# Biological Sciences Section

Pakistan J. Sci. Ind. Res., Vol. 14, Nos. 4–5, August–October 1971

## FRACTIONATION OF PECTINS

NASEEM AKHTAR and MAHBOOB UDDIN

*Department of Chemistry, University of Karachi, Karachi 32*

(Received January 22, 1970; revised October 31, 1970)

Samples of pectins were extracted from ethanol–water-extracted orange peels and turnips (*Brasseca napiformis*) in a stepwise manner with water, ammonium oxalate and ethylenenediaminetetraacetate (EDTA) solutions. Pectin samples were examined for (a) uronic acid anhydride contents, (b) specific rotations, and (c) constituent sugars, and the results showed no marked difference in the chemical compositions of the polysaccharides. The hydrolysates of the pectin samples were found to contain degraded galacturnans, D-galacturonic acid and its oligomers, varying amounts of D-galactose and L-arabinose and traces of D-xylose and L-rhamnose. Some of the samples of polysaccharides were fractionated by (a) column chromatography on diethylaminoethylcellulose (phosphate form) column[1] and (b) electrolyte precipitation[2,3] with sodium acetate, sodium chloride and potassium chloride. The polysaccharide was eluted in a single broad band from diethylaminoethylcelluose and was found to contain all the sugars present in the original polysaccharide sample. The fractions obtained by electrolyte precipitation were analysed and showed no marked difference in chemical composition. The results of this fractionation have been discussed in this paper.

Citrus fruits are one of the chief sources of pectins.[4] Similarly, turnip has been reported to contain appreciable quantities of this group of polysaccharides.[5] The samples of pectins were extracted from these two sources in a stepwise manner,[6,7] with ethanol–water (80:20) to remove colouring matter and soluble sugars, with water to extract soluble polysaccharide(s), with ammonium oxalate solution to extract pectins as ammonium pectate and finally with EDTA solution to recover rest of the pectin still present in the plant material as calcium salt.[7,8]

Pectins are very susceptible to degradation under various conditions.[9] They are easily degraded by acids, alkalis, enzymes and even in neutral solutions.[10] This property of pectic substances presents a number of problems during extraction and fractionation of the polysaccharide. In view of this fact the polysaccharide samples were extracted under mildest possible conditions.[7]

The samples of pectins were purified via insoluble calcium salt. Calcium chloride solution was added to a solution of polysaccharide and the precipitated calcium pectate after centrifugation was washed with water. The supernatant solution and washings were found to contain no neutral polysaccharide(s). Calcium pectate was converted to ammonium pectate by heating at 90°C with ammonium oxalate solution.

The analyses of the samples of pectins are given in Table 1, which shows that all the samples have similar uronic acid anhydride contents and specific rotations. Samples of the polysaccharides were hydrolysed and the hydrolysates gave the similar paper chromatographic pattern, indicating the presence of sugars shown in Table 1 in the approximate proportions given in paranthesis.

*Fractionation*

The samples of pectic substances were fractionated by two methods: (i) DEAE-cellulose column chromatography[1] and (ii) graded precipitation with electrolytes.[2,3]

(1) The homogeneity of samples of pectinic acids was examined by DEAE-cellulose column chromatography.[1] All the polysaccharide preparations gave the same elution pattern on this ion-exchange resin. More than 95% of the polysaccharide was eluted in a single broad band with NaOH solution and only traces of polysaccharide were eluted with 0.5M sodium phosphate buffer and found to contain the same constituent sugars as the original pectinic acid.

(2) Various pectic acids have been fractionated by graded precipitation with electrolytes such as sodium acetate[2,3] and potassium chloride[9] and in some cases pure galacturonans have also been isolated.[12]

Pectin samples were de-esterified to give pectic acids, under very mild conditions.[13] Fractionation of these pectic acids by Bishop's method[2] gave only one major fraction, which precipitated when the molarity of sodium acetate in the solution was 0.18. Traces of pectic acid were precipitated at lower concentrations of sodium acetate, and the analysis of these polysaccharide samples showed the presence of all the constituent sugars present in the original polysaccharide. Neither pure galacturonan nor neutral polysaccharide(s) was obtained.

TABLE 1.—ANALYSIS OF PECTIN SAMPLES.

| Sample | Specific rotation | —$OCH_3$ content (%) | Uronic acid anhydride (%) | Constituent sugars |
|---|---|---|---|---|
| *A. Orange Peel Pectins* | | | | |
| (i) Pectin extracted with cold water | +249 | 9.1 | 88 | Galacturonic acid, galactose(+), arabinose(++), and rhamnose(tr) |
| (ii) Pectin extracted with ammonium oxalate | +250 | 9 | 86 | galacturonic acid, galactose(+), arabinose(++), and xylose(tr). |
| (iii) Pectin extracted with EDTA | +220 | — | 83 | galacturonic acid, galactose(+), and arabinose (++). |
| *B. Turnip Pectins* | | | | |
| (i) Pectin extracted with water | +225 | 8 | 80 | galacturonic acid, galactose(+), arabinose(++), xylose(tr), and rhamnose(tr). |
| (ii) Pectin extracted with ammonium oxalate | +220 | — | 80 | galacturonic acid, galactose, arabinose, and xylose(tr). |
| (iii) Pectin extracted with EDTA | +220 | — | 88 | galacturonic acid, galactose, and arabinose. |

The results of this fractionation show that the polysaccharide fractions formed a series of related molecular species with no apparent discontinuities in the composition. Fractionation with sodium chloride and potassium chloride gave only one fraction which ensures the homogeneity of the polysaccharide.

These results of fractionation of pectins also show that various neutral sugars are integral constituents of this class of polysaccharides, which is not a mixture of a galacturonan, a galactan and an arabinan.[14] On the contrary, pectins are a group of very complex polysaccharides[15] containing D-galacturonic acid as a major constituent sugar with varying amounts of neutral sugars like L-arabinose, D-galactose, L-rhamnose and D-xylose, present as chemical constituents of the pectin molecule.

## Experimental

Paper chromatography was carried out on Whatmann filter papers No. 1 and No. 4, using the following solvent systems:[16] (a) ethyl acetate–pyridine–water (10:4:3), (b) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (c) butanol–ethanol–water (4:1:5; upper layer) and (d) butanol–acetic acid–water (4:1:5; upper layer). The chromatograms were developed by spraying with *p*-anisidine hydrochloride, aniline oxalate or aniline phthalate and the sugars were identified by either $R_{GalA}$ values or by comparing with reference sugars.

Column chromatography of samples of polysaccharides was carried out on DEAE-cellulose (phosphate form) columns,[1] which was eluted in a stepwise manner with 0.05, 0.1, 0.25 and 0.5M sodium dihydrogen phosphate solutions (pH 6) and lastly with 0.3M NaOH. Cations were removed from the sugar solutions with Amberlite resin IR 120 (H) and anions with IR 45 (OH). All concentrations were done at reduced pressure below 50°C.

The samples of polysaccharides were hydrolysed in N $H_2SO_4$ and the hydrolysates were neutralized with BaOH and $BaCO_3$. The insoluble inorganic salts were removed by filtration. The filtrates were deionized, concentrated and examined by paper chromatography. The uronic acid anhydride contents (u.a.a.) of the samples of polysaccharides were determined by Anderson's decarboxylation method[17] and methoxyl contents by Ziesel method.[18]

### *Extraction and Purification of Pectins*

*Brasseca (Turnip) Pectins.*—Fresh turnips (2 kg) were sliced in small pieces, washed with water and extracted with ethanol–water (4:1; 3 times) to inactivate the enzymes and remove soluble sugars, colouring matter and proteins. The plant material was then dried and extracted in a stepwise manner[6] with water (4×3 l), ammonium oxalate solution (0.5%, 2×3 l; at 90°C for 1 hr) and EDTA (2%; 2×2 l; boiled for 30 min). The polysaccharide from each extract was precipitated with acetone (1:1), filtered and dried by solvent exchange method. Each pectin sample was purified via insoluble calcium salt.[16] Calcium chloride solution (10%) was added to the pectin solution (1%) which precipitated the polysaccharide as calcium pectate. The precipitate was filtered, washed with water and reconverted to ammonium pectate by treatment with ammonium oxalate (0.5%). Regenerated ammonium pectate was precipitated with ethanol (1:1), filtered and dried by solvent exchange method. In this way three samples of brasseca pectin were obtained and analysed (Table 1).

*Pectins of Orange Peels.*—Dried and powdered orange peels (200 g) were first extracted with ethanol–water (4:1). The plant material was then extracted in a stepwise manner with water (4×3 l), ammonium oxalate (0.5%; 3×3 l; at 90°C for 1 hr) and EDTA (pH 6; 2%, 2×2 l). The polysaccharide from each extract was recovered as described previously and purified via

insoluble calcium salt. The analyses of the samples of pectins are given in Table 1.

*Fractionation*

(a) *Column Chromatography on Diethylaminoethyl-cellulose*

DEAE-cellulose column (10×1.5 cm; 5 g; phosphate form) was prepared as described in general methods and samples of brasseca and citrus pectins were chromatographed by Neukoms method.[1]

(i) *Brasseca Pectin (water-extracted polysaccharide).*—A small sample (0.2 g) of brasseca pectin was adsorbed on the above-mentioned column, which was then eluted in a stepwise manner with 0.05, 0.1, 0.25, and 0.5M sodium dihydrogen phosphate (pH 6; 100 ml each) and finally with NaOH (0.3M; 200 ml). The eluates were dialysed to remove inorganic material and the polysaccharides were precipitated with ethanol (1:1). It was observed that about 95% of the polysaccharide was eluted with NaOH solution. Hydrolysis of this polysaccharide and paper chromatographic examination of the sugars present in hydrolysate, in solvent systems A, B and C showed the presence of D-galacturonic acid, D-galactose and L-arabinose. Traces of polysaccharide eluted with 0.5M sodium dihydrogen phosphate solution, on hydrolysis, showed the same paper chromatographic pattern as the original pectin.

(ii) *Orange Peel Pectins.*—A sample (0.3 g) of water-extracted pectin of orange peel was chromatographed on DEAE-cellulose column, as described before. Most of the polysaccharide was eluted with NaOH solution and only traces of it were eluted with 0.5M sodium dihydrogen phosphate. Both the samples of polysaccharide on hydrolysis and paper chromatographic examination of hydrolysates in solvents A, B and C showed the presence of D-galacturonic acid, D-galactose and L-arabinose.

(b) *Fractionation by Graded Precipitation*

(i) *Sodium Acetate Fractionation of Brasseca (Turnip) Pectic Acid.*—Brasseca pectin was first converted to pectic acid under mildest possible conditions.[13] The aqueous pectic acid solution (4 g in 400 ml water) was kept at pH 12 (pH being adjusted by dropwise addition of 2M NaOH) at 0°C for 2 hr. The de-esterified pectic acid was then precipitated with 18% HCl, washed with acidified ethanol–water (3:2; containing 5 ml acetic acid/l of solution), ethanol and ether.

The dried pectic acid (3.5 g) was dissolved in water (400 ml) and its pH was adjusted at 6.5 with NaOH. Sodium acetate (2M, 25 ml) was added to this pectic acid solution at 0°C so that the molarity of sodium acetate in the solution was 0.12M. The mixture was stirred and kept at 0°C for 18 hr. The precipitated polysaccharide (0.02 g) was removed at centrifuge, washed with sodium acetate solution (0.12M) and dried by solvent exchange method. A sample of the polysaccharide was hydrolysed and the hydrolysate was examined by paper chromatography in solvents A and B. Galacturonic acid, galactose and arabinose were identified.

A further amount of sodium acetate (4.7 ml; 2M) was added to the mother liquor from first fractionation so that the molarity of sodium acetate became 0.14M in the resulting solution. The mixture was kept at 0°C for 18 hr. The precipiated polysaccharide (0.03 g) was removed at centrifuge, washed with sodium acetate solution (0.14M) and ethanol–water (1:1) and dried by solvent exchange method. Hydrolysis of a sample of this polysaccharide and paper chromatographic examination of the hydrolysate in solvent systems A and B indicated the presence of galacturonic acid, galactose and arabinose. Another addition of sodium acetate (2M; 9 ml) to the mother liquor from second fractionation and recovery of the precipitated polysaccharide (as described previously) gave a third fraction of the polysaccharide (3.2 g; sp. rot.,+225; u.a.a. 80%) paper chromatographic examination (in solvents A and B) of sugars obtained by hydrolysis of a sample of this polysaccharide, showed the presence of galacturonic acid galactose and arabinose. Addition of more sodium acetate and of alcohol to the mother liquor from third fractionation gave no further fractions of pectic acid.

(ii) *Sodium Acetate Fractionation of Orange Peel Pectic Acid.*—Water-extracted orange peel pectin (5 g) was converted to pectic acid (4.1 g), as described previously. The pectic acid (4 g) was fractionated by portionwise addition of sodium acetate(2M) in such a way that the molarity of sodium acetate was 0.12, 0.14, 0.18 and 0.2M in first, second, third and fourth fractionation, respectively. Only traces of polysacchairde were precipitated as first and second fractions and the major pectic acid fraction (3.6 g) was precipitated at 0.18M sodium acetate. No further fractions were obtained by addition of more sodium acetate and finally by addition of alcohol (1:1).

The hydrolysis of all the fractions and paper chromatographic examination of the hydrolysates in solvents A and B showed the presence of galacturonic acid, galactose and arabinose.

(iii) *Graded Precipitation of Orange Peel Pectic Acid with Sodium Chloride.*—De-esterified orange peel pectic acid (2 g) was dissolved in water (200 ml) and the pH of the solution was adjusted at 6.5 by dropwise addition of N NaOH. Sodium chloride solution (2M; 13.5 ml) was added to the pectic acid solution with stirring and the mixture was kept at 0°C for 18 hr. The precipitated poly-

saccharide was then removed at the centrifuge, washed with NaCl (0.12 M), acidified ethanol–water (1:1; containing 4 ml acetic acid/l of solution) and ethanol and dried by solvent exchange method. The analysis of this fraction of polysaccharide is as follows: sp. rot. $+250 \pm 5$; u.a.a. 86%; constituent sugars (observed by paper chromatography in solvents A and B): galacturonic acid, galactose and arabinose.

Addition of more NaCl to the mother liquor gave no further fractions of pectic acid and no polysaccharide was precipitated by the addition of alcohol to the mother liquor.

(iv) *Graded Precipitation of Orange Peel Pectic Acid with Potassium Chloride.*—Potassium chloride was tried for the graded precipitation of pectic acid (2 g), but all the polysaccharide was precipitated at 0.12M KCl concentration in the solution and no further fractions were obtained by addition of either more KCl or alcohol. The fraction obtained, resembled the original polysaccharide having the same sp. rot., u.a.a. content and constituent sugars.

**Acknowledgement.**—The authors thank Professor Salimuzzaman Siddiqui, F.R.S., and Dr. M. Ghaziuddin Ahmad for their help and cooperation in carrying out this research work.

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