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*Full Length Research Paper*

# Characterization of the polysaccharide material that is isolated from the fruit of *Cordia abyssinica*

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**Treatment of aqueous extracts of the fruit of *Cordia abyssinica*, containing 0.25% sodium chloride with three volumes of ethanol produced an acidic polysaccharide with a 2% yield, on a fresh weight basis. Upon precipitation of the polysaccharide with acid, the yield of the polysaccharide decreased to 1.2% and the polymer showed some level of degradation. With protein content between 2.6 and 4.6% for the acid and ethanol precipitates respectively, the polymer contained 0.29% hydroxyproline. Treatment of the ethanol precipitated polysaccharide with pronase E resulted in a decrease in viscosity of polysaccharide solutions and high performance size exclusion (HPSEC) chromatograms of the protease treated samples showed peaks that had slightly shifted to low molecular weight. Uronic acid content, determined using the m-hydroxydiphenyl method was 9%. Some uronic acid residues along the polymer chain were methyl esterified, with the methoxyl content being 38%. With an ash content of 17% the polymer had a mineral ion content of Ca, 0.3%, Mg, 0.3%, Na, 0.2% and K, 4.8%. The optical rotation of a 0.25% solution was -50°.**

**Key words:** *Cordia abyssinica*, polysaccharide, yield, viscosity, hydroxyproline, protein content.

## INTRODUCTION

Polysaccharides or polyglycans are polymers of monosaccharide residues that are joined together by glycosidic bonds, which are formed by the elimination of elements of water, between the hemiacetal hydroxyl group of one residue and a primary or secondary hydroxyl group of an adjacent residue (Laere et al., 2000). The monomer species may be simple monosaccharides or sugar derivatives such as N-acetylaminosugars, uronic acids or ester sulphate sugars. Uronic acids are constituents of hemicellulose, pectin, gums, mucilages and other plant polysaccharides. Uronic acids occur widely in nature and much of the carbohydrate materials in plants contain this important component (Ridley et al., 2001). Typically, in the polyuronide molecule the neutral monosaccharide and uronic acids are joined by glycosidic linkages to form complex acidic polymers. Many polyuronides contain methyl groups that are linked through ether bonds to the uronic acid. In hemicelluloses and gums, the content of uronic acids is low but in pectic acids, uronic acid units may constitute essentially the entire polysaccharide chain (Figure 1).

The ability of polysaccharides to produce high viscosity in water at low concentrations is a major property of polysaccharides that gives them valuable and widespread use in the food and non-food industries. Polysaccharide gums have mainly been used for thickening, modification of texture, gelling, formation of protective films, and for stabilization of emulsions, foams and suspensions (Kossori et al., 2000, Euston and Hirst, 2000). *Cordia abyssinica*, a member of the family Boraginaceae, is a small to medium sized tree that grows to about 9 m in height. The tree is fast growing and occurs in medium to low altitudes in woodland and bush. The tree is found in warm moist riverine areas, often along riverbanks (Van Wyk and Van Wyk, 1998). *C. abyssinica* grows in north-eastern Africa, extending southwards to Angola, Mozambique, Zimbabwe and the Limpopo province of South Africa (Palmer and Pitman, 1972). In Zimbabwe, the tree is commonly found in the South-Eastern parts of the country, such as Masvingo province and near the boarder with Mozambique.

The fruit of *C. abyssinica* is a drupe, about 10 to 30 mm in diameter, which has a globose shape and a sharp tip. Green when unripe the fruit turns yellow to orange on

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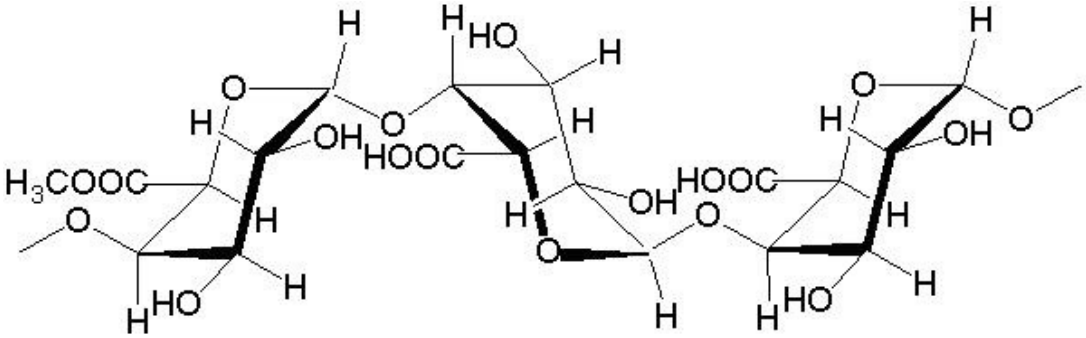


**Figure 1.** Structure of pectin showing uronic acid residues and methylated groups (http://www.kjemi.uio.no/Polymerkjemi/Research/pectin.jpeg).

ripening, which occurs between December and April. The shell encloses a sweet mucilaginous flesh, which is highly viscous and sticky. Rural school children often use the mucilage from the fruits of *C. abyssinica* as glue. Although edible, the fruit of *C. abyssinica* is not normally consumed by humans but is eaten by wild animals. The fruit of *C. abyssinica* was chosen as a suitable candidate for study because of the unique adhesive properties that the mucilage of the fruit possesses and high viscosity of the solutions formed when the polymer is dissolved in water. The polysaccharide from *C. abyssinica* has potential for application as a thickener (Benhura and Katayi-Chidewe, 2000), emulsion stabiliser (Benhura and Chidewe, 2004), and as a binding agent in the food industry and as an effective adhesive in the non-food industry. The polysaccharide that was isolated from *C. abyssininica* is made up of the sugars, mannose, glucose, galactose, arabinose, xylose, rhamnose, fucose and an unidentified methyl sugar (Benhura and Chidewe, 2002).

The objectives of the study were to determine some of the physical properties of the polysaccharide of *C. abyssinica*.

## EXPERIMENTAL

### Collection of the fruit

Mature but unripe fruit of *C. abyssinica* were picked from trees in Bikita, South Eastern Zimbabwe. The fruit was collected when in season during the period between December and April. Fruits were collected as available from trees occurring alongside streams in the same area. Harvested fruit, with their calyces on, were stored at room temperature and processed within 72 h from the time of collection.

### Extraction of pulp from the fruit

The pulp was extracted by squashing the fruit by hand to release the stones, in which was most of the fruit pulp. The stones, in a strong plastic or stainless steel container, were vigorously agitated with a robust wooden rod during which process the pulp separated as a thick sticky mass. The separated stones were removed and the pulp, where necessary, was stored frozen at -20°C until required.

### Precipitation of polysaccharide using 0.25 M sodium chloride and ethanol

Water was added to the sticky freshly prepared or thawed pulp in order to make a workable mixture, which was centrifuged in a BHG Hermle ZK 401 centrifuge at 6000 rpm for 30 min to remove insoluble material. To the supernatant, solid sodium chloride was added to make a 0.25 M solution. In routine preparation of the polysaccharide, four volumes of ethanol were then added to the supernatant to precipitate the polysaccharide. The polysaccharide was dried in a pre-heated oven set at 100°C or freeze dried in a Christ-Alpha 2 to 4 freeze drier. The dry gum was stored at room temperature until required.

### Precipitation of polysaccharide using 0.2 M HCl

Sodium carbonate (1 M, 120 ml) was added to the sticky pulp (400 ml) of *C. abyssinica*. When the sample dissolved, it was diluted to a final volume of 1200 ml with water so that the final concentration of sodium carbonate in solution was 0.1 M. The mixture was centrifuged in a BHG Hemle ZK 401 centrifuge at 6000 rpm for 30 min, to remove insoluble material. To precipitate the polysaccharide, 0.2 M HCl (500 ml) was added to the supernatant with stirring. The precipitated polysaccharide was washed five times with water and freeze- dried in a Christ Alpha 2 to 4 freeze-drier.

### Determination of yield of polysaccharide

A known number of fruits were de-capped, weighed and the pulp was extracted from the fruit. After the polysaccharide was precipitated using sodium chloride-ethanol or HCl the freeze-dried mass of the polysaccharide was determined. Yield was expressed as percentage of the mass of the dry precipitate against the mass of the whole fresh de-capped fruit (James, 1995).

### Determination of moisture, ash and mineral ion content of the polysaccharide

The dry polysaccharide (2 g) of *C. abyssinica* was weighed into a previously ignited, cooled and weighed porcelain crucible and the sample heated to constant weight in a pre-heated oven at 100°C. To determine ash content, the sample, dried at 100°C was first charred at 200°C for two hours in order to prevent the foaming that

is likely to occur as a result of too rapid rise in temperature (James, 1995). The charred mass was ashed at 550°C in a Phoenix MRB2-017-8 furnace. To determine the mineral ion content, the ash was dissolved in 5 ml of concentrated HCl and the mixture boiled for 5 min on a hot plate in a fume cupboard, with acid being added as necessary in order to maintain constant volume. The mixture was transferred to a beaker and the crucible washed with distilled water pouring the washings into the beaker containing the sample. The volume was adjusted to about 40 ml and the mixture boiled for 10 min. The mixture was cooled and filtered through glass wool into a 100 ml volumetric flask and the beaker was rinsed into the volumetric flask.

The solution, cooled and made up to 100 ml, was used for the determination of the individual mineral ions including, sodium, calcium and magnesium using a Perkin Elmer 500 atomic absorption spectrophotometer and potassium was determined using a Corning 400 flame ionisation photometer (Rojas et al., 2004).

**Determination of specific optical rotation of the polysaccharide**

The optical rotation for solutions of polysaccharide at concentrations up to 0.5% was measured at room temperature on an Otago Polax-D polarimeter using the D-line of polarised sodium light and a 100 mm cell (Saka and Msonthi, 1994).

**Determination of uronic acid content of the polysaccharide**

Uronic acids were determined using the p-hydroxydiphenyl-sulphuric acid method with galacturonic acid as the standard (Chaplin and Kennedy, 1986).

**Determination of the methyl ester content**

Sodium hydroxide (0.75 M, 0.25 ml) was added to aliquots (0.5 ml) of the polysaccharide (50 to 200 µg) and the tubes gently swirled. After 30 min at room temperature, the samples and methanol standards (2 to 40 µg) were acidified with 2.75 M $H_2SO_4$ (0.25 ml) and cooled in an ice-water bath, for permanganate oxidation. To aliquots (1 ml) of the saponified polysaccharide or methanol standards aqueous potassium permanganate (2% w/v, 0.2 ml) was added, taking care not to splash liquid onto the sides of the tube. The mixture was agitated by swirling gently and the tubes held in an ice bath for 15 min. Sodium arsenate (0.05 M, 0.2 ml), followed by water (0.6 ml) was added, and the thoroughly mixed solution left for 1 h at room temperature. After addition of pentane-2,4-dione (2 ml) and thorough mixing, the tubes were closed with marbles, heated at 60°C for 15 min, and cooled to room temperature.

Absorbance at 415 nm was determined in a Spectronic 20 Genesys spectrophotometer, using a blank of water (1 ml) treated identically to the samples.

**Determination of the protein content of the polysaccharide**

Crude protein content of the polysaccharide preparations was determined using the Kjeldahl method with the nitrogen content being multiplied by a factor of 6.25 (Rodriguez et al., 2004).

**Treatment of the polysaccharide with protease**

Pronase E (52.5 mg), from Sigma, was dissolved in phosphate buffer (13.82 ml, pH 7.5). The solution of enzyme (300 µl) was mixed with solution of the polysaccharide (1%, 17.7 ml) and incubated at 37°C. In the control tube, an equal volume of the phosphate buffer was used instead of the solution of enzyme. The action of the enzyme was followed by measuring the viscosity at 37°C using a Cannon Fenske routine viscometer # 350, from PSL Ltd, England. In order to determine the nature of interaction between the protein and the polysaccharide, the native and protease treated solutions of the polymer were analysed by HPSEC using a Zorbax GF 250 size exclusion column and water as the mobile phase at a flow rate of 2 ml/min. A 1% solution of sample (50 µl) was injected into the column.

**Determination of hydroxyproline residues in the polysaccharide chains**

Solutions (100 µg/ml) were prepared by dissolving 0.05 g of the standard amino acids in water (400 ml). Concentrated HCl (11 M, 20 ml) was added to the solutions to prevent microbial degradation and the solutions were made up to 500 ml with distilled water. Working solutions of standards, concentrations of up to 20 µg/ml were prepared by diluting the 100 µg/ml standard solutions with water. Polysaccharide that had been prepared by precipitation with acid or ethanol were used for determination of hydroxyproline, with the ethanol precipitate being dissolved in water and the acid precipitate was dissolved in acetate buffer, pH 5.5, to make 0.5% solutions. Copper sulphate solution (0.05 M, 1 ml) was added to samples and standards (1 ml), in duplicate, in rimless Pyrex tubes each tube followed by 2.5 M sodium hydroxide (1 ml), and the tubes were agitated by gentle swirling. The tubes were placed in a water bath at 40°C for 5 min after which hydrogen peroxide (6%, 1 ml) was added with immediate mixing by swirling of the tubes while still in the bath. The tubes were left in the bath for a further 10 min with occasional swirling. Tubes were cooled to room temperature with tap water and sulphuric acid (1.5 M, 4 ml) was added followed by 5% p-dimethylaminobenzaldehyde (1 ml).

The contents of the tubes were mixed on a vortex mixer after each addition. The tubes were capped with marbles and placed in a water bath at 70°C for 16 min. After this time, the solutions were left to cool to room temperature and the mixtures were agitated thoroughly on a vortex mixer and the absorbance at 555 was measured using a Genesys Spectronic 20 spectrophotometer from Spectronic instruments, USA.

## RESULTS AND DISCUSSION

### Precipitation of the polysaccharide of *C. abyssinica* using sodium chloride and ethanol, and acid

Initial attempts to precipitate the polysaccharide out of aqueous extracts using ethanol alone were not successful. When sodium chloride was added to the extract before adding ethanol, a white fibrous precipitate that could be spooled onto a glass rod was produced. On addition of acid to extracts of *C. abyssinica* fruit, a white particulate precipitate was obtained. On analysis of the acid precipitate by HPSEC using water as the mobile phase, the elution pattern was similar to that of the ethanol precipitates, but the peaks had shifted to lower molecular weight (Chidewe, 2004). It is possible that precipitation of the polysaccharide using acid had resulted in degradation of the polymer to some extent. Such degradation would give rise to polymers of reduced

molecular weight. The degradation of *C. abyssinica* polysaccharide would be similar to that observed for other polysaccharides.

Thomas and Coworkers (2003) have pointed out that when precipitating pectin using acid it is difficult to avoid some degradation of the polymer that takes place.

## Characterisation of some properties of the polysaccharide of *C. abyssinica*

More polysaccharide material was recovered when ethanol was used for precipitation than when acid was used. The difference in yield, expressed as percent fresh weight, could arise if acid did not precipitate neutral polysaccharides that would be precipitated using salt and ethanol.

At less than 1%, the ash content of the polymer precipitated with acid was much lower than that for the polymer precipitated using salt and ethanol at 17.4%. It can be concluded that the polysaccharide of *C. abyssinica* was associated with metal ions. As shown in Table 1, potassium was the most abundant ion, with levels of Ca, Mg and Na being ten times lower. When ethanol was added to the extracts, the metal ions would have been precipitated together with the ionised polysaccharide. Addition of acid would have replaced metal ions associated with the polymer with hydrogen ions, leading to a reduced metal ion content upon precipitation. The ash content can be taken as a measure of the ions or salts that were associated with the polymer (James, 1995).

There is no obvious explanation for the observation that the protein content of polysaccharide precipitated with acid was just over half of that precipitated with ethanol. The uronic acid content of 9% for both the acid and ethanol precipitates is consistent with the acidic nature of the polysaccharide.

Some of the uronic acid groups in the polysaccharide were methyl esterified as indicated by the methoxyl content of 38% for both the acid and ethanol precipitates. The methoxyl groups would be expected to have an effect on the functional properties of the polysaccharide such as gel formation, with different gel forming mechanisms being observed for high and low methoxy pectins (Barnavon et al., 2001).

## Effect of protease treatment of the polysaccharide of *C. abyssinica*

When solutions of the polysaccharide that had been precipitated with ethanol were treated with pronase E, a decrease in viscosity was observed as shown in Figure 2. The decrease in viscosity would result from hydrolysis of protein portions occurring in the polysaccharide, by pronase E. Polysaccharides that have been isolated are often associated with proteins (Sims and Furneaux, 2003). The protein could be free protein that was co purified with the polysaccharides during isolation or protein that was covalently bound. Proteins that are non-covalently bound may be removed by physical methods such as gel chromatography, density gradient centrifugation or treatment with dissociating agents. The HPSEC profile for the native and protease treated samples was similar but the peaks in the protease treated sample shifted to low molecular weight, as shown in Figure 3. On the basis of these results it was not possible to conclude whether the protein digested by protease was free or covalently bound to the polysaccharide.

## Determination of hydroxyproline residues in the polysaccharide chains

The hydroxyproline content obtained for both the acid (0.28 ± 0.01) and ethanol (0.29 ± 0.01%) precipitates, was low compared to the hydroxyproline content reported for arabinogalactan-peptide preparations isolated from wheat endosperm which contained 15 to 20% hydroxyproline and a protein content of 6 to 8% (Strahm et al., 1981). The relatively low content of hydroxyproline could be related to low levels of integrated protein occurring in the polysaccharide of *C. abyssinica*. The proteoglycans isolated from various plant tissues have been shown to contain arabinose linked covalently to the hydroxyl group of hydroxyproline. For example, gum arabic is believed to be a member of the arabinogalactan-protein group of proteoglycans with 25% hydroxyproline content (Osman et al., 1993). The gum exudate from *Acacia robusta* has been found to contain protein (18%) bound to arabino-galactan (Churms and Stephen, 1984).

From the characteristic presence of hydroxyproline in the polysaccharide from *A. robusta* it has been suggested that hydroxyproline occurs in the polysaccharide- protein linkages. The hydroxyproline o-arabinosyl linkage in cells provides cross-links in the polysaccharide network (Vidal et al., 2003).

## Conclusion

The polysaccharide of *C. abyssinica* is an acidic polymer that is associated with protein, some of which appears to be covalently bound. Further work will be done to try and determine the nature and types of linkages between the monosaccharide constituents of the polysaccharide and ultimately determine the actual structure of polysaccharide using techniques such as mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

## ACKNOWLEDGEMENTS

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**Table 1.** Some properties of the polysaccharide isolated from the fruit of *C. abyssinica*. The uncertainties shown are standard deviations for at least three determinations. ND indicates that the measurement was not made.

| Parameter | Sample<br>Ethanol precipitate (%) | Acid precipitate (%) |
|---|---|---|
| Yield (fresh weight) | 2.0 ± 0.4 | 1.2 ± 0.5 |
| Moisture | 10.3 ± 0.6 | 9.1 ± 0.1 |
| Ash | 17.4 ± 0.6 | 0.7 ± 0.5 |
| Specific optical rotation | -50.0°± 0.0 | -50.0° + 0.5 |
| Protein | 4.6 ± 0.6 | 2.6 ± 0.4 |
| Uronic acids | 9.2 + 0.4 | 8.7 ± 0.8 |
| Methoxyl content | 38.3 ± 0.5 | 38.0 ± 0.9 |
| | | |
| Mineral ion content | | |
| Sodium | 0.2 ± 0.1 | ND |
| Calcium | 0.3 ± 0.1 | ND |
| Magnesium | 0.3 ± 0.1 | ND |
| Potassium | 4.8 ± 0.1 | ND |

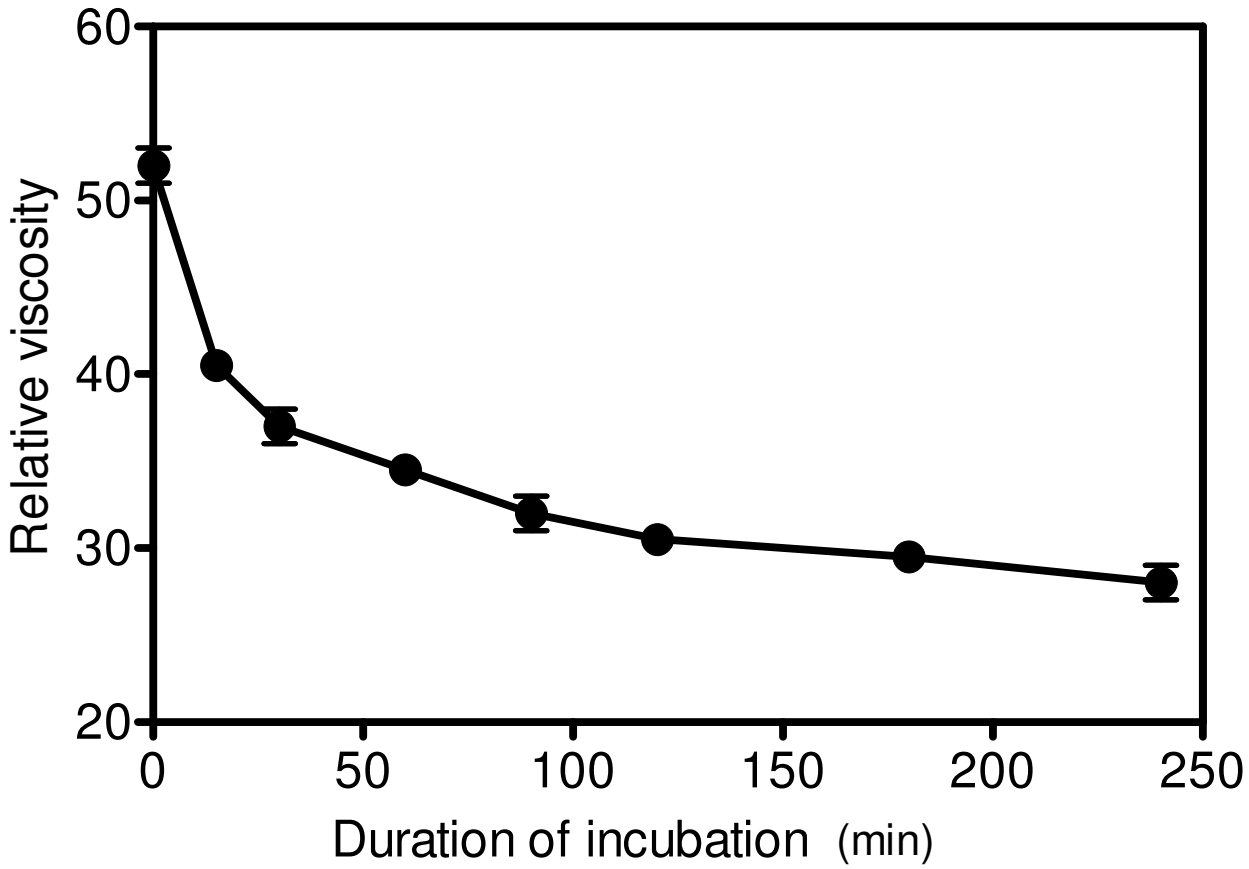


**Figure 2.** Change in viscosity of the polysaccharide of *C.abyssinica* during treatment with pronase E at 37°C. The uncertainties shown are standard deviations for at least three determinations. The points were standard deviation seem to be missing mean that SD was negligible.

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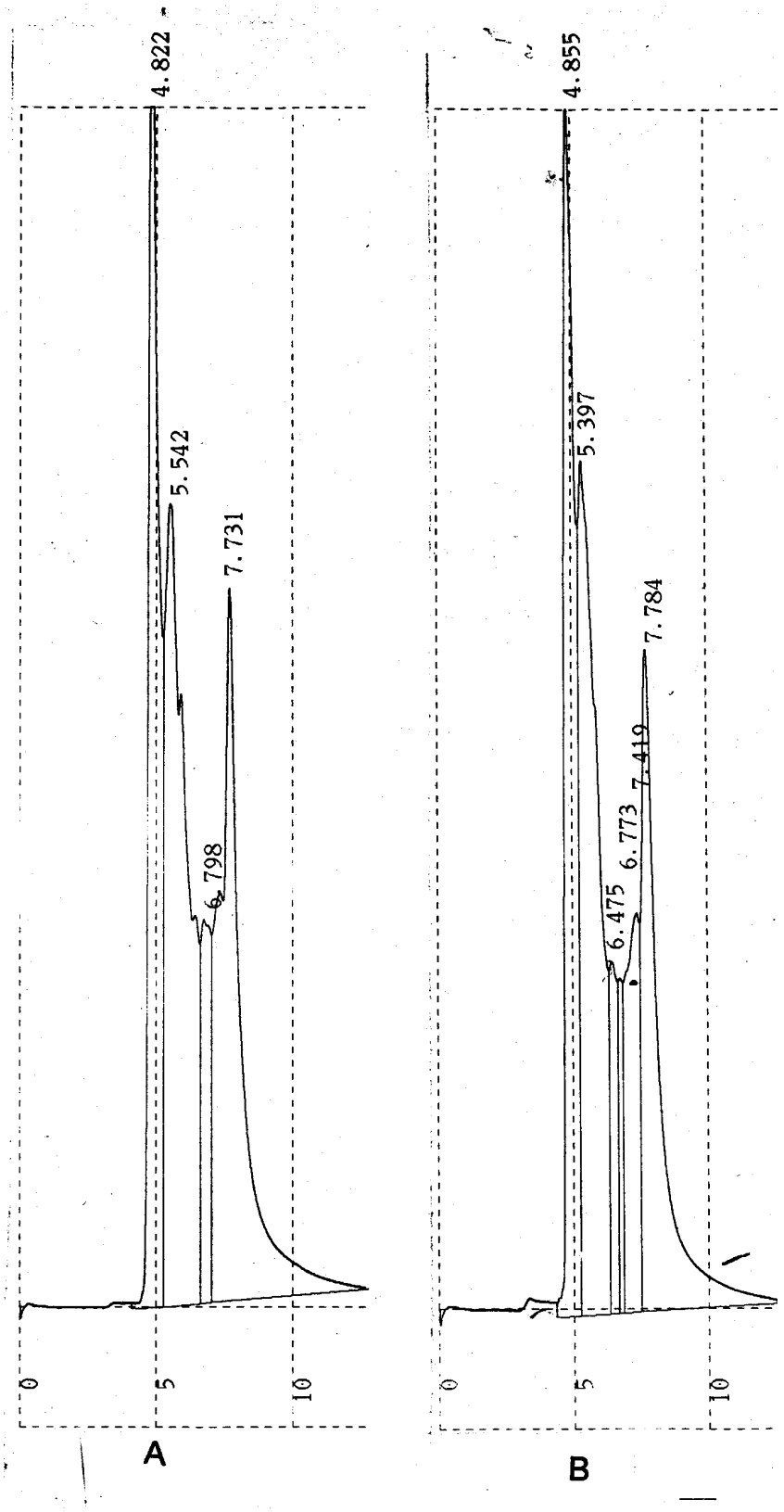


**Figure 3.** HPSEC profiles of the native (A) and protease treated (B) polysaccharides of *C. abyssinica*. HPSEC was done using a Zorbax GF 250 column and water as the mobile phase at a flow rate of 2 ml/min. Summary of retention times of substances was as follows: A (4.822, 5.542, 6.798 and 7.731) and B (4.855, 5.397, 6.475, 6.773, 7.419 and 7.784).

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