is a device where the molecules migrate under the influence of an applied electric field which has found wide applications for judging the homogeneity of a polysaccharide. 1% solution of the purified and fractional polysaccharide in 0.05 ml sodium tetraborate decahydrate buffer (pH = 9.2) was examined on Laboratorium Felzerelsk model DE 201 apparatus, using Whatman no. 1 mm filter paper sheet. After spraying with staining reagent the electrophoretic pattern showed only single spot thereby establishing the homogeneity of the sample. (Fig.3.1).

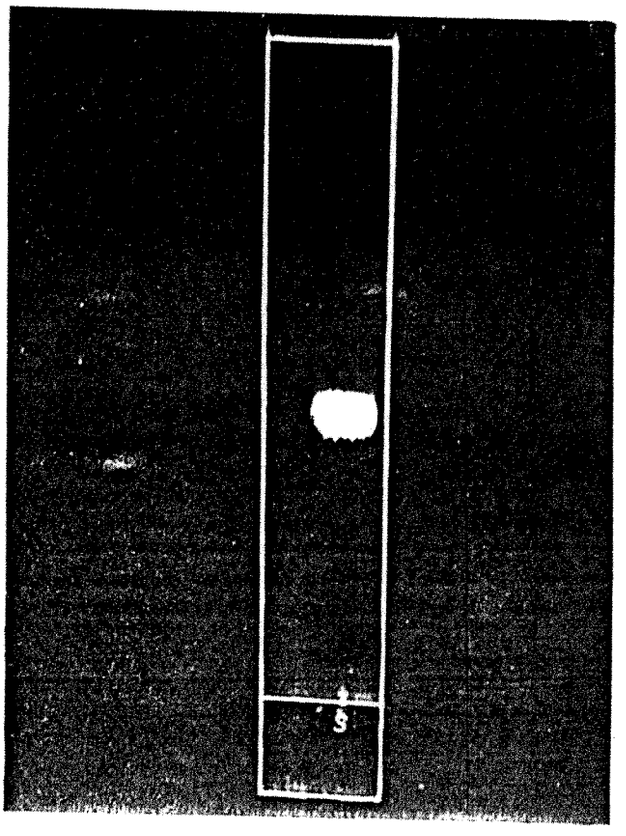
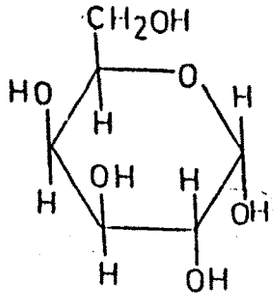


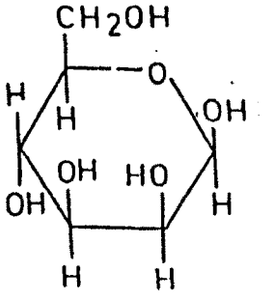
Fig.3.1 : Paper Electrophoresis

IR Spectral Analysis of the Polysaccharide :

This analysis of the neutral polysaccharide was used to determine the anomeric configuration of the constituent sugars. H.C.Srivastava[7] and his co-workers have isolated D-galactose and D-mannose in the ratio of 1:2 from the seeds of Sesbania grandiflora. The IR spectrum (KBr pellets)(Fig.3.2) of the polysaccharide obtained from Sesbania grandiflora showed the broad band around 3400 cm^{-1} which indicated the presence of OH groups. The two absorption bands at 817 and 875 cm^{-1} are indicative of the presence of α linked D-galactopyranose and ß linked D-mannopyranose units.



α-D-galactose



ß-D-mannose

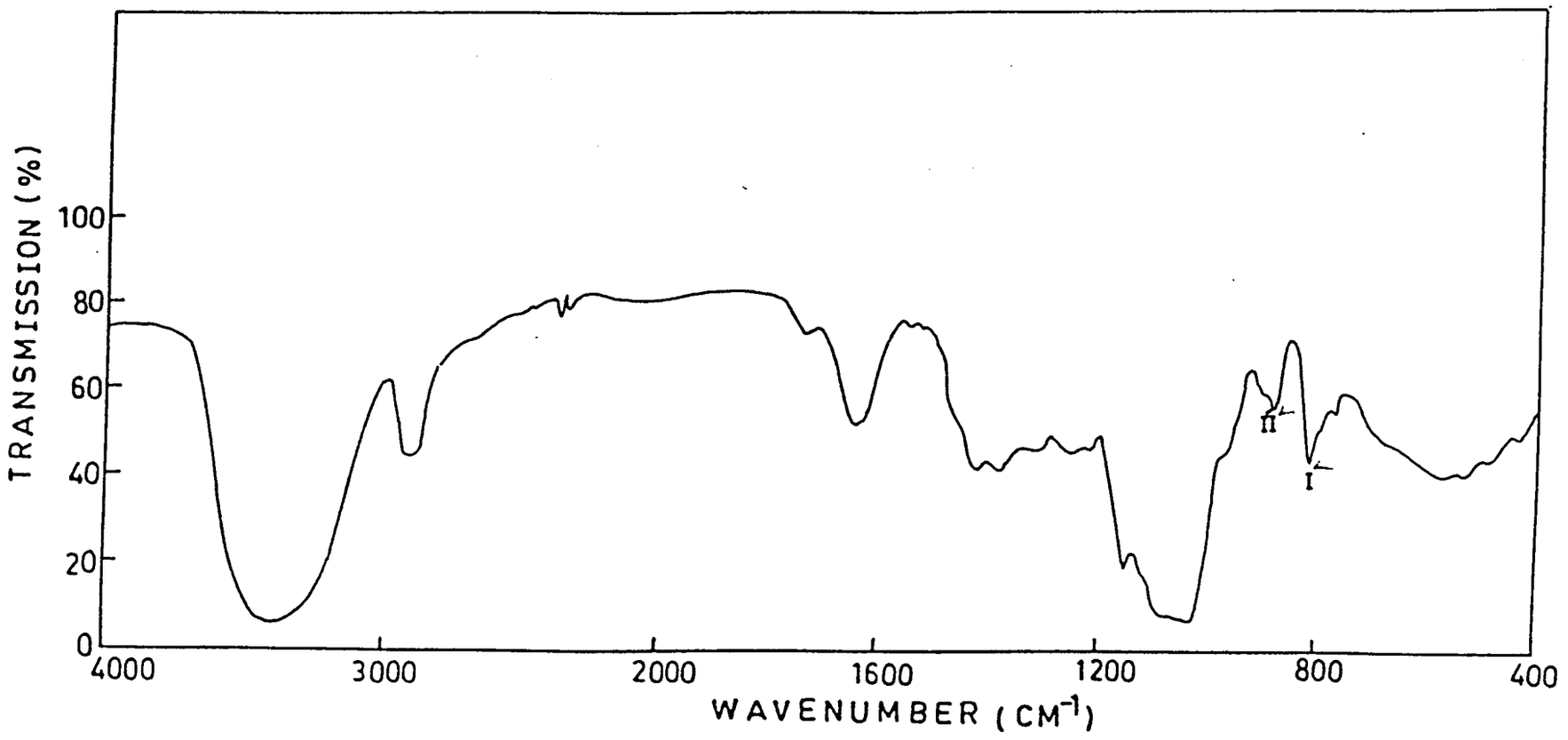


Fig.3.2 : I.R.Spectrum of Sesbania Grandiflora Seed Polysaccharide (KBr Pellets)

I : α-Linked - Galp, II : ß-Linked - Manp

ISOLATION OF SESBANIA GRANDIFLORA SEED POLYSACCHARIDE

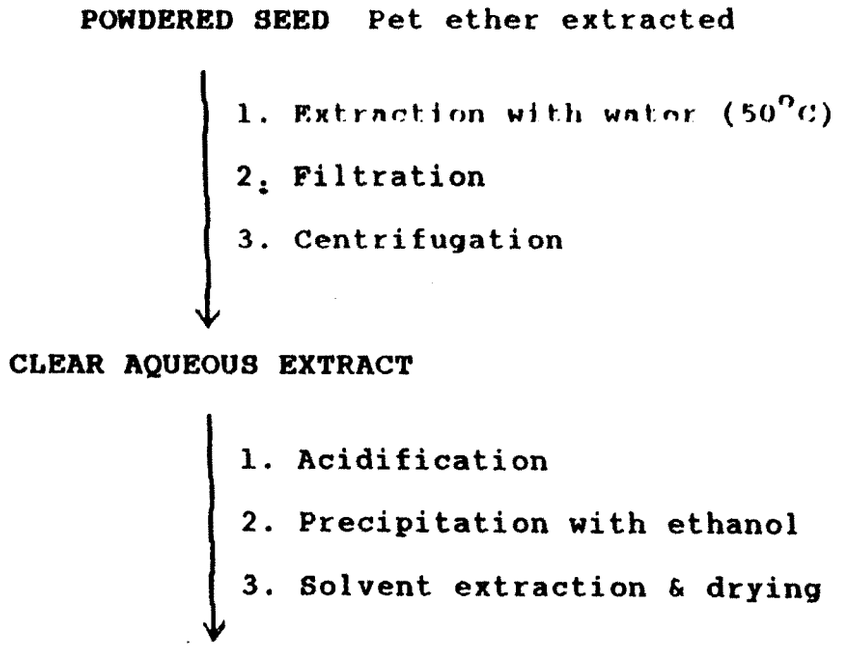


CHART 3.1

PURIFICATION AND FRACTIONATION OF

SESBANIA GRANDIFLORA SEED POLYSACCHARIDE

CRUDE POLYSACCHARIDE

1. Cation Exchange [IR-120(H^+)]
2. Dialysis
3. Precipitation with ethanol (4 volume)
4. Solvent exchange and drying

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PURIFIED PREPARATION

1. Fractionation via Cu complex process
2. Ion Exchange resin treatment
   Amberlite IR - 120 (H^+)
   Dowex 3(OH^-)
3. Dialysis
4. Precipitation with ethanol (4 volume)
5. Solvent exchange & drying

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FRACTIONATE PREPARATION

CHART 3.2