Introduction

1.1 Polysaccharides

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.

Polysaccharides are present in abundance in mammalian fluids, tissues and organs, in plant flowers, seeds, leaves and stems, in microbial organelles and in viral particles (Deters *et al*., 2001; Missang *et al*., 2001). Polysaccharides occur extracellularly or intracellularly and are present at concentrations varying from 1 % to over 90 % in plant cell walls (Ridley *et al*., 2001).

Polysaccharides can be classified according to their chemical composition and structure. In this classification, polysaccharides such as glucan, arabinan, galacturonan and xylan, that release a single monosaccharide on hydrolysis are termed homoglycans whereas polysaccharides hydrolysing to two, or more monosaccharide types are termed heteroglycans (Lee *et al.*, 1998). Examples of heteroglycans are glucomannans, arabinogalactan, galactomannans and galactoglucomannans.

In plant polysaccharides, it is common to encounter branched structures in which linear chains with a more or less uniform type of linkage carry variable proportions of rather short side chains. In branched plant polysaccharides, of which arabinoxylans and galactomannans are examples, there is usually no apparent regularity in the pattern of branching. From the irregularity of branching, it appears that the attachment of side chains to the extent desired for the modification of properties of a

given polymer occurs as a separate process after completion of synthesis of the main polysaccharide chain.

Uronic acids are constituents of hemicellulose, pectin, gums, mucilages and other plant polysaccharides. Uronic acids occur widely in nature and much of the carbohydrate material 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.

All polyuronides are non-crystalline and insoluble in strong alcohol. Many polyuronides are soluble in water and those that are insoluble in water are often soluble in solutions of sodium, potassium or ammonium hydroxides or carbonates (Vidal *et al*., 2003). Many polyuronides become sticky, slimy or gelatinous when moistened with water. Most carboxylated polysaccharides will form an insoluble salt upon addition of cetyltrimethylammonium halide to their aqueous solution, thus allowing their separation from neutral polysaccharides and other extraneous substances. Multivalent cations will combine with uronic acid carboxyl on polysaccharides and can form cross links with carboxyl groups on other polysaccharide molecules to prevent dissolution of the polysaccharide causing gelatinisation or precipitation of polysaccharides from solution.

D-glucuronic acid is the commonest naturally occurring uronic acid but D-galacturonic acid constitutes the units of pectic acids and is present in many plant gums and animal polysaccharides (Goubet *et al*., 2002). D-glucuronic acid units occur in polysaccharides both within polymer chains and at the reducing ends. The D-glucuronic acid units in polysaccharides are not usually esterified but exist in salt form with common inorganic cations and are normally ionised and highly hydrated. The extensive hydration, coupled with the formal negative charge of the ionised carboxyl groups, enhances

solubility of the polysaccharide in water over a wide range of pH. However, if the pH of the solution is lowered to such an extent that ionisation of carboxylic acid groups is repressed, much of the hydration is lost, repulsion between molecules is diminished, and the molecules can more easily associate to form a gel or precipitate.

Although occurring as the major sugar unit of alginates, together with L-guluronic acid, D-mannuronic acid is infrequently found in polysaccharides (Holme *et al*., 2003).

1.1.1 Glycoconjugates

Whereas the polysaccharides are composed of only carbohydrate units, glycoconjugates are composed of carbohydrate and protein or lipid moieties, with carbohydrates linked to the other moieties by covalent bonds.

Proteoglycans are conjugates of protein and carbohydrate (Vidal *et al*., 2003). The glycosylaminoglycan chains of proteoglycans are high molecular weight, unbranched heteropolymeric molecules, consisting of repeating disaccharides that are highly substituted with carboxyl or sulphate ester groups. Proteoglycans are the most abundant class of glycoconjugates and are widely distributed in biological materials (White, 1991).

The linkages between the reducing unit of carbohydrate chains and one of several types of amino acid residues of the protein, in proteoglycans may be N-glycosidic or O-glycosidic in type. The carbohydrate residues involved in formation of the glycosidic bonds are usually N-acetylgalactosamine, xylose, arabinose, galactose or mannose. In N-glycosidic proteoglycans, the carbohydrate chains are joined to the amide group of asparagine while in O-glycosidic proteoglycans the carbohydrates are joined to the hydroxyl group of serine, threonine, hydroxyproline or hydroxylysine. In both N- and O-glycosidic proteoglycans, only a few of those amino acids present in the protein are involved in linkage formation.

1.1.2 Functions of polysaccharides

The functions of polysaccharides generally cannot be uniquely assigned but it is evident that the polymers act as storage molecules, structural components and as protective agents (Barnavon *et al*., 2001). Starch, glycogen, some β glucans, fructans and some galactomannans are well known reserve polysaccharides that may be rapidly metabolised and vary in amount with the state of development of the organism (Sims *et al*., 2001; Busato *et al*., 2001).

Structural polysaccharides fall into two distinct classes, the fibrous and matrix polysaccharides. Fibrous polysaccharides include cellulose, chitin and the less frequently occurring mannans and xylans. Matrix polysaccharides, which include pectins and hemicelluloses, are characterised by their capacity to form gels. Polysaccharides that form gels can adopt regular chain conformations for substantial parts of their structures. Interruptions in regularity of structure permit disruption of ordered structures thus preventing precipitation (Laere *et al*., 2000). Formation of gels, therefore, is a balance between the tendency to precipitate out that derives from the presence of regular linear sections and the tendency to go into solution that results when the formation of ordered structures is prevented.

Antigenic and immunogenic extracellular polysaccharides from microorganisms, are examples of protective polysaccharides. In *xanthomonas*, xathan appears to protect from excess loss of water. The exudate gums from *Acacia* species appear to provide a similar protective role in sealing injured parts of the plant from microbial infection.

1.1.3 Extraction and Purification of Polysaccharides and glycoconjugates

Polysaccharides occur as heterogeneous mixtures that are associated with or chemically bonded to other cellular macromolecules, which are often other polysaccharides, proteins or lipids. The desired

polymers need to be isolated and purified before structure, biosynthesis, metabolic functions, and potential applications can be studied.

Numerous and varied techniques for isolating soluble polysaccharides and glycoconjugates have been described (Marry *et al.,* 2000). Many solvents have been used for the extraction of polysaccharides under different conditions of temperature, pH, agitation and duration of treatment. Selection of the solvent and the conditions of extraction depend on the chemical nature of the polymer, the ease of dissociation of the polymer from the other cell constituents and on the types of contaminants present in the tissue homogenate.

Solvents that are used to extract polysaccharides include water, acids, alkali, buffers and detergents. The simplest extraction methods for polysaccharides are those in which water alone is used at room temperature (Dong *et al*., 2003). Extraction of polysaccharides under acidic conditions is usually undesirable as chain cleavage could occur under these conditions (Thomas *et al*., 2003). Dilute alkali has been used extensively for extraction of polysaccharides but it is possible that structural modification or base catalysed degradations may occur. The O- glycosidic linkage of glycosaminoglycans to protein, for example, is split under alkaline conditions (Aspinall, 1982). The extraction of polymers may be done at cold temperature, room temperature and at boiling temperature depending on the material to be isolated.

When polysaccharides have been made soluble, fractionation is based either on selective precipitations of the polysaccharides themselves, their salts and complexes, or on chromatographic procedures. The complete fractionation of mixtures of polysaccharides by precipitation methods is rarely achieved, although one component may be isolated in reasonably homogenous form after reprecipitation. The polysaccharides can be isolated from aqueous solution by the addition of a water miscible solvent such as acetone or a lower alcohol. Carefully controlled fractional precipitation by the

addition of non-solvent to aqueous mixtures may occasionally give separations of mixtures of polysaccharides but the method is not highly selective (Vidal *et al*., 2003).

Fractional precipitation of polysaccharides can be done by selective precipitation using cationic reagents. The components that will be precipitated are those that form a complex with the reagent added, from which the complexed polysaccharide can be regenerated. An example of selective precipitation with cationic reagents such as cetyltrimethylammonium bromide is the preferential precipitation of the more highly acidic components of gum tragacanth and *Anogeissus leiocarpus* gum (Stephen, 1983).

Precipitation methods for polysaccharide fractionation are generally suitable for isolation of large quantities of material.

1.1.4 Criteria for homogeneity or purity of polysaccharides

A carbohydrate or carbohydrate-containing polymer can be considered pure if the polymer can be reisolated and the resulting preparations possess the same chemical and physical properties.

Separations of polysaccharides may be based on differences in molecular weight, or monomer composition. Differences in molecular weight can be determined by gel filtration chromatography whereas separations based on composition, such as presence of acidic groups, can be achieved by ion exchange chromatography. Ion exchange chromatography on DEAE-cellulose, DEAE sephadex and other types of ion exchangers has been used for isolating carbohydrate polymers and glycoconjugates and for determining chemical homogeneity of acidic polysaccharides (Missang *et al*., 2001; Suni *et al.*, 2000).

The essential evidence for the absence of heterogeneity for a polysaccharide, is the demonstration of constancy in monosaccharide composition on repeated fractionation. In order to use constancy of monosaccharide composition analytically, methods must be available for selectively

determining each of the monosaccharide components of the glycan. Methods that are widely used for determination of composition of monosaccharides are thin layer chromatography and high performance liquid chromatography (Aspinall, 1982). Ideally absence of heterogeneity should be demonstrated by as many criteria as possible rather than chemical composition alone.

1.2 Methods used in the study of polysaccharides

The determination of properties of polysaccharides, which depends on the distribution of molecular size, molecular shape and the distribution of ionisable acidic residues, is important for the proper assessment of polysaccharides. The presence of protein components in polysaccharides may exert a disproportionate influence on the properties of the polymers (Euston and Hirst, 2000). In gum arabic, for example, it is the protein fraction that is responsible for the emulsifying properties of the gum (Chanamai and McClements, 2002).

1.2.1 Estimation of molecular weight of polysaccharides

The measurement of the molecular weight of polymers is an important method for characterising the polymers and assessing their purity. Despite the fundamental nature of molecular weight, the molecular weights of polysaccharides have proved very difficult to determine (White, 1991). A single determination of the average molecular weight or its distribution is often a difficult undertaking that takes a long time. Inability to determine molecular weight is a hindrance as knowledge of the molecular weights of polysaccharides is important for the understanding of their biotechnological applications and their role in living systems.

The difficulty in the determination of molecular weight of glycans arises because polysaccharides are polydisperse in nature. Although no discontinuities in structure and properties may

be observed, the polymers consist of molecules with a range of sizes, which makes it difficult to determine the molecular conformation of polysaccharides in solution.

Finally, at high concentrations some polysaccharide molecules may associate to form aggregates that give rise to an overestimation of molecular size.

Molecular weights of polysaccharides can be estimated by gel chromatography and viscometric methods. Gel filtration chromatography is a separation based on size, also called molecular exclusion or gel permeation chromatography. The stationary phase consists of porous beads with a well defined range of pore sizes (Motlagh *et al.*, 2000). The stationary phase for gel filtration is said to have a fractionation range, meaning that molecules within that molecular weight range can be separated. Polymers that are small enough can fit inside all the pores in the beads and are said to be included. These small polymers have access to the mobile phase inside the beads as well as the mobile phase between beads and elute last in a gel filtration separation. Polymers that are too large to fit inside any of the pores are said to be excluded. They have access only to the mobile phase between the beads and therefore elute first. Polymers of intermediate size are partially included, meaning they can fit inside some but not all of the pores in the beads. These polymers will then elute between the large and the small polymers. In gel chromatography, standards of known molecular weight are needed for preparing the calibration curves that are used in estimating the molecular weight of new polymers. Additionally, it is important to establish that the polymer under investigation behaves on the column in a manner that is similar to the behaviour of the standards, a requirement that is not easy to attain for a new polysaccharide.

The intrinsic viscosity of a polysaccharide in dilute solutions can be used to estimate the viscosity average molecular weight of the polysaccharide (Togrul and Nurhan, 2003). Intrinsic viscosity is a convenient index of the size or hydrodynamic volume of isolated polymer coils. Experimentally, intrinsic viscosity is determined by direct comparison of solution viscosity over a

range of low polymer concentrations with that of the solvent and extrapolating to infinite dilution (Avallone *et al*., 2000). For each polymer solvent system, intrinsic viscosity increases with molecular weight according to the Mark- Houwink relationship,

$$[\eta] = kM^a$$

Where the parameters k and *a* must first be determined experimentally by calibration against a primary molecular weight technique such as light scattering and then subsequently used for the routine determination of molecular weight from intrinsic viscosity measurements. The Mark- Houwink exponent *a* is directly related to the degree of molecular expansion and hence to chain flexibility (Hokputsa *et al*., 2003).

1.2.2. Determination of structure of polysaccharides

Acquiring information on the structure of polysaccharides is necessary in order to exploit the polymers most effectively (Fares *et al*., 2001). The determination of the complete covalent or primary structure of a polysaccharide is always a complex task in which answers must be given to questions of component monosaccharides, type of glycosidic bonds, pattern of branching and configuration of the monomers. Determination of the composition of a polysaccharide requires initial identification and quantitative estimation of sugar constituents. In addition, the sequence of monosaccharide units needs to be determined. It is also essential to determine patterns of branching especially in multi branched polysaccharide structures.

No one method of determining structure will give answers to all the questions for determination of the structure of polysaccharides. In addition, each method has limitations so it is always desirable to obtain information on particular aspects of structure using more than one method.

1.2.3 Analysis of the composition of polysaccharides

Determination of the monosaccharides that make up complex heteropolysaccharides, glycan chains and glycoconjugates is important for studying the biological and physicochemical properties of these polymers. Chromatographic methods such as thin layer chromatography, gas liquid chromatography and high performance liquid chromatography have been used in investigating the composition of complex carbohydrates, but these methods must be preceded by an enzymic or chemical hydrolysis step (Lu and Yoshida, 2003). In many cases, the limiting factor in the reliability of compositional analysis is the method that is used to hydrolyse the specific polymer. Polysaccharides can be made up of neutral sugars, uronic acids, amino sugars or other derivatives of monosaccharides such as sulphates. Because of the diversity in monosaccharide composition and consequent variation in the stability of associated glycosidic bonds, it is difficult to find optimal hydrolysis conditions.

1.2.3.1 Hydrolysis of polysaccharides with acids

Analysis of the composition of polysaccharides has typically been based on hydrolysis procedures using hydrochloric, sulphuric or trifluoroacetic acid, at high temperatures (Goubet *et al*., 2002; Dourado *et al*., 2000). Trifluoroacetic acid has become the preferred acid for most carbohydrate analyses due to its effectiveness in hydrolysing glycosidic bonds without causing extensive destruction of the resulting monosaccharide components and due to its volatility, which makes removal of acid after hydrolysis easy. Factors that affect the release of monomers during hydrolysis include the nature of the monomer, temperature, and duration of treatment.

The presence of carboxyl groups makes the hydrolysis of glycosidic linkages more difficult because of the unusual stability of the glycosyl uronic acid linkage (Jung *et al*., 2000). The uronic acids liberated upon hydrolysis are themselves much more susceptible to degradation, which makes identification of the uronic acids after hydrolysis difficult. Methyl groups, which often occur ether-linked to the uronic acid especially in plant gums and hemicelluloses, resist hydrolysis by mineral

acids, even in the autoclave. Such groups resist hydrolysis conditions that are usually chosen for hydrolysis of polyuronides. The furanoside bond of glycosides is easily split whereas the pyranoside bond survives the mild treatment that breaks the furanoside bonds. Glycosidic linkages of α configuration are seemingly to be more easily ruptured than the β linkages.

Procedures that are commonly applied for identification and quantification of the liberated sugars have disadvantages, mainly originating from the necessity for derivatisation of the monosaccharide to alditol acetates and trimethyl silyl ethers for gas chromatography, or inadequate separation of the monosaccharides by HPLC. In addition, the effectiveness of the method used for hydrolysis of a polysaccharide cannot be assessed after derivatisation, as oligomers resulting from incomplete hydrolysis often escape detection. An improvement in the sensitivity of analysis of monosaccharides can be achieved using high performance anion exchange chromatography. With this method carbohydrates can be analysed accurately without derivatisation with a sensitivity of 0.1 nmol per injected amount (Suni *et al*., 2000).

1.2.3.2 Hydrolysis of polysaccharides with enzymes

In investigating the structure of polysaccharides, enzymes may be used to cleave acid resistant linkages in order to generate oligosaccharides whose bonds are more susceptible to acid treatment than the parent polymer. When hydrolysis with enzymes is combined with fractionation and analyses by chromatographic methods, quantitative recovery, characterisation of fragments and determination of glycan structure is possible (Fares *et al*., 2001).

Endo-enzymes degrade polysaccharides by cleaving internal positions in an essentially random fashion yielding oligosaccharides. The proportion and size of released oligosaccharides, which range from small oligosaccharides to those approaching the starting material, depends on the enzyme,

substrate and conditions of the reaction (McCleary and Matheson, 1986). Endo-acting enzymes may act on unbranched or branched regions as substrates because they require a number of binding sites.

In contrast to endo-enzymes, exo-acting enzymes degrade polysaccharides from the non-reducing end releasing mono or oligosaccharides until a structural feature, such as branching, that prevents further action is reached. Glycanases are exo-enzymes that act on polysaccharides to sequentially release di-, tetra- and hexasaccharides (Acebes and Zarra, 1993). The rate of hydrolysis with glycanases increases with the degree of polymerisation of the substrate. Glycosidases primarily recognise the terminal sugar whose glycosidic bonds will be cleaved by their action (Matheson and McCleary, 1985).

The action of enzymes that degrade polysaccharides may be followed by measuring the increase in reducing activity, which is proportional to the number of monosaccharide residues released or by measuring the decrease in viscosity (Schmeter *et al*., 2002). The rate of decrease in viscosity depends on the position of hydrolysis with cleavage of bonds near the middle of the polymer chain giving rise to a greater change in viscosity than cleavage at the end of the chain.

Care is required when using enzymes for analysis or modifying polysaccharides for technical use as the enzyme of interest may be contaminated with other carbohydrate degrading enzymes. Crystallinity is no guarantee for single function.

The enzyme may be inhibited by the products of the reaction, which may lead to incomplete reaction. Inhibition by reaction products may be minimised by working out a suitable dilution and, if one product is still polymeric, by dialysing and ultrafiltration during reaction.

In procedures that require long incubation periods, microbial and fungal contamination should be stopped by the addition of suitable agents. Denaturation by heat, pH extremes, heavy metal ions as well as microbial infection of stored enzyme preparations should be avoided. For optimum action of enzyme, any activators should be included in the reaction mixture.

A further complicating factor in the use of enzymes is that the products of the hydrolysis of polysaccharides may, at high concentrations, undergo transglycosylation reactions that lead to products not originally present. Upon subsequent analysis, products of transglycosylation give rise to misleading results concerning the nature of the native polymer.

In their role of protecting plants from bacterial or fungal attack plant polysaccharides are expected to be resistant to enzymatic degradation (Stephen, 1995). It is not surprising, therefore, that attempts to hydrolyse polysaccharides with enzymes, most of which are of microbial origin, have not been entirely successful.

1.2.3.3 Analysis of carbohydrates by Thin Layer Chromatography (TLC)

Thin layer chromatography is a method of separating a mixture into its various components by making use of a heterogeneous equilibrium that is established when a solvent flows over a fixed stationary phase. In TLC, capillary action in the finely divided particles of the stationery phase causes the mobile phase to move up the plate. The layers in the stationery phase usually contain water that is adsorbed onto the surface during manufacture or exposure to the atmosphere. The bound water acts as the stationary liquid phase, partitioning the sample components between it and the mobile phase (Touchstone, 1992). The mobile phase is usually a cocktail of organic solvents with a greater or lesser proportion of water. The separation of a compound takes place based on non-bonding interactions in the stationary and mobile phases. Generally, a solvent or a solvent mixture of the lowest polarity consistent with a good separation is used. Suitable mixing of solvents gives mobile phases of intermediate eluting power, but it is best to avoid mixtures of more than two components as much as possible, as more complex mixtures readily undergo phase changes with changes in temperature. When mixtures of solvents are used, care is necessary over equilibrium. Excessive volatility in a

solvent is undesirable, as great care is then required during equilibration of the mobile phase. On the other hand high volatility makes for easy removal of the solvent from the sheet after the separation run.

> The purity of solvents is of much greater importance in TLC than in most other forms of chromatography because of the small amounts of material involved. The solvents that are used in TLC separations should be reasonably inexpensive, as large amounts are often consumed and must be obtainable in high levels of purity.

The sorbent is applied to a support as a coating to obtain a stable layer of suitable thickness. The most common support is a glass plate, but other supports such as plastic and aluminium foil are also used. The four sorbents most commonly used are silica gel, alumina, kieselguhr and cellulose. Silica gel, slightly acidic in nature, is the most popular layer material. In order to hold the silica gel firmly onto the support, a binding agent such as calcium sulphate is commonly used. The binding agent may be omitted if the silica gel employed has a very small particle size. Fine particles will adhere well to the support without a binder, as in high performance TLC.

Alumina, which is basic, is also widely used as a sorbent. For a given layer thickness, alumina will not separate quantities of material as large as can be separated on silica gel. Alumina is more chemically reactive than silica gel and care must be exercised with some compounds and compound classes in order to avoid decomposition or rearrangement of the substances during sample application or development. The amount of water in the support material greatly affects the chromatographic behaviour of alumina, for control, the plates may be heated at specific temperatures before use.

Diatomaceous earth or kieselguhr is a chemically neutral sorbent that usually have wide pores and does not separate or resolve mixtures as well as either alumina or silica gel.

Cellulose is used as the sorbent in TLC when it is convenient to perform a given paper chromatographic separation by TLC so as to decrease the amount of time necessary for the separation and increase sensitivity of detection. Many separations achieved by paper chromatography can be directly transferred to TLC on cellulose. Thin layer chromatography is useful when only a small amount of sample is available, as TLC does not generally require as much sample as paper

chromatography. The primary separation mechanism is partition, where the cellulose becomes a support for a stationary phase of water adsorbed from the atmosphere. Cellulose is usually coated onto a plate without a binder as its particles adhere well to the support without a binder.

Two ultraviolet indicators, which aid in the location of separated substances, can be incorporated either singly or together in silica gel or other layer materials. Zinc silicate fluoresces when exposed to ultraviolet light of 254 nm wavelength, so that substances absorbing this wavelength will contrast sharply by appearing dark through quenching of the greenish yellow fluorescing background.

The manner in which substances and eluents behave in a given chromatographic system is determined by their polarity (Hans and Robyt, 1998). Non-polar substances are held only loosely by the stationery phase and, consequently, can be chromatographed with slightly polar solvents such as pyridine and butanol, while the separation of highly polar substances requires use of more polar solvents, for example methanol and water. The polarity of solvents in TLC is typically expressed in an eluotropic series in which the solvents are arranged in order of increasing polarity as indicated by their dielectric constant as shown in table 1 (Claus and Fischer, 1988).

Table 1: **Eluotropic series for TLC solvents**

Solvent	Dielectric constant (at 25 ^{o}C)
Hexane	1.89
Cyclohexane	2.02
Carbon tetrachloride	2.24
Benzene	2.28
Toluene	2.38
Acetonitrile	3.88
Diethylether	4.34
Chloroform	4.87
Formic acid	5.00
Ethyl acetate	6.02
Acetic acid (glacial)	6.15
Dichloromethane	10.9
Pyridine	12.3
Butan-2-ol	15.8
Propan-2-ol	18.3
Acetone	20.7
Ethanol	24.3
Methanol	33.6
Water	78.3

1.2.3.4 Analysis of carbohydrates by High Performance Liquid Chromatography (HPLC)

HPLC is a non-destructive technique that can be used to obtain high-resolution separations of complex carbohydrates. The method can serve as a preparative method for the efficient isolation of molecules that can subsequently be identified by chemical or instrumental means. The liquid chromatography technique has the advantage that no sample derivatisation is required.

HPLC separations can be carried out on a variety of stationary phase columns such as cation exchange resins, amine bonded microparticulate silica gels, reverse phase alkylated silica or a hydroxylated polymeric support. Developments in the instrumentation, automation and computerisation methods for the detection and quantitation of sugar peaks separated by HPLC have transformed the method into a versatile, sensitive, rapid analytical technique (Hicks, 1988).

Effective separations of simple oligosaccharides have been achieved on columns packed with aminopropyl bonded silica gel, aminocyanopropyl bonded silica gel, pure silica gel, amine modified silica gel, reverse phase silica gel, polystyrene based and anion exchangers. The polystyrene based anion exchangers are used in partition, ion-chromatographic mode and cation exchange resins in Ca^{2+} or Ag^{+} forms (Marry *et al.*, 2000). Aminopropyl and cyanopropyl silica gel columns both allow the rapid separation of neutral oligosaccharides in ascending order of molecular weight when aqueous acetonitrile is used as mobile phase.

When silica gel columns that are modified with amine are eluted with a mobile phase of acetonitrile-water, monosaccharides are separated based on normal phase partitioning (Berthod, 1991). Increasing the water content of the mobile phase speeds up the elution of the sugars but results in reduced resolution of peaks.

A useful silica derivative, diol modified silica gel, appears to function like aminopropyl silica gel but is more robust than normal silica and can be used for the separation of pentoses and hexoses (Goff *et al*., 2001).

For analysis of mixtures of simple uronic acids, methods based on ion-exchange chromatography are rapid (Missang *et al*., 2001). When several uronic acids exist in the same sample, separations are performed on resin or silica based, strong anion exchange columns (Suni *et al*., 2000). For the separation of mannuronic and guluronic acids, which exist in hydrolysates of alginates, liquid chromatography on strong anion exchange silica gel columns has been recommended (Suni *et al*., 2000).

Complex, ionic oligosaccharides and glycopeptides include, sialylated or phosphorylated oligosaccharides, glycopeptides, acidic aminoglycan and acidic plant cell wall oligosaccharides. Strong anion exchange silica gels, and strong anion exchange resin beads are used in the separation of sialylated oligosaccharides according to the number of sialic acid groups bound (Volpi, 2004).

Alkylated or reverse phase silica gels are useful for the analysis and preparation of carbohydrate derivatives. When water is used as the mobile phase, reverse phase silica gel columns may be used for the analysis of the more non-polar or higher molecular weight carbohydrates, such as methyl glycosides, chitin or starch derived oligosaccharides and glycopeptides (Martin and Briones, 2000). Reverse phase columns are generally very stable and do not dissolve in the aqueous mobile phases that are used (Hicks, 1988). The disadvantage of reverse phase columns is that they resolve the anomers of reducing sugars, which leads to complex peaks.

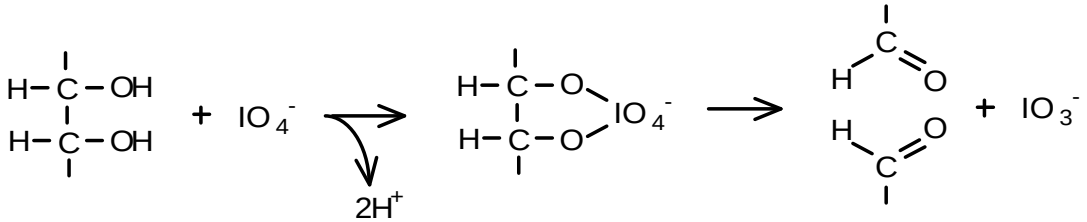
1.2.3.5 Refractive index detection during HPLC

Refractive index detectors, are the most commonly used detectors for liquid chromatography analysis of substances such as, simple sugars, alditols and oligosaccharides, which do not absorb in the UV region. Refractive index detectors detect differences in the refractive index between the sample and the solution reference in the cells by measuring the degree of change in the angle of an incident light beam (Sanchez *et al*., 2000). As the light beam is deflected across the detector, an electrical signal is generated and the degree of deflection is proportional to the concentration of sample. There is a facility that allows the user to move the beam to zero signal before analysis.

Refractive index detectors are relatively inexpensive and are regarded as universal detectors because they detect almost all classes of molecules present in a mixture and are especially useful in preparative chromatography. Despite their common use, refractive index detectors may be sensitive to temperature and pressure.

1.2.4 Oxidative hydrolysis of carbohydrates by periodate

When molecules that contain vicinal hydroxyl groups are treated with periodate or its salts, cleavage of the carbon chain takes place with the formation of two aldehyde groups. In the process one molecular proportion of periodate is consumed. The selectivity of the reaction probably arises from the ability of the periodate to form a cyclic intermediate with the vicinal groups, as illustrated below (Fredon *et al*., 2002)



Oxidation with periodate is limited to 1,2 glycols, 2-amino alcohols, α hydroxy ketones and aldehydes, α-amino aldehydes, 1,2 diketones, and certain activated metylene groups. The common features of all periodate reactions are cleavage of C-C bond between the vicinal groups, oxidation of each carbon atom to the next higher oxidation state and reduction of one mole of periodate to iodate for each bond cleaved.

In practice, the polysaccharide is usually oxidised in a dilute solution of sodium periodate at temperatures below 5 ^{o}C, with the production of formic acid and the consumption of periodate being followed at intervals. The concentration of periodate is followed by titrimetric procedures or by a spectrophotometric method. Formic acid is determined by direct titration with standard alkali or by manometric procedures or spectophotometric procedures. The mild conditions of the reaction with periodate are especially well adapted for application to the sensitive carbohydrate structures. The aqueous solvent conditions used in periodate oxidation lend themselves well to use with the water-soluble carbohydrates. Because inorganic salts of periodate are insoluble in water, their removal from the reaction mixture is simplified thereby facilitating isolation of the organic products.

Oxidation of a polysaccharide, quantitative determination of the periodate consumed, the formic acid generated, and the determination of the proportion of surviving sugar units will give information concerning the nature and proportion of the glycosidic linkages present in a polysaccharide. Aldoses are oxidised to give formaldehyde from terminal groups and formic acid from the other carbon atoms. In the case of αβγ-triols, which contain three neighbouring hydroxyl groups, a double cleavage of the carbon chain occurs with the formation of two aldehyde groups, consumption of two moles of periodate and the liberation of one mole of formic acid. Units that do not possess adjacent hydroxyl groups such as units involved in branching at C-2 and C-4 are not affected by periodate (Dervilly *et al*., 2004).

The kinetics of the reactions of polymers with periodate are often very complex because the reaction of one unit or linkage in a given chain can modify the reactivities of other units or linkages in the same chain (Strli *et al*., 2003). In flexible coils, the modification of reactivity is likely to be short ranged, and the reactive sites immediately adjacent to those attacked will be most affected (Aman and Bengtsson, 1991). When amylose, xylan and guaran are oxidised in aqueous sodium metaperiodate, the aldehyde groups of oxidised sugar residues spontaneously form six membered hemiacetal rings with the nearest hydroxyl groups on neighbouring unoxidised residues in the same chain (Christensen *et al*., 2001; Dervilly *et al*., 2003, Ishak and Painter, 1974). These hemiacetals exist in rapid equilibrium with the free or solvated aldehydic forms and with intra residue hemiacetals or hemialdals that may be formed. Periodate oxidation takes place in two stages, that consist of an initial rapid stage producing chains in which every oxidised unit has at least one unoxidised unit in an adjacent position. In the slow final stage, the remaining unoxidised units are oxidised at a rate that is diminished to an extent determined by the position of the equilibrium of the reaction (Christensen *et al*., 2001).

With guar gum, which contains residues of galactose and mannose in the ratio of 1:2, complete oxidation of the galactose residues was accomplished fairly rapidly but only about half of the mannan backbone was oxidised rapidly (Ishak and Painter, 1974). The remaining 50% of the mannose residues were extremely resistant to oxidation, with prolonged treatment with a 12 fold molar concentration of periodate failing to raise the oxidation limit to above one mol/hexose residue. The oxidation limit remained constant as the oxidised mannose residues were forming stable hemiacetals with adjacent unoxidised mannose residues making them unavailable for oxidation.

1.3 Plant gums of commercial importance

Gums are high molecular weight polysaccharides that are soluble or dispersable in water usually with a thickening, stabilising or gelling effect (Diaz and Navaza, 2003). The polymers are made up of a selection of monosaccharide units linked as glycosides in a limited number of modes of anomeric configuration and position of attachment. Glucose, galactose, mannose, arabinose and xylose are the most commonly occurring monosaccharides in plant polysaccharides. Incorporation of acidic sugars in gums results in improved solubility and the ability to bind cations. In addition introduction of charged sugars has effects on viscosity, and ability to form gels resulting from changes in pH of the dispersing medium such as water. Gums may be found associated with protein or terpenoid material (Sims and Furneaux, 2003).

One of the many ways of classifying commercially important gums of plant origin is shown in table 2. Pectins and starches are obtained from suitable plant materials by extraction with water or other appropriate aqueous solvents. Gums arabic, tragacanth, karaya and ghatti are obtained as exudates by tapping. Seed gums are obtained from the endosperms of seeds and tend to be expensive because of the labour involved in processing the seeds. Alginate, agar and carrageenan are obtained from seaweeds by extraction with water and appropriate aqueous solvents.

Table 2: Classification of plant gums (Lazaridou *et al*., 2001)

Plant extracts	Plant exudates	Seed gums	Seaweed extracts
Pectin	Arabic	Locust bean gum	Agar-agar
Starches	Tragacanth	Guar gum	Alginate
	Karaya	Tamarind	Carrageenan
	Ghatti		

1.3.1 Plant extracts

Pectin, comprises a family of complex polysaccharides that are present to a greater or lesser extent in primary cell walls of plants. These anionic polysaccharides have 1,4 linked α -D-galactosyluronic acid as the basic structural unit (Thomas *et al*, 2003; Ridley *et al*., 2001). Three pectic polysaccharides as shown in figure 1, homogalacturonan, rhamnogalacturonan I (RGI) and substituted galacturonans (SG) have been isolated from primary cell walls and structurally characterised (Fares *et al.*, 2001). Homogalacturonan is a linear chain of galacturonic acid residues in which some of the carboxyl groups are methyl esterified.

Rhamnogalacturonan I is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide -4-α-D-galacturonosyl -(1,2)-α-L-rhamnopyranosyl residues. The backbone galactopyranosyluronic acid residues may be O-acetylated on C2 or C3 (Ridley *et al*., 2001).

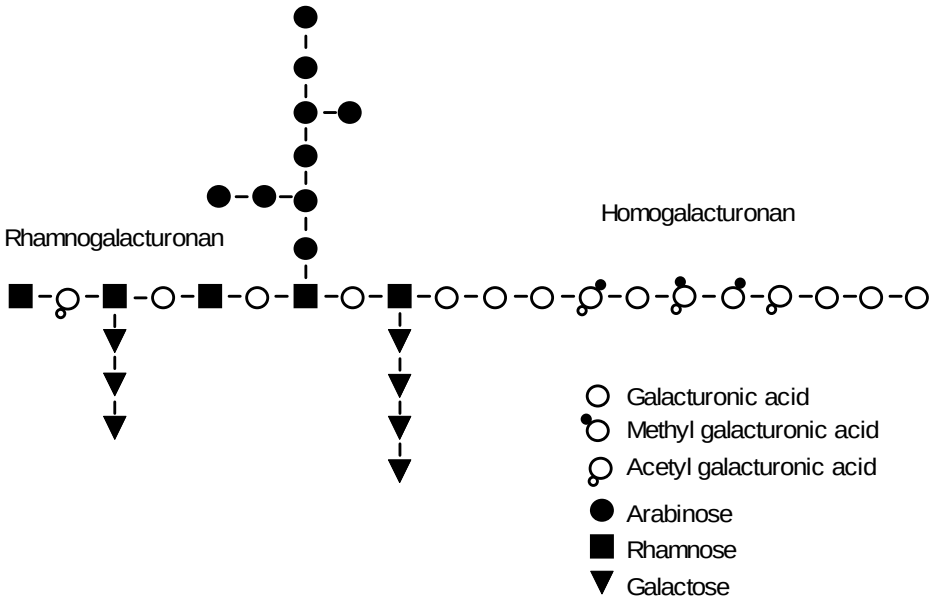


Figure 1: Schematic diagram showing the homogalacturonan and rhamnogalacturonan regions of pectin Redrawn after E. Bonnin, INRA..

The predominant side chains of RGI contain linear and branched α-L-arabinofuranosyl and/or β-D-galactopyranosyl residues, although the relative proportions and chain lengths may depend on the plant source. The glycosyl residues α-L-fucosyl, β-D-glucuronosyl and 4 -O-methyl β-D-glucuronosyl may also be present in the side chains.

Substituted galacturonans is a diverse group of pectic polysaccharides that contain a backbone of linear 1,4-linked α-D-galactopyranosyluronic residues. The locations on the backbone of SG of side chains with respect to one another have not been established with certainty. An octasaccharide side chain A, and a nonasaccharide side chain B, are attached to C2 of some of the backbone galactopyranosyluronic acid residues and two structurally different disaccharide side chains C and D are attached to C3 of the backbone (Ridley *et al*., 2001).

Pectin can be obtained by extraction with aqueous solutions at low pH, from appropriate plant material such as the peel of lemon and lime and, to a minor extent, orange and grapefruit (Panchev and Karageorgiev, 2000).

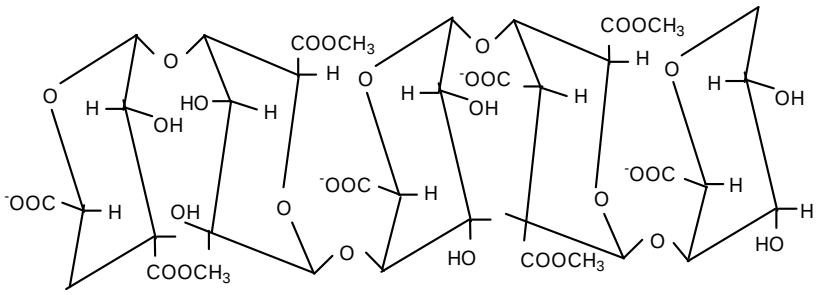


Figure 2: A segment of the pectin molecule

Pectins are partly esterified as shown in figure 2, and the ratio of esterified galacturonic acid groups to total galacturonic acid groups, termed the degree of esterification, greatly influences the properties of pectin, including the solubility and gel forming properties (Barnavon *et al.,* 2001). Commercial low-methoxy pectins are obtained by treatment of highly esterified pectin with acid or ammonia after extraction and have a degree of methylation of less than 50% whereas high-methoxy pectins have a degree of methylation greater than 50%.

High methoxy pectins form gels under conditions of low pH and when the amount of soluble solids present is high (Avallone, 2000). In contrast, the gelation of low-methoxy pectins can take place over a wide range of pH and soluble solids but requires the presence of calcium ions or other polyvalent cations (Cardoso *et al*., 2003). The gels formed by low-methoxy pectins are softer and more elastic than those produced with high-methoxy pectins.

High-methoxy pectins have been used in conventional jams and have gained ground in confectionery jellies at the expense of agar. Low-methoxy pectins have been used in jams with

reduced sugar content (Schmelter *et al*., 2002). Jams with reduced sugar content are becoming increasingly popular with nutrition-conscious consumers.

Sold in powder form and mainly used as a gelling agent pectin must be completely dissolved to ensure full utilisation and to avoid formation of gels that are not uniform (Zhou *et al*., 2000). Solutions of pectin show lower viscosity than that of other plant gums and thickeners.

1.3.2 Plant exudates

Many plants, particularly those that grow under semi-arid conditions, produce gummy exudates when their bark is damaged with such exudates apparently serving to seal the wound. The exudate often dries up to a hard glassy solid and is produced in sufficient abundance by some species of trees or shrubs for collection and commercial utilisation (Biswas and Phillips, 2003). Gum exudates, are unique among hydrocolloids in that they are readily obtainable in relatively pure, undegraded form, in contrast to most of the related hemicelluloses, which often require drastic conditions for isolation and purification, with resultant degradation. It is still necessary, however, to purify exudates further to ascertain homogeneity prior to chemical investigation. Commercial gum exudates that have been used industrially and studied include gum arabic, ghatti, tragacanth and karaya.

1.3.2.1 Gum arabic

Commercial gum arabic is a highly branched, uronic acid type heteropolysaccharide produced as an exudate from *Acacia senegal*. As shown in figure 3, the gum contains a (1-3) β-D-galactan core with some alternate branch points. Neutral side chains of D-galactopyranose and L-arabinofuranose or arabinopyranose oligosaccharides are located at positions 3 of the galactopyranose residues. Side chains consisting of acidic residues are attached at positions 6 of D-galactopyranosyl residues (Sanche *et al*., 2002). Considerable variation in sugar composition, structure and properties of gum arabic exists between gums from different geographical regions. Complete structural elucidation of *Acacia* gums has proven difficult due to their highly complex structure in which branches may themselves, in turn, be branched.

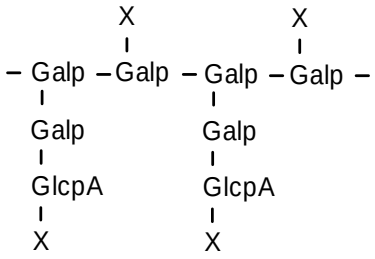


Figure 3: Partial structure of gum arabic where X represents L-rhamnose or L-arabinofuranose

Gum arabic has a molecular weight of around 250 000 Daltons. Both the molecular weight and composition of the gum depend on the species from which the gum is obtained. In solution, the gum arabic molecule is thought to exist as a somewhat rigid spiral, the length of which varies with the charge on the molecule (Sanche *et al*., 2002). Gum arabic is unique among the natural hydrocolloids because of the combination of its extremely high solubility in water and low viscosity of its

concentrated solutions. The highly branched nature of the gum accounts, in part, for the low viscosities of solutions of gum arabic despite the high molecular weight. The viscosity of solutions of gum arabic rises sharply with increasing pH to a maximum at about pH 5 to 7, after which the viscosity decreases.

The solubility, viscosity properties and protein content of gum arabic impart to the gum its well known stabilising and emulsifying properties (Chanamai and McClements, 2002). In ancient Egpty gum arabic was used in the embalming of mummies (Motlaph *et al*., 2000). Historically gum arabic has been used as a binder and medium for colourants and inks. Gum arabic is used in the pharmaceutical industry as an adhesive and a binder in tablets. In the cosmetics industry the gum is used as a stabilizing and emulsifying aid in protective creams and lotions. In the textile industry gum arabic is used in finishing silk and rayon.

Currently gum arabic is used in the food industry for emulsification and for stabilisation in prepared flavouring products, such as microencapsulated citrus flavours and in health foods. Gum arabic is used as a coating for confectionery and bakery preparations and in soft drinks as a suspension (Sanche *et al*., 2002).

1.3.2.2 Gum ghatti

Gum ghatti or Indian gum is an exudate from the tree *Anogeisus latifolia* a large tree growing widely in India and Sri Lanka and a member of the family combretacae (Glicksman, 1988).

The structure of gum ghatti is based on a central chain of 1,2-linked β-D-mannopyranose and 1,4- linked β-D- glucuronic acid residues, as shown in figure 4. Attached to D-mannose residues of the central chain at C3 and C6 positions are short chains of 6-O-substituted β-D-galactopyranose residues linked through 3-O- substituted L-arabinopyranose units and side chains composed of L-arabinofuranose units. Gum ghatti also contains xylose and minute amounts of rhamnose.

The properties of gum ghatti are similar to gum arabic but its solutions are more viscous and less adhesive. Although gum ghatti is non-gelling it can be dispersed in hot or cold water to give colloidal sol. Solutions of gum ghatti have maximum viscosity between pH 5 to 7.

Gum ghatti is not widely applied in food systems because the supplies and quality are unreliable and because only a small amount of the gum is available worldwide.

Food uses of gum ghatti are based primarily on the emulsifying properties of the gum. The gum has been used in combination with lecithin in pancake that contain butter and waffle syrups (Glicksman, 1988). In waffle syrups the gum helps to produce a clear product by modifying the refractive index of the syrup.

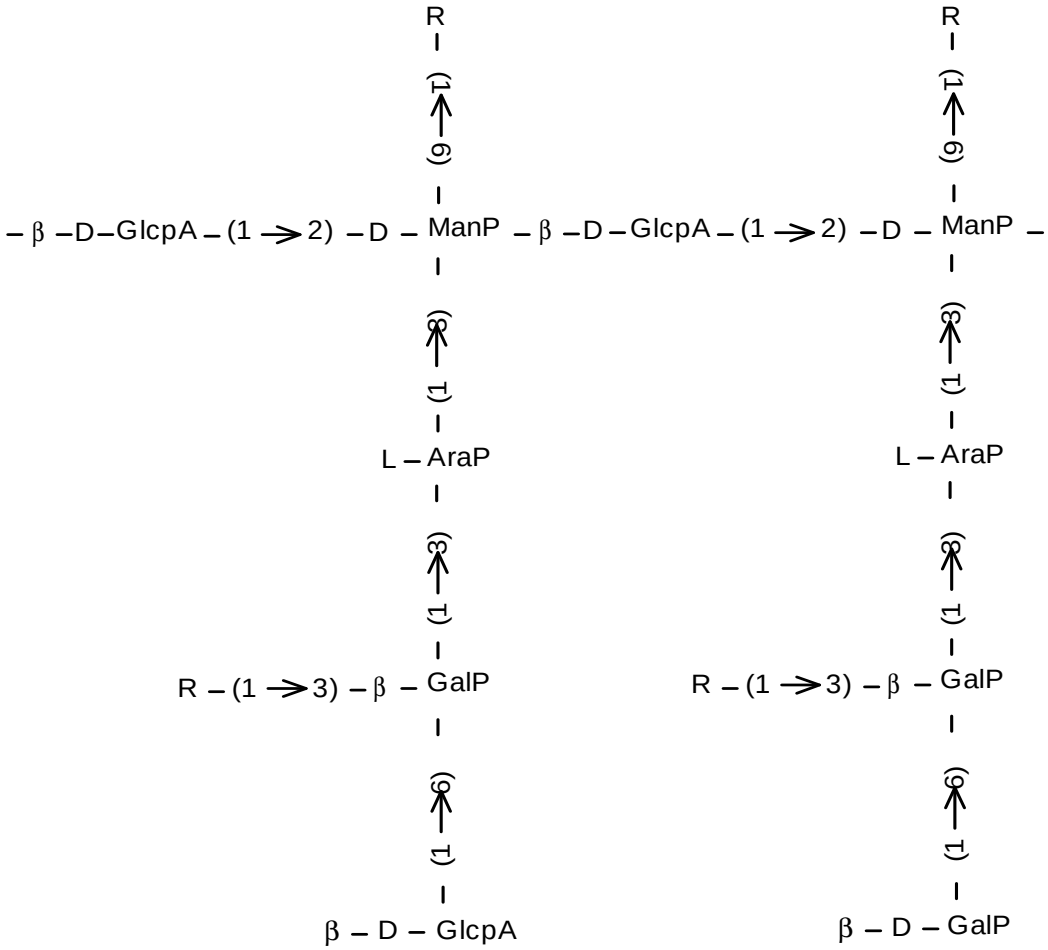


Figure 4: Repeating unit of gum ghatti where R represents L-arabinose or L-rhamnose

1.3.2.3 Gum tragancanth

Gum tragacanth is an exudate from *Astragalus gummifer*, a bush plant that is found in the dry mountainous regions of Iran, Syria and Turkey. The gum is obtained in the form of ribbons and flakes, the latter being of inferior quality as well as of smaller particle size (Stauffer and Andon, 1975).

Gum tragacanth is composed of a mixture of polysaccharides, tragacanthic acid, a water insoluble component, that confers water swelling properties of the gum and arabinogalactan, a water soluble component that gives the gum its solubility (Dziezak, 1991). A shown in figure 5, tragacanthic acid is composed of D-galacturonic acid, D-xylose, D-fucose, D-galactose and trace amounts of L-arabinose. The L-arabinogalactan component is composed of L-arabinose and D-galactose and contains small amounts of L-rhamnose and galacturonic acid. Tragacanthic acid is structurally related to the pectic D-galacturonans in having a galacturonic acid core.

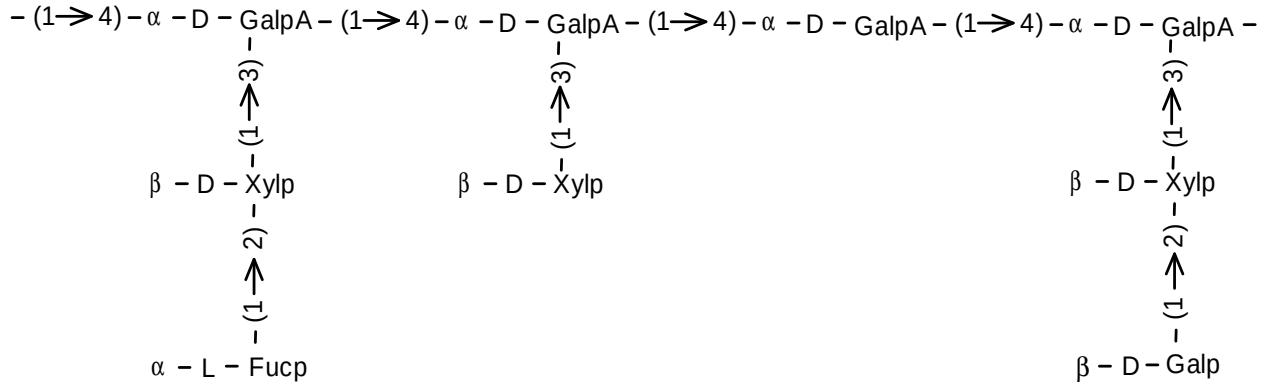


Figure 5: Structure of tragacanth acid

Gum tragacanth is used as a thickener that swells and dissolves in cold water and has the two outstanding characteristics of being able to form solutions of very high viscosity and being stable under strongly acid conditions. Gum tragacanth is an effective stabiliser and thickener in highly acid food

products such as salad creams and dressings. The gum is used as a binder and emulsion stabiliser in pharmaceutical products. Gum tragacanth is a suspending agent with extremely long shelf life. The gum is used in toilet creams, lotions, dental creams and cigars. In the textile industry the gum is used to stop wet ink from running by acting as a stabiliser.

Partly because it is not easy to prepare gum tragacanth of sufficiently low bacterial loads, the gum has been steadily replaced by xanthan gum and derivatives of starch in various applications.

1.3.2.4 Gum Karaya

Gum Karaya is obtained from *Sterculia curens*, a tree native to India (Silva *et al*., 2003). The gum, whose structure is shown in figure 6, consists of a main chain comprising D-galacturonic acid, L-rhamnose and D-galactose units with side chains containing D- glucuronic acid. The D-glucuronic acid side chain units generally occur singly attached to the main chain via a D-galactose residue and are also present as the 4-O-methyl ether derivative. Gum karaya contains about one O-acetyl group for every three sugar units.

Gum Karaya is less soluble than the other exudate gums but is the most adhesive (Silva *et al*., 2003). Gum karaya is often regarded as a replacement for gum tragacanth although its heat and acid stability properties are less than for gum tragacanth. The maximum viscosity of gum karaya is reached at about pH 8.5 and decreases when acids or electrolytes are added.

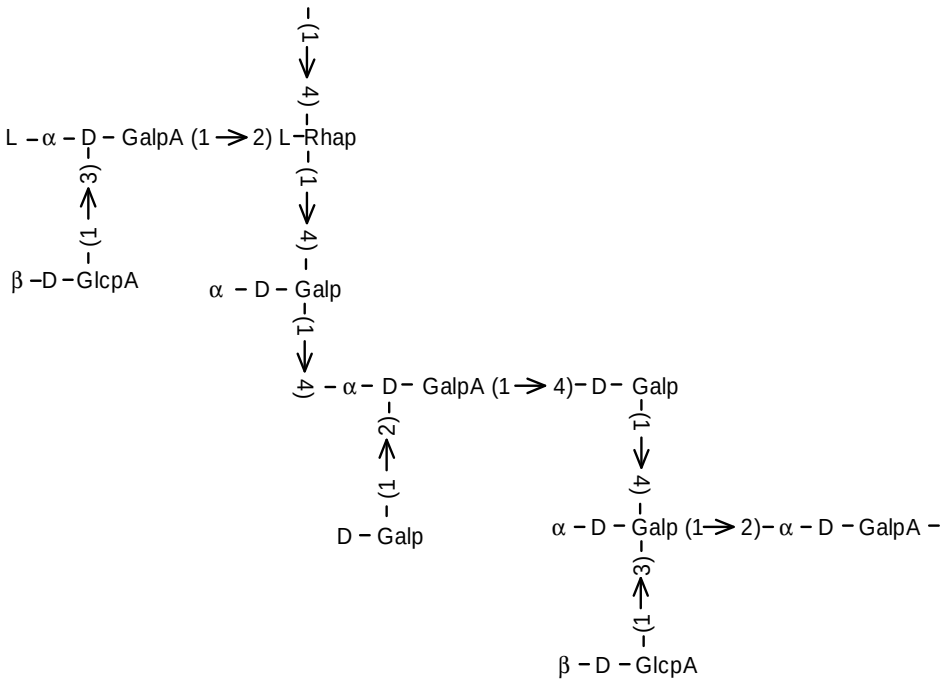


Figure 6: Proposed structure of gum karaya

Gum Karaya has been used as an emulsifying agent and, at times, has been used with gum arabic and gum tragacanth to improve emulsifying ability through the synergistic effects of the separate gums. The gum is used to a large extent in the medical field in laxatives. The gum has been used in hair wave lotion and in ice creams. The gum has been largely replaced by alginates, agar and carboxymethylcellulose.

1.3.3 Seed gums

The endosperm of seeds of leguminous plants often contains substantial amounts of D-galacto-D-mannans, which function as food reserve material. The galactomannans present in guar seeds and in carob or locust bean are valued for their thickening and emulsifying properties (Patmore *et al.*, 2003).

1.3.3.1 Guar gum

Guar gum is derived from the seeds of the guar plant *Cyanopsis tetragonolobus*, a pod-bearing legume grown commercially in Pakistan and India. Guar gum, whose structure is shown in figure 7, is a galactomannan, of molecular weight of about 220 000 Daltons, that comprises a straight chain of D-mannose units, joined by β-D-1,4- linkages with a D-galactose side chain on approximately every other mannose unit (Singh *et al*., 2003). Although the ratio of mannose to galactose has long been known to be 2: 1, the fine details of the sequence of galactose residues are still uncertain.

The most important characteristic of guar gum is the ability to hydrate rapidly in cold water to form a very viscous colloidal dispersion (Wang *et al*., 2003). Hydration of the gum is improved by heating. Solutions of guar gum are non-gelling and the gum is used chiefly as a viscosity builder, stabiliser and water binder. Solution of the gum show a pseudoplastic behaviour on its own and in the presence of carrageenan.

Guar gum is stable over a wide range of pH from 3.5 to 10.5. Although the gum is recognised as a safe food additive and can be used without restriction, bacterial contamination is a regular problem. Guar gum is used in pet food, soups and sauces, to improve texture in ice cream, baby food, canned foods, soft cheeses, pie and pudding fillings, instant mixes and bakery products (Ribotta *et al*., 2004; Patmore *et al*., 2003). Guar gum is used in pharmaceutical products such as medicinal

suspensions and tablets, as an appetite depressant, for management of peptic ulcers and in cosmetics and diabetic products (Rayment and Ellis, 2003).

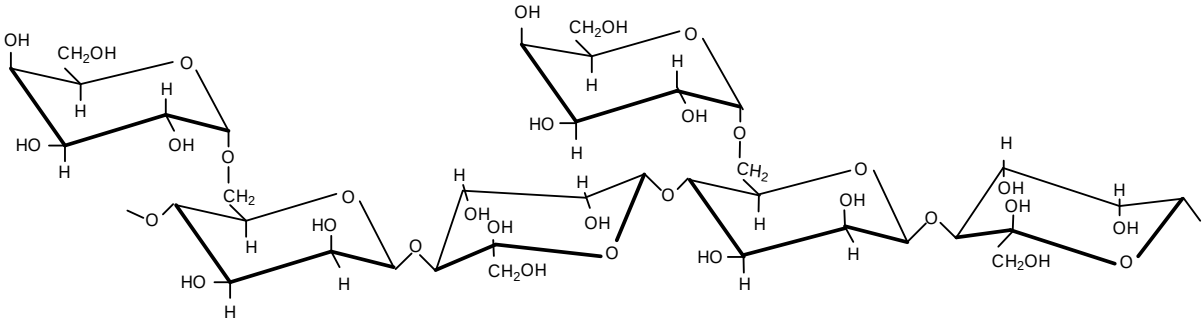


Figure 7 : Partial structure of guar gum

Estimated technical non-food applications of guar gum account for about 65% of gum consumption, in textile, printing, papermaking, water treatment and explosives (Sandford and Baird, 1983). Food applications account for about 30% use and pharmaceuticals, and cosmetics for 5%. In the main countries of production particularly Pakistan and India, guar is used as cattle feed.

1.3.3.2 Locust bean gum

Locust bean gum, also known as carob bean gum, is a galactomannan that is refined from the endosperm of the seed of the carob tree, *Ceratonia siliqua* of the family leguminosae and grows well in poor, rocky soil in which many other plants would not survive (Lazaridou *et al*., 2000). Carob bean gum, with a molecular weight of 310 000 Daltons, is a neutral polysaccharide consisting of a β-(1-4)-mannan backbone to which single D-galactopyranosyl residues are attached via (1-6) linkages, as shown in figure 8.

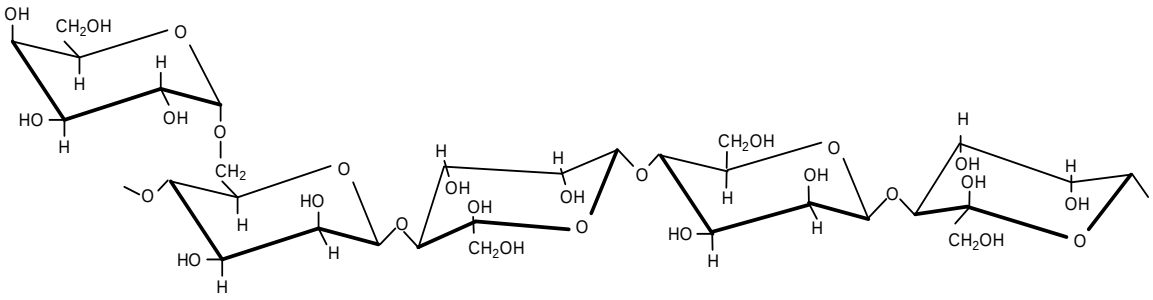


Figure 8 : Structure of locust bean gum

Locust bean gum produces solutions of high viscosity at low concentrations and has synergistic action with gelling agents, such as kappa carrageenan and agar (Diaz and Navaza, 2003). Locust bean gum is not totally soluble in cold water and is fully soluble only at temperatures above 85 ^{o}C. Solutions of locust bean gum are hardly affected by pH changes in the range of 5 to 8, but viscosity falls outside this range.

Locust bean gum possesses excellent stabilising, thickening and emulsifying properties and is used in ice creams, cream cheeses, instant soups, baby foods and canned pet foods (Patmore *et al*., 2003). The texturising properties, in particular, are of great value and hard to replicate fully by using other products (Lazaridou *et al.,* 2000). A solution of locust bean gum with xanthan gum in certain proportions has both thickening and gelling properties.

Although technically superior to guar and other gums, locust bean gum has been substituted by gums such as guar because it is too expensive.

1.3.4 Seaweed polysaccharides

Marine algae contain polysaccharides as components of their cell walls and membranes or in intracellular regions where the polymers serve as structural and food reserve material (Lee *et al*., 1998).

The commercially interesting seaweed gums of the Rhodophaceae, red seaweeds and phaeophyceae, brown seaweeds are used in a variety of applications, including foods and pharmaceuticals.

1.3.4.1 Alginates

Alginate is the dried colloid obtained from species of Phaeophyceae or brown algae or seaweeds that are found along rocky coasts of Europe, Japan and North America. Each company produces a unique type of (Roberts *et al*., 2000).

Alginates consist of D-mannuronic acid and L-guluronic acid residues that are arranged in regions composed solely of one unit or the other and regions where the two units alternate, as shown in figure 9. The ratio of mannuronic acid to guluronic acid in the structure of the polymer determine the solution properties of the alginate.

The physical and chemical properties of alginic acid are greatly influenced by the strong anionic nature imparted by the carboxylic acid groups. Owing to the general structural features of the molecule and its high molecular weight, which ranges from 500 000 to 2 000 000 Daltons, and extended random coil chain conformation, solutions of alginate are highly viscous even at low concentration (Larsen *et al*., 2003). The viscosity of a solution of alginate is dependent on temperature, concentration, and the presence of polyvalent cations. A solution of alginate and calcium ions is thixotropic becoming a thin fluid when shaken and more viscous again when allowed to stand.

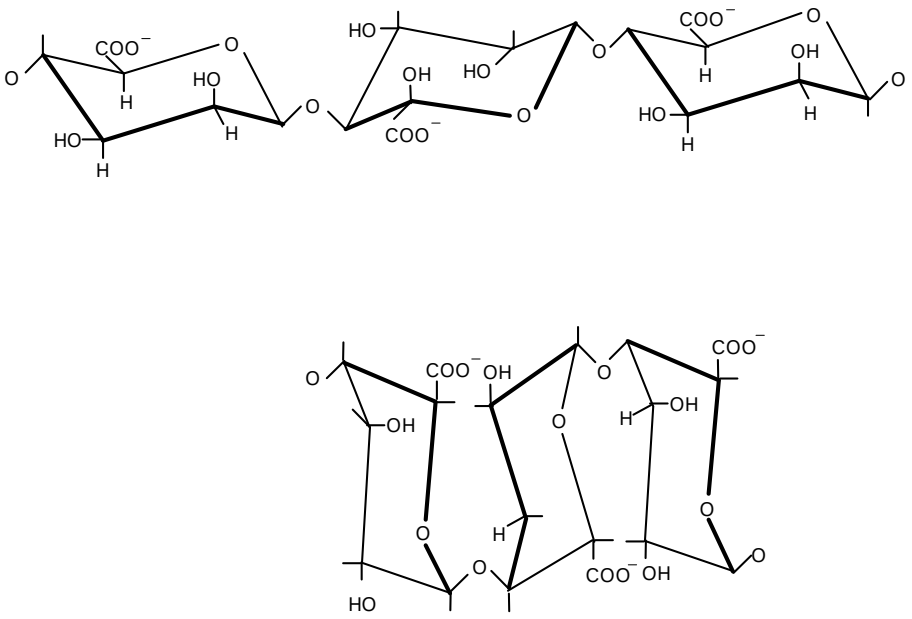


Figure 9: β - 1, 4 - linked mannuronic acid (A) and α - 1 , 4 - linked L - guluronic acid (B)

Alginates are best known for their ability to form irreversible gels in cold water in the presence of calcium ions (Walkenstrom *et al*., 2003). The sources of calcium are usually calcium chloride or tartrate. The rate of gel formation as well as the quality and texture of the resultant gel can be modified by the controlled addition of the calcium salts.

The gelling properties of alginates make them extensively suited for the preparation of food products such as fruit and meat analogues (Roberts *et al*., 2000). Alginates are widely used in biotechnology as immobilisation agents of cells and enzymes (Chavez *et al*., 1994). The largest quantity of alginate is used in the textile industry to prevent the migration of dyestuffs, such that fine line prints are produced with good definition and excellent wash out. Alginates are compatible with most dyes used in printing of textiles.

1.3.4.2 Carrageenan

Carrageenan is obtained from the seaweed *Chondrus crispus* or Irish moss, which grows abundantly along the North Atlantic coasts of the USA, Canada, the Iberian Peninsula and France. Other important sources are *Euchemia* and *Gigartina*, which are harvested along the coasts of East Africa, the Phillipines and Japan (Falshaw *et al*., 2003).

Carrageenans are a family of structurally similar galactans that carry various proportions of half-ester sulphate groups linked to one or more of the hydroxyl groups of the galactose units. The galactans consist of alternating units of 3-linked β-D-galactose and 4-linked α-D-galactose, as illustrated in figure 10.

Three types of carrageenan are commercially available, kappa, iota and lambda, all of which are obtained by aqueous extraction (Falshaw *et al*., 2003). Kappa carrageenan has one sulphate group for every two galactose units and one anhydro-bridge. The sulphate groups increase solubility of carrageenans while the anhydro-bridge tends to decrease their water solubility.

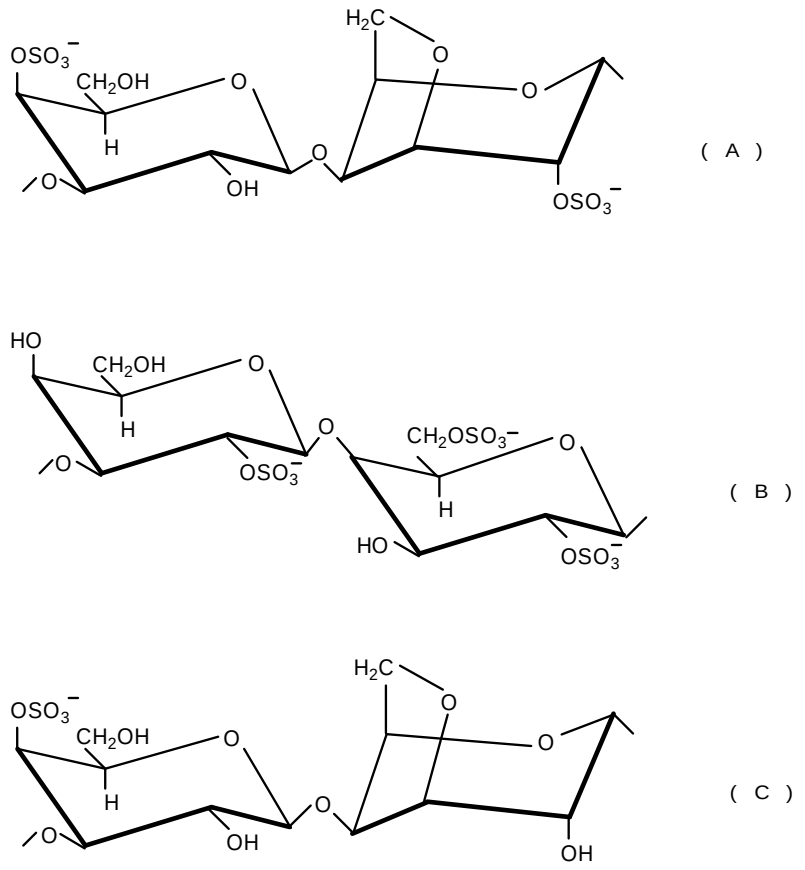


Figure 10: Repeating unit of iota carrageenan (A), lambda carrageenan (B) and kappa carrageenan (C)

Iota carrageenan has two sulfate groups and one anhydro-bridge for every two galactose residues and is more soluble than kappa carrageenan. Lambda carrageenan, the most soluble form, has three sulphate groups and no anhydro-bridge for every two galactose residues.

Solutions and gels of carrageenans are fairly stable over a wide range of pH at room temperature or lower but the polymers are rapidly degraded under conditions of low pH and high temperature. Kappa and iota carrageenan form gels with potassium and calcium ions respectively whereas lambda carrageenan does not form gels.

The shape or conformation of carrageenan molecule has been determined and related to the gel forming mechanism. The structure of kappa and iota carrageenans allows the formation of double helices in the sol-gel transformation, whereas the structure of lambda carrageenan inhibits formation of the double helices. Iota carrageenan gels strongly with calcium ions to form a clear, elastic gel that sets after shear. Kappa carrageenan is soluble in hot water and is used mainly as a gelling agent (Yuguchi *et al*., 2003). Kappa carrageenan forms gels in the presence of potassium ions, producing a strong, rigid gel, which tends toward syneresis. Gels that are formed from both kappa and from iota carrageenans are thermoreversible, the sol state increases in viscosity and sets to form a gel upon cooling.

Carrageenan has been used for its gelling, thickening, stabilising, emulsifying and suspending properties. Because of its reactivity with proteins, the gum has found use at low concentrations in a number of milk based products such as chocolate milk, ice cream, puddings and cheese analogues. Some low fat and no-fat salad dressings incorporate lambda carrageenan for its ability to give good suspension of seasonings while stimulating the sensory qualities of an oil based dressing (Torres *et al*., 2003). Carrageenans are used in jams, jellies and confections that have low sugar content.

1.3.4.3 Agar

Agars, polysaccharides that are extracted from certain red sea weeds, including *Gracilaria*, *Gelidium* and *Pterocladia* species are marketed in bars, strips, flakes and in powdered form. Japan and the USA are the largest producers but agar is also produced in Morocco, Mexico, Namibia, Portugal, Spain and Denmark.

Agar is composed of two polysaccharides, agarose and agaropectin (Sandford and Baird, 1983). Agarose, present in proportions of 55 to 66%, is an alternating copolymer of 3- linked β-D-

galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose units, as illustrated in figure 11. Small amounts of D-xylose are present in agarose. Agaropectin, comprising of approximately 40% of agar, has essentially the same structure as agarose except the fact that various units of the copolymer are replaced by sulphated or methylated sugar residues. The replacement occurs in such manner that the alternating sequence of 3-linked β-D- units and 4-linked α-L- units is maintained. Agar contains a spectrum of molecules that have a similar but varying chemical structure with the structures described above being the most common (Sandford and Baird, 1983).

Agarose, insoluble in cold water and soluble in boiling water, has the ability to gel at temperatures much lower than the gel-melting temperature (Deszczynski *et al*., 2003). Agarose, the gelling portion of agar, has a double helical structure in which the helices aggregate to form a three dimensional framework, which holds the water molecules within the interstices of the framework. Agarose produces thermoreversible gels and many of its uses depend on this characteristic. The strength of gel formed varies according to the source of agar (Normand *et al*., 2003).

The viscosity of solutions of agar is dependent on temperature and pH but is fairly constant in the range of pH 4.5 to 9.

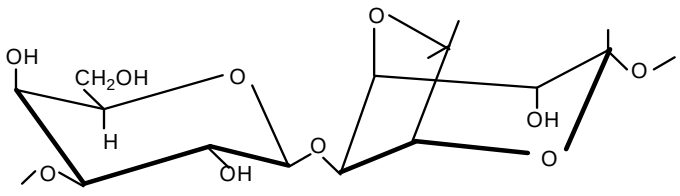


Figure 11: Basic repeating unit of agar

The use of agar as a bacteriological medium is the most important application. A well defined shape of gel is a characteristic of agar that is difficult to match with any other substance that forms gels. Agar performs efficiently as a medium for growing microorganisms since agar does not contain high levels of inhibitory or metabolisable substances, debris or thermoduric spores. Agar is stable to most organisms although there are a few organisms that metabolise agar by producing agarolytic enzymes. Except for its microbiological applications, agar has been replaced by pectin and, to a lesser extent, by starches. Most of the agar produced is used in the pharmaceutical industry and in research laboratories for the culture of microorganisms and analysis of nucleic acids and proteins.

Locust bean gum has synergistic effects with agar, on gel strength, whereas both alginates and starches reduce gel strength (Normand *et al*., 2003). Agar is not absorbed by the body during digestion and can, therefore, be used in low-calorie confections.

1.4 Functional properties of polysaccharides

The ability of polysaccharides to produce high viscosity in water at low concentrations is a major property of the polysaccharides that gives them valuable and widespread use in the food and non-food industries. Viscosity has special importance for colloidal food systems because viscosity influences characteristic textural and behavioral characteristics of many natural, formulated and processed dietary foods (Guizani *et al*., 2001). Polysaccharide gums have been mainly used for the thickening, modification of texture, gelling, formation of protective films and for stabilisation of emulsions, foams and suspensions (Kossori *et al*., 2000; Garcia *et al*., 2000; Euston *et al.*, 2000). Typically used at low concentrations, the polymers do not contribute to the nutritional value, taste or smell of the finished product.

1.4.1 The flow properties of polysaccharide solutions

For polymer solutions, viscosity may be expressed as the ratio of the viscosity of a solution to that of pure solvent at the same temperature and shear rate. The dimensionless ratio is known as the relative viscosity

$$\eta_{rel} = \eta/\eta_0$$

where η is the viscosity of the solution and η_0 is the viscosity of solvent.

The fractional increase in viscosity due to the presence of the solute is defined as the specific viscosity, η_{sp}, and is given by

$$\eta_{sp} = (\eta - \eta_0) / \eta_0 = \eta_{rel} - 1.$$

As the degree of viscosity enhancement is dependent on the amount of dissolved material as well as molecular size, another quantity that can be used is reduced viscosity, η_{red} which is given by

$$\eta_{red} = \eta_{sp}/c$$

where c is the concentration of solution.

In the limit of infinite dilution, reduced viscosity characterises the fractional increase in viscosity due to isolation of each solute molecule and is then known as the intrinsic viscosity $[\eta]$,

$$[\eta] = \lim_{c \to 0} (\eta_{sp}/c).$$

Intrinsic viscosity may be obtained from the intercept of a graph of η_{sp}/c against c, which is known as the Huggins plot and from extrapolation to infinite dilution of $(\ln \eta_{rel})/c$ against c, known as the Kraemer plot. Combined application of both Huggins and Kraemer extrapolations allows intrinsic viscosity to be measured with greater precision than by either technique alone (Burkus and Temelli, 2003). Extrapolation to zero polymer concentration is intended to eliminate polymer intermolecular interactions. The intrinsic viscosity should be independent of the fluid shear rate.

The properties of solutions of polymers are controlled by molecular characteristics such as molecular weight, intrinsic viscosity and chemical structure (Lazaridou *et al*., 2003). When a polymer is dissolved in a liquid, the dimensions of the polymer coils depend on the solution concentration and on the nature and strength of interactions between the solvent and the polymer. Because the size of the polymer molecule is much larger than that of the solvent molecules, a drastic increase in the viscosity of the solvent is observed when even a small amount of polymer is dissolved. Staudinger showed that the relative increase in solution viscosity could be related to molar mass average or viscosity average molecular weight of the polymer chains (Tirtaatmdja *et al*., 2001). The Mark-Houwink- Sakurada (MHS) equation relates intrinsic viscosity [η] and the molecular weight MW as shown below

$$[\eta] = KM^a$$

where both K and *a* are constants for a given polysaccharide solvent system.

The constants K and *a* must first be determined by calibration against a primary molecular weight technique, such as light scattering, and may then be used for routine determination of molecular weights from intrinsic viscosity measurements. The Mark-Houwink exponent *a*, is directly related to the degree of molecular expansion and hence to chain flexibility (Tirtaatmdja *et al*., 2001).

When a fluid system is in motion, it demonstrates a viscosity or resistance to shear that can be studied in terms of internal friction as described by Isaac Newton who postulated that the rate of flow (D) was directly related to the applied stress (τ) and the constant of proportionality was the viscosity (η),

$$\tau = \eta D$$

Simple fluids that obey the relationship described by Newton are called Newtonian fluids. However, many concentrated colloidal systems such as food gels, pastes, and creams that contain asymmetric particles, however, do not follow these Newtonian principles of fluid flow and are described as non-

Newtonian fluids (Sandeep *et al*., 2000). Non-Newtonian fluids can be subdivided into five discrete patterns of flow based on shear rate and shear stress behaviour, which include Bingham plastic, pseudoplastic, dilatant, thixotropy and rheoplastic flows.

Wide ranges of instruments that may be used to determine the flow properties of fluids exist. A capillary viscometer can be used to determine viscosity if the fluid is Newtonian and the flow is stream-lined (Aulton, 1988). The rate of flow of the fluid through the capillary is measured under the influence of gravity or an externally applied pressure. Examples of capillary viscometers include the Oswald U-tube viscometers, suspended level viscometers and falling sphere viscometers.

For non-Newtonian fluids the viscosity should be measured at more than one shear rate because the viscosity of the fluid varies with the rate of shear. Though determination of viscosity at one shear rate would be acceptable for a Newtonian fluid, it could lead to completely erroneous comparative results, if done for non-Newtonian fluids. Determination of viscosity at different shear rates is achieved by the use of rotational viscometers. Rotational viscometers rely on the viscous drag exerted on a body when it is rotated in the fluid, to determine the viscosity. An advantage of such instruments is that wide ranges of shear rate can be achieved and often a programme of shear rates can be produced automatically.

Usually an increasing shear rate, which is effected by increasing the speeds of the rotational viscometers, results in a decrease in viscosity a phenomenon known as shear thinning and the system is described as pseudoplastic (Lazaridou, 2000). The vast majority of natural and synthetic gums display pseudoplastic flow properties. Pseudoplastic behaviour is important in controlling mouth feel, which is directly related to viscosity of the fluid at the shear rate encountered in the mouth. If, on removal of the shearing force solutions revert to their original state the system is described as being thixotropic. Thixotropic solutions are related to pseudoplastic systems but require time to recover viscosity after removal of the shearing force.

The viscosity of polysaccharide solutions depends on the degree of polymerisation, molecular extension, rigidity and shape of the solvated polymer chain (Shim and Mulvaney, 2001). In solution, molecules oscillate at a minimum energy state as a result of collisions and thermal energy between molecules of the polysaccharide. The shapes of polysaccharide molecules in solution are a function of oscillations of molecules around the bonds of the glycosidic linkages. The greater the internal freedom at each glycosidic linkage, the greater the number of conformations available to each individual segment of the chain and the less likely it would be for the chain as a whole to adopt a particular shape. Linear molecules in solution gyrate and flex, sweep out a large volume and frequently collide with each other, consuming energy, creating friction and thereby producing viscosity.

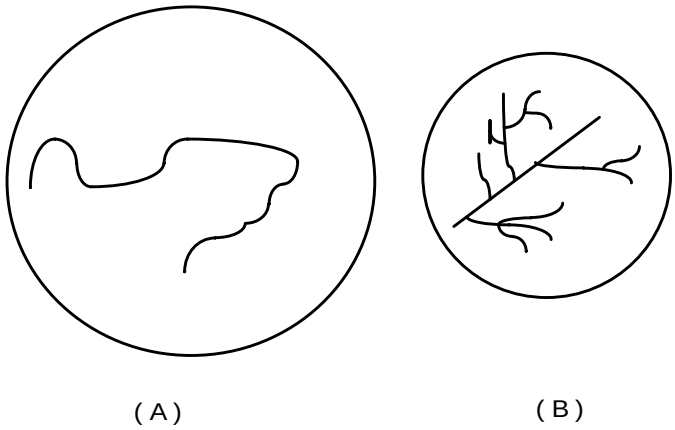


Figure 12: Relative volumes occupied by a linear polysaccharide (A) and a highly branched polysaccharide (B) of the same molecular weight (Lauray, 1986)

Highly branched molecules sweep out less space, collide less frequently and produce a lower resistance to flow than linear molecules of the same degree of polymerisation at the same concentration, as illustrated in figure 11.

1.4.2 Gel forming properties of polysaccharides

A gel is a continuous, three-dimensional network of connected molecules or particles that trap a relatively large volume of a continuous liquid phase (Walkenstrom *et al.*, 2003). A continuous network of solid material forms the gel matrix and enmeshes a finely dispersed liquid phase. Gels are usually produced by direct intermolecular association and binding of short segments of otherwise soluble polysaccharide chains resulting in the formation of junction zones. Three-dimensional networks are formed through the establishment of junction zones through covalent bonds, ionic interactions, intermolecular hydrogen bonds and hydrophobic interactions. The formed junction has stability that depends mainly on its length and the number of intermolecular bonds that develop. Extension of the junction may be induced by movement of the chains allowing adjacent segments of the molecules to align and leading to growth of the junction in a zippering fashion. Further binding of segments is aided by restricting the amount of water that is available for solvation of the polysaccharide molecules.

Food gels are semisolid systems that show various degrees of elasticity, brittleness and rigidity depending on the gelling substance employed (Panchev and Karageorgiev, 2000). The type and degree of cross-linking give gels their characteristic strength, elasticity and flow behavior. Some properties of gels, such as rigidity with elastic response to pressure, correspond to properties of solids whereas vapour pressure and electrical conductivity correspond to properties of liquids.

Usually the strength of a gel depends on the concentration of the polysaccharide. In cases where proteins are found in the mixture containing a gelling polysaccharide, the particular proteins present also influence gel strength. Other substances influence the rigidity or strength of the gel by competing with water for the binding loci, competing with solid phase for the liquid, as sugar does in high-methoxy pectin.

Factors that affect the formation of gels include types and quantity of interactive groups available in the polymer molecules, the molecular weight and composition of the polymer-polymer mixture. Those factors, in turn, influence the time and temperature of gel formation as well as the

minimum concentration of the dispersed phase required for the formation of gel. In general solutions of polysaccharides will form gels at relatively low concentrations of the gelling material. Firm gels can be prepared from a small number of polysaccharides, for example, pectins, carrageenans, agar and alginates, at levels of 1% or lower (Cardoso *et al*., 2003; Torres *et al*., 2003; Walkenstrom *et al*., 2003).

Non- covalent forces are believed to be responsible for gel formation in high methoxy pectins. High methoxy pectins form gels when the pH is below 3.5 and a cosolute, typically sucrose at concentration greater than 55% by weight is present. In the formation of gels of high methoxy pectins, sugar is believed to stabilise junction zones by promoting hydrophobic interactions between ester methyl groups. The effect of sugars thus depends specifically upon the molecular geometry and interactions of the sugar with neighbouring water molecules.

Gel structures can be produced by chemically cross-linking glycan chains with difunctional reagents such as calcium ions (Walkenstrom *et al*., 2003). Alginate and low methoxy pectin can produce gels with divalent or polyvalent cations, such as calcium ions, which bind individual chains together. The interaction of calcium with the polymer chains forms bridges with the polymer chains, thus altering the charge distribution on the polymer molecules and influencing gel strength. Interactions between calcium ions and carboxyl groups of pectins are described by the egg box model involving a two stage process of initial dimerisation and subsequent aggregation of preformed egg boxes, as illustrated in figure 13. The pH should be high in the gelation of low methoxy pectins because only dissociated carboxylic groups take part in the cross linkages.

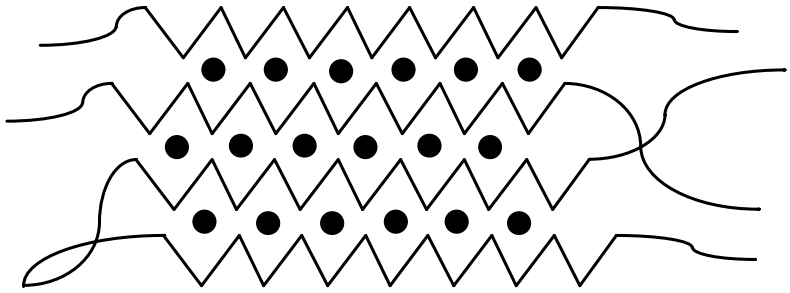


Figure 13: Egg box model for polysaccharide gels proposed by Grant and Coworkers, 1973. The junction zones are stabilised by calcium ions (●)

Kappa and iota carrageenans, known as the gelling carrageenans, contain anhydro D-galactose units that are essential for their ability to form thermoreversible gels (Yuguchi *et al*., 2003). Carrageenan gel networks are formed by a series of polymer chain associations that give rise to a three-dimensional helix framework. In the sol state, above 50 ^{o}C, the chains are present as random coil conformation and cooling will develop a helix. A gel develops when enough of the helix has been formed to provide cross-links. During cooling, kappa carrageenan aligns two helical coils in such a way as to focus its four sulphate groups toward each other and the negative charges are then neutralised by potassium ions. For iota carrageenan, the process of forming a gel is similar except that there is no additional hydrogen bonding or aggregation of the carrageenan helices because the two sulphate groups present in iota carrageenan project outward from the double helix. The sulphate groups interact with divalent cations, commonly calcium, to form ionic bridges between helices. Iota carrageenan form gels with special properties of flexibility and good water binding capacity.

In thermoirreversible gels, gels that do not reform on cooling, heat destroys the bonds that sustain the gel matrix. As temperature rises, the bonds that are responsible for maintaining the gel become increasingly labile until the semisolid state of the gel assumes viscous liquid properties. In thermoreversible gels conversion can be reversed upon subsequent cooling (Normand *et al*., 2003). As

a rule, the melting points for reformed gels, such as agar systems, are higher than the original temperatures at which the gels form.

1.4.3 Formation of edible films and coatings

Edible films and coatings are promising systems for the maintenance of food quality and preservation during storage (Diab *et al*., 2001). Environmental issues related to disposal of conventional synthetic food packaging materials and the need to develop more environmentally-friendly and biodegradable materials, are becoming increasingly important. Edible films are considered environmentally friendly and knowledge of edible polymers can be used for the development of these biodegradable packaging materials.

Although many functions of edible films and coatings are identical to those of synthetic films, there are additional requirements pertaining to their use in foods such as, acceptable sensory characteristics, appropriate barrier properties for gas, water and oil, good mechanical strength and adhesion, reasonable microbial, biochemical and physiochemical stability and freedom from substances that are harmful to health. Much of the research efforts done on packaging of foods aim at one or more of the above mentioned issues, all relevant to the performance of edible films and coatings.

Edible films and coatings are designed using biological materials such as proteins, lipids and polysaccharides (Lazaridou *et al.*, 2003). Depending on their composition, the functionality of materials used in making films may vary, as each component offers different properties to the composite matrix. Films that are made of polysaccharides or proteins usually have suitable mechanical and gas barrier properties but may be highly sensitive to moisture and may not act as effective barriers to water vapour (Sothornvit *et al.*, 2003).

In contrast, to films made from polysaccharides or proteins films composed of lipids provide effective barriers against water vapour but tend to be inflexible and are susceptible to rancidity or

oxidation. When polysaccharides or proteins and lipids are combined in the formation of films, physical or chemical interaction may produce films and coatings with improved properties. However, compatibility of constituents is an important issue when dealing with mixtures of biopolymers as the combination might drastically alter the performance of composite films.

The development of films and coatings from water-soluble polysaccharides has led to promising new types of materials for preservation of fruits and vegetables partially because these biopolymers are selectively permeable to oxygen and carbon dioxide (Zevailos and Krochta, 2003). The ability of water-soluble polysaccharides to reduce levels of oxygen and increase carbon dioxide levels in internal atmospheres of coated fruits and vegetables reduces respiration rates, thereby extending the shelf life of fresh produce in a manner similar to modified controlled atmosphere storage (Lee *et al*., 2003). Nussinovich (1997) reported that pears and apples coated with a carboxymethyl chitosan film ripened later than uncoated fruit. Use of chitosan -based coatings was observed to increase the shelf life of strawberries and tomatoes (Nussinovich, 1997).

1.4.3.1 The manufacture of edible films

The formulation of films and coatings requires the use of at least one component that is able to form a structural matrix of sufficient (Diab *et al*., 2001). Edible films may be made from several components in order to determine the effect of each component and arrive at the formula with the most desirable properties. Most of the composite films that have been studied include hydrophobic compounds, such as lipids, with a hydrocolloid structural matrix.

In research, films have been obtained by laying or spreading a film forming solution on support, drying and detaching the resulting film. In industry the techniques used for the making of edible and biodegradable films are usually those traditionally used for plastic films.

1.4.3.2 Inclusion of additives in the formation of edible films

Various materials can be incorporated into edible films to influence mechanical, protective, sensory or nutritional properties. Formation of films using carbohydrate or protein often results in films that are quite brittle due to the presence of extensive intermolecular forces involving interaction between chains (Sothornvit and Krochta, 2000). One approach to overcoming film brittleness is the addition of food grade plasticisers to the formulation of the film. Plasticisers function by weakening intermolecular forces between adjacent polymer chains resulting in a decreased tensile strength and increased film flexibility. Commonly used food grade plasticising agents are polyols such as glycerol, sorbitol, mannitol, sucrose, propylene glycol, and polyethylene glycol. In addition to improving the mechanical properties of films, plasticisers also increase film permeability to moisture, which is usually undesirable. Plasticisers, therefore, need to be used in minimal amounts.

An edible film or coating may be used as a vehicle for incorporating additives such as antioxidants and antimicrobial agents onto the surface of the food products, where deterioration of many solid foodstuffs by oxidation or microbial growth begins (Lee *et al*., 2003). The inclusion of a preservative in an edible coating covering the product places the preservative at the site of greatest susceptibility to deterioration. While food-coatings can effectively be used as carriers of food additives, additives may alter adversely the resistance of the film to the movement of vapour, gas or solutes. The influence of a given additive on film properties will depend on its concentration in the film and to the extent of its interaction with the polymer.

1.4.3.3 Evaluating performance of edible packagings

The particular application of a given film depends on the food product that is to be coated the primary mode of deterioration of the food and the qualities of the film (Sothornvit *et al*., 2003). When the integrity of the product and resistance to water are important, insoluble films may be used whereas water soluble films are desirable in situations where the food is consumed without further processing.

When the food or a coated component of a heterogeneous food system is high in polyunsaturated fat, a film extremely resistant to oxygen transport is desirable. However, when an edible film is applied to fresh fruits and vegetables, a certain degree of oxygen and carbon dioxide permeability is necessary in order to avoid anaerobic respiration, which would result in physiological disorders and a rapid loss of quality.

For many food applications, the most important functional characteristic of an edible film or coating is resistance to the migration of moisture. Critical levels of water activity must be maintained in many foods if the product or a certain component of a multiphase food is to exhibit optimum quality and acceptable safety. Deteriorative chemical and enzymatic reactions are also strongly influenced by water activity or moisture content.

An edible film with effective barrier properties could be inefficient if its mechanical properties are such that its integrity during handling, packaging and carrying processes is not maintained. In order to ensure applicability under variety of conditions the mechanical resistance or deformability of edible coatings has to be determined.

The choice of edible packaging depends on the specific characteristics of the product that requires protection and on storage conditions. Edible films have been applied on, poultry, sea food, fruits, vegetables, grains, candies, heterogeneous and complex foods or fresh, cured, frozen and processed foods (Wu *et al*., 2001; Diab *et al*., 2001; Pen and Jiang, 2003; Sebti and Coma, 2002).

1.4.4 Emulsifying properties of polysaccharides

Emulsions are intimate mixtures of two immiscible liquids in which one phase is dispersed throughout the other as small discrete droplets whose mean diameter can vary from less than 2 μm to greater than 100 μm (McClements and Decker, 2000). Emulsions are used as vehicles for adding flavour to food, diluting ingredients, and hiding objectionable odours or tastes (Ribotta *et al*., 2004). Although foods such as egg yolk, milk and cream exist naturally as emulsions, prepared foods such as salad dressings and margarine, depend upon the development and maintenance of an emulsion for their consistency or structure.

The formation of emulsions depends on the reduction of interfacial tension either by mechanical agitation or action of surface-active agents (Wu Victor, 2001). Because the contact between water and oil is energetically unfavorable, when a simple emulsion is allowed to stand, the dispersed droplets will quickly rise and coalesce to form a floating layer. In stable emulsions, the various processes that result in the separation of oil and water phases takes place only slowly. The processes involved in separation, which may occur singly or in combination, include creaming, flocculation, coalescence and oiling off.

Emulsifiers are substances that facilitate the formation of fine dispersions and the emulsions produced may be stabilised against creaming, flocculation and coalescence by several methods. Combinations of emulsifiers may be used to achieve optimal stability of emulsions. Substances that have been used as emulsifiers include gum arabic, proteins and phospholipids (Rodriguez *et al*., 2002; Galazka *et al*., 2000; McClements and Decker, 2000). The ability of an emulsifier to facilitate formation of an emulsion is related to its ability to adsorb onto and stabilise the oil-water interface. Emulsifiers reduce interfacial tension and the amount of work that is necessary to create new surfaces.

In addition to enhancing the formation of small droplets emulsifiers reduce the rate at which droplets coalesce (Galazka *et al.,* 2000).

Mechanisms of increasing the stability of emulsions include impediment of molecular movement, increase of electrostatic repulsion, formation of mechanically strong interfacial films and setting up of physical barriers with ingredients (Chanamai and McClements, 2002). In food systems, decreased coalescence of droplets may be accomplished by impeding molecular motion through the addition of water dispersible hydrocolloids that result in increase in viscosity (Leroux *et al*., 2003).

The stability of emulsions is commonly measured in terms of the amount of oil separating from an emulsion during a specified period at a stated temperature and gravitational field. The time required for a specified degree of breakdown to occur is used as a measure of emulsion stability (Chove *et al*., 2001).

1.5 *Cordia abyssinica*

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 *Cordia abyssinica,* known as Matobvu in Shona, is a drupe, about 10 to 30 mm in diameter, which has a globose shape and a sharp tip, as shown in figure 12. Green when unripe the fruit turns yellow to orange on 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 a glue. Although edible, the fruit of *C. abyssinica* is not normally consumed by humans but is eaten by wild animals.

The fruit of *Cordia abyssinica* was choosen 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, emulsion stabiliser, and as a binding agent in the food industry and as an effective adhesive in the non-food industry.

Figure 14: Life size photograph of the ripe fruit of *C. abyssinica*. Photograph by Professor M. A. N. Benhura.

1.6 Objectives of the project

1.6.1 Main Objectives

1. The work, on the polysaccharide isolated from the fruit of *C. abyssinica,* that is described in this report was done in the context of a worldwide trend towards sustainable exploitation of plant resources. Technical exploitation of the polysaccharide material from *C. abyssinica* would help to encourage sustainable exploitation of the plant material in rural communities and the unnecessary cutting down of the tree could be avoided.

2. In order to make effective use of a given polysaccharide, its chemical and physical properties are to be evaluated after extraction of the plant material. No information is available on the nature,

composition and structure of the mucilaginous polysaccharide that occurs in the fruit of *C. abyssinica*. A study of the properties of the polysaccharide from *C. abyssinica* would help to identify the most suitable application and the optimum conditions for its use. Any need for modification of the original polysaccharide before useful application can be identified.

1.6.2 Specific objectives

1. Isolate the polysaccharide material from the fruit of *Cordia abyssinica*
2. Attempt to purify the polysaccharide
3. Determine some of the physical and chemical properties of the polysaccharide
4. Estimate the molecular weight of the polysaccharide
5. Determine the partial structure of the polysaccharide
6. Characterise the flow properties of solutions of the polysaccharide
7. Investigate the ability of the polysaccharide to form gels
8. Evaluate the emulsifying properties of the polysaccharide
9. Determine the ability of the polysaccharide to form useful edible films

2. EXPERIMENTAL

2.1 Collection of the fruit

Mature but unripe fruit of *C. abyssinica* were picked from trees in Bikita, South Eastern Zimbabwe, in 1997 and every two years thereafter. 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 hours from the time of collection.

2.2 Extraction of pulp from the fruit

The pulp was extracted by squashing the fruit by hand to release the stones, on 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 ^{o}C until required.

2.3 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 minutes 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 ^{o}C or freeze dried in a Christ-Alpha 2-4 freeze drier. The dry gum was stored at room temperature until required.

2.4 Precipitation of the polysaccharide using the ionic detergent hexadecyltrimethylammonium bromide (CTAB).

An aqueous solution (0.125%, 800 ml) of the polysaccharide that had been precipitated with ethanol was prepared. An aqueous solution of CTAB (0.1 M, 100 ml) was added slowly to the solution of polysaccharide with stirring. Addition of the ionic detergent was continued until no more polysaccharide could be precipitated. In order to regenerate the polysaccharide, the quaternary ammonium ion was removed by exchanging it with sodium ions, by shaking the CTAB- polysaccharide complex for several hours with a saturated solution of inorganic electrolyte, made by mixing sodium chloride (46 g) in 25 ml water and 100 ml ethanol, with slight warming of the solution. The polysaccharide was left in the saturated solution overnight in a shaking incubator. The insoluble polysaccharide was collected by centrifugation and the supernatant discarded. Fresh ethanolic solution (100 ml) of inorganic electrolyte was added and the ion exchange was allowed to proceed for up to seven hours more by which time the ion exchange process was virtually complete as indicated by solubility of the precipitate in distilled water. The precipitates were washed four times with ethanol (20 ml) to remove inorganic salt. After drying, the precipitates were dissolved in water to make 0.25% concentration and analysed by High Performance Size Exclusion Chromatography (HPSEC).

2.5 Precipitation of polysaccharide using 0.2 M HCl

Sodium carbonate (1 M, 120 ml) was added to the sticky pulp of *C. abyssinica* (400 ml). 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 minutes, 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 2-4 freeze-drier.

2.6 Analysis of the polysaccharide by HPSEC

For HPSEC, a Shimadzu liquid chromatograph, model CR4AX that was equipped with a refractive index detector and a Zorbax GF-250 size exclusion chromatography column was used for analysis.

Dextran standards, T10, T40, T70, T500 and T2000 whose corresponding molecular weights ranged from 10 000 to 2 000 000 Daltons respectively were obtained from Pharmacia (Uppsala, Sweden). Solutions (1%) of the standards and samples (0.25 % to 1 %) were prepared in distilled water and 20 µl aliquots were injected for analysis. Both the acid and ethanol precipitates were analysed by HPSEC.

In order to find the most suitable mobile phase for eluting the polysaccharide, water, 0.5 M sodium carbonate, 0.5 M sodium bicarbonate, 0.1 M sodium chloride, and an ion exchange buffer used by Barth and Regnier (1993), were tried. The mobile phases were filtered under vacuum by use of a 0.45 µm membrane filter, from Gelman Sciences, USA.

The ion exchange buffer was prepared by adding 60 ml of a 4M solution of sodium acetate and 440 ml of 4 M acetic acid to a 1L volumetric flask and filling the volume with water, to give a pH 3.7 buffer of 0.24 M ionic strength. The ionic strength of the buffer was then increased to 1.42 by adding 57 g of sodium sulphate to 1 L of the 0.24 M acetate buffer. The solution was diluted two times with water and used as the mobile phase. When water was used as mobile phase a flow rate of 2 ml/min was used otherwise a flow rate of 1.5 ml /min was used. The refractometer attenuation was set at X6 (Supelco, 1985).

2.7 Determination of yield of polysaccharide

A known number of fruits were decapped, 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 decapped fruit (James, 1995).

2.8 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 ^{o}C.

To determine ash content, the sample, dried at 100 ^{o}C was first charred at 200 ^{o}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 ^{o}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 minutes 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 minutes. The mixture was cooled and filtered through glasswool 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).

2.9 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).

2.10 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).

Reagents:

Reagent A: solution of sodium tetraborate (0.0125 M) in concentrated sulphuric acid.

Reagent B: solution of p-hydroxydiphenyl (0.15% w/v) in 0.5% (w/v) NaOH. The reagent was stored at 4 ^{o}C and discarded after one month.

Procedure

To a 0.2 ml solution of the standards (100 μg/ml uronate) or polysaccharide (0.25%), 1.2 ml of reagent A was added, the tubes cooled in crushed ice and shaken vigorously. The tubes were removed from the ice bath and maintained in a boiling water bath for 5 minutes after which they were cooled in an ice-water bath and 20 μl reagent B added. The tubes were shaken and absorbance of the solutions at 520 nm was immediately measured.

2.11 Determination of the methyl ester content

Preparation of Reagents

Reagent A*:* aqueous potassium permanganate 2% (w/v) was prepared and filtered through medium porosity sintered glass which had been cleaned with chromic acid, water, dilute potassium permanganate, and water.

Reagent B: sodium arsenite (0.5 M) in 0.06 H_2SO_4.

Reagent C: Freshly distilled 0.02 M pentane-2,4-dione was dissolved in a solution of ammonium acetate (2 M) in acetic acid (0.05M).

Preparation of samples and standards

Sodium hydroxide (0.75 M, 0.25 ml) was added to aliquots (0.5 ml) of the polysaccharide (50-200 μg) and the tubes gently swirled. After 30 minutes at room temperature, the samples and methanol standards (2-40 μg) were acidified with 2.75 M H_2SO_4 (0.25 ml) and cooled in an ice-water bath, for permanganate oxidation.

Procedure for determination of methanol

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 minutes. Sodium arsenate (0.05 M, 0.2 ml), followed by water (0.6 ml) was added, and the thoroughly mixed solution left for 1 hour at room temperature. After addition of pentane-2,4-dione (2 ml) and thorough mixing, the tubes were closed with marbles, heated at 60 ^{o}C for 15 minutes, 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.

2.12 Determination of the protein content of the polysaccharide (Connolly *et al.,* 1988)

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

Dry *C. abyssinica* polysaccharide (2 g) was added to a 500 ml conical flask to which powdered copper (10 g) was added and sodium sulphate, which raised the boiling temperature of the mixture. Concentrated sulphuric acid (25 ml) was added and the flasks were placed in a fume hood while heating. Two blanks with everything except the sample were run at the same time. After heating the mixtures for 30 minutes, tap water (00 ml) was added to the resultant green solutions. Sodium hydroxide (40%, 75 ml) was added to the mixture resulting in the formation of a blue colour. The flasks were connected to distillation apparatus and heated using Bunsen burners. One hundred millilitres of the distillate was collected in flasks containing 50 ml of 4% boric acid. The boric acid mixture was titrated against 0.1402 M HCl to a light pink end point.

2.13 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 ^{o}C. In the control tube, was included phosphate buffer instead of the solution of enzyme. The action of the enzyme was followed by measuring the viscosity at 37 ^{o}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 water as the mobile phase at a flow rate of 2 ml/min.

2.14 Determination of hydroxyproline residues in the polysaccharide chains

Reagents

Solution A: copper sulphate pentahydrate (0.05 M) in water

Solution B: sodium hydroxide (2.5 M) in water

Solution C: H_2O_2 (6%) (this was made by diluting 30% H_2O_2 with water

Solution D: H_2SO_4 (1.5 M)

Solution E: p-dimethylaminobenzaldehyde (5%) in redistilled propan-1-ol

Standards: L-hydroxyproline, tyrosine, tryptophan, threonine, proline or glutamate standard solutions

Preparation of solutions of standards

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 at concentrations of up to 20 μg/ml were prepared by diluting the 100 μg/ml standard solutions with water.

Preparation of solutions of samples

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.

Procedure (Vidal *et al*, 2003)

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 ^{o}C for 5 minutes 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 minutes 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 ^{o}C for 16 minutes. 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.

2.15 Determination of intrinsic viscosity of solutions of polysaccharide

Stock solutions of the polysaccharide (1%) were prepared by dissolving the polymer in water, 0.1 M NaCl, 0.1 M Na_2CO_3, 0.1 M NH_3, and 0.1 M NaOH, at room temperature. Working solutions were prepared by diluting stock solutions with the appropriate solvents up to 0.1 g/dl. Viscosity of the solutions was determined using a Cannon-Fenske Routine # 200, capillary viscometer from PSL Ltd.,

England. The samples were equilibrated at room temperature for 15 minutes and the flow times, from which the relative specific and reduced viscosities were calculated, measured in quadruple. The intrinsic viscosity was obtained by combined application of Huggins (η_{sp}/c against c) and Kraemar (ln η_r/c against c) plots (Nishida *et al*., 2002). Extrapolation of the two lines to zero concentration of polysaccharide was used to evaluate intrinsic viscosity.

2.15.1 Estimation of viscosity average molecular weight of the polysaccharide.

A crude estimate of the viscosity average molecular weight of the polysaccharide was attempted from the Mark -Houwink-Sakurada equation,

$$[\eta] = KM^a$$

where $[\eta]$ is the intrinsic viscosity, M is the viscosity average molecular weight and K is the proportionality constant which is characteristic of the polymer and solvent and the exponent *a* is a function of the shape of the polymer coil in solution. An estimation of the viscosity average molecular weight of the polysaccharide is shown in appendix B.

2.16 Determination of the monosaccharide composition

The polysaccharide that was precipitated using acid was used for all structural studies.

2.16.1 Hydrolysis with sulphuric acid

Three methods of hydrolysing the polysaccharide were used. In the first method, the polysaccharide (1.5 g) was suspended in 120 ml of 2.5% sulphuric acid and the mixture refluxed for 24 hours (Quach *et al*., 2000). After 7, 15, and 24 hours samples (40 ml) were withdrawn, neutralised with barium

carbonate, filtered and concentrated to 10 ml in a rotary evaporator at 50 ^{o}C. The sample that was collected after 7 hours of hydrolysis was passed through cation and anion exchange resins respectively in order to remove interfering ions.

In the second method, the polysaccharide (5 mg) was suspended in 2 M H_2SO_4 (2 ml), in a screw capped vial and the mixture heated at 100 ^{o}C for up to 7 hours. The hydrolysates were neutralised with barium carbonate and, after centrifugation to remove barium sulphate, the supernatants were concentrated by freeze-drying. The freeze dried samples were redissolved in 50 µl distilled water and analysed by high performance liquid chromatography (HPLC).

The standard Saeman method was also used for hydrolysis of the polysaccharide (Avallone *et al*., 2000). The sugars were released by treatment of 2 mg of polysaccharide with 225 µl of 12 M H_2SO_4 (72% w/v) in a screw capped vial at 30 ^{o}C for 1 hour. Distilled water was added to give 1 M H_2SO_4 and the mixture further heated for 3 hours at 100 ^{o}C. The samples were cooled, neutralised with barium carbonate. After centrifugation the supernatants were freeze-dried. The dried samples were redissolved in 50 µl of distilled water and analysed by TLC and HPLC.

2.16.2 Hydrolysis of polysaccharide using 2 M trifluoroacetic acid (Stephen, 1988).

The polysaccharide (10 mg) was suspended in 1 ml of 2 M trifluoroacetic, made by mixing 850 µl of water with 150 µl of concentrated trifluoroacetate acid, in a screw capped vial. The vial was firmly sealed and heated for 1 hour in an autoclave, 121 ^{o}C. After cooling the vial, the contents were centrifuged for 5 minutes at 2000 g. The TFA was evaporated off from the supernatant in fume hood and the remaining mixture was freeze dried, after which the dried material was dissolved in 50 µl of distilled water.

In order to determine the most suitable duration for hydrolysis with TFA samples were hydrolysed for 30 minutes, 1 hour, 2 hours and 3 hours.

Samples of the hydrolysed mixtures were analysed by TLC and HPLC. The concentration of uronic acids in the hydrolysates was determined by the m-hydroxydiphenyl-sulphuric acid assay (Chaplin and Kennedy, 1986).

2.16.3 Thin layer chromatography of hydrolysates (Chaplin and Kennedy, 1986)

The samples were analysed on Whatman linear K silica gel plates, size 20 x 20. Ethyl acetate , pyridine, water (20:7:5) was used as the developing solvent and diphenylamine, aniline, phosphoric (5:5:1) as the detection reagent. Xylose, galactose, rhamnose, arabinose, mannose, fucose and glucose were used as standards.

2.16.4 HPLC of hydrolysates (Nikolov *et al.,* 1985; Supelco, 1985).

HPLC was done on a Shimadzu C-R4AX model equipped with a refractive index detector. Samples or standards (30 µl) were injected into a Zorbax-NH_2 column and acetonitrile/ water (85:15) as the mobile phase at a flow rate of 2 ml/min. The first mixture of standards contained fucose, rhamnose, arabinose, glucose and xylose. The second mixture of standards contained fucose, rhamnose, galactose and mannose.

2.17. Hydrolysis of the polysaccharide with pectinases and Gamanase.

Pectinex Smash (Poly (1,4-β-D-galactosiduronate glycanohydrolase) from *Aspergillus aculeatus* and *Aspergillus oryzae*) and Gamanase (1,4 β-D-mannan mannohydrolase) were a generous donation from

the enzyme division of Novo Nordisk Ferment Ltd., Switzerland. Pectinase EC 3.2.1.15 from *Aspergillus niger* was obtained from Sigma.

The polysaccharide, dissolved in 0.02 M sodium acetate buffer, pH 5.0 (1%, 8 ml) was dispensed into each of four 50 ml conical flasks. To each flask, 0 to 2 ml acetate buffer was added to make the final volume of the reaction mixture 10 ml after the addition of enzyme. After thorough mixing, the solutions were incubated for 15 minutes at 30 ^{o}C and 37 ^{o}C for Pectinex Smash and Gamanase respectively, before addition of 0 to 2 ml of the solution of the enzyme as supplied. The reaction mixture was added into a # 200 Cannon Fenske Routine viscometer, which was maintained at 30 ^{o}C and 37 ^{o}C for Pectinex Smash and Gamanase respectively. The viscosity of the reaction mixture was determined at intervals.

Alternatively, the reaction mixtures were incubated at the respective temperatures for 48 hours before adding 5 ml ethanol to precipitate unhydrolysed material. The mixtures were centrifuged in a Selecta Centronic centrifuge at 3000 rpm for 5 minutes and the supernatant frozen at -80 ^{o}C and freeze-dried. The samples, reconstituted in 50 µl of water and analysed for neutral and acidic components by thin layer chromatography.

2.17.1 Treatment of the polysaccharide with pectinases

To a 50 ml conical flask, 0.01 g of polysaccharide and 10 ml of glycine-NaOH buffer, pH 9 (Matsuura *et al*., 2000) were added. After the solutions were allowed to stand at 45 ^{o}C for 15 minutes, 3 ml of the solution was removed from each flask and added to large test tubes. Pectinex Smash or pectinase (Sigma), (0.18 ml) was added to the solution of polysaccharide and the mixtures incubated at 45 ^{o}C in a water bath for up to 8 hours. Aliquots of the reaction mixture (100 µl) were withdrawn at intervals for the determination of reducing sugars.

2.17.2 Treatment of the polysaccharide with Gamanase

The polysaccharide solution (0.5%, 15 ml) prepared in 0.02 M sodium acetate buffer, pH 5.5, was equilibrated in a water bath at 37 ^{o}C. After addition of Gamanase (2 ml), aliquots of the reaction mixture (100 µl) were withdrawn at intervals for measurement of reducing sugars using the dinitrosalycylic acid (DNSA) method (Chaplin and Kennedy, 1986).

2.17.3 Determination of reducing sugars by the Dinitrosalycylic acid (DNSA) method (Chaplin and Kennedy, 1986)

Preparation of Reagent

DNSA (0.25 g) and sodium potassium tartrate (75 g) were dissolved in 50 ml of 2 M sodium hydroxide. The reagent was stored in the dark at 4 ^{o}C for up to six weeks.

Procedure

To hydrolysed samples of polysaccharide, standards and controls (100 µl), 1 ml of DNSA reagent was added with thorough mixing. The mixtures were heated in a boiling water bath for 10 minutes. The samples were rapidly cooled and absorbance at 570 nm was measured.

2.17.4 Thin layer chromatography of hydrolysed samples

Samples were analysed on Whatman K5F 10 x 5 cm silica gel plates, which were first activated by heating at 120 ^{o}C for 20 minutes and cooled to room temperature before applying the samples (Kim *et al*., 2002). The plates were developed three times with acetonitrile/ water (85:15) was used for

developing the plates. After development, the plates were dried and the spots visualised by dipping into a solution containing 0.3% (w/v) N-(-1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heated at 110 ^oC for 10 minutes.

2.18 Periodate oxidation of the polysaccharide

The polysaccharide was prepared from alkaline extracts by treatment with acid to obtain a precipitate that was washed with water and freeze dried. The polysaccharide (50 mg) was dissolved in 20 ml of 0.1 M NaOH. The base was neutralised with 0.2 M HCl and the volume made up to 50 ml with water.

To 25 ml of polysaccharide solution, 25 ml of sodium metaperiodate solution at concentrations up to 250 mM was added and the reaction vessel covered with aluminium foil and kept at 4 ^oC in the dark. To stop the reaction, ethylene glycol (10 ml) was added to the reaction mixture in order to destroy periodate that had not reacted. Substances of low molecular weight were removed by dialysis against distilled water for 48 hours at 4 ^oC with the dialysis water being changed four times. After dialysis, the oxidised polysaccharide was lyophilised to dryness and kept at room temperature.

The oxidised polysaccharide (20 mg) was dissolved in water (10 ml) and sodium borohydride (5 g) added. The reaction mixture was kept at room temperature for 24 hours before dialysis against distilled water for 24 hours at 4 ^oC. The dialysed solution was lyophilised to dryness and the product hydrolysed with 2 M trifluoroacetic acid for 2 hours at 121 ^oC in a vial fitted with a teflon lined cap. Sugars in the hydrolysed samples were identified by thin layer chromatography TLC (Chaplin and Kennedy, 1986).

During oxidation, samples were removed at intervals for the determination of formic acid and formaldehyde produced (Fredon *et al*., 2002; Keleti and Lederer, 1974). The amount of periodate

consumed was determined from the amount of iodate produced, as one mole of periodate will produce one mole of iodate on oxidation (Benhura and Marume, 1994).

2.18.1 Oxidation of polysaccharide that had been reduced with sodium borohydride

The polysaccharide (30 mg) was dissolved in 0.01 M NaOH (10 ml). Sodium borohydride (0.5 g) was added to the solution and the mixture kept in the dark for 48 hrs at room temperature. Excess borohydride was decomposed with dilute acetic acid, the pH was adjusted to 5.5 and the volume of the solution made up to 25ml with water. To the reduced polysaccharide sodium metaperiodate (25 ml, 0.05 M) was added and the mixture incubated in the dark at 4 ^{o}C for 5 days. Samples (10 □l) were withdrawn at intervals and the amount of iodate produced was measured.

2.18.2 Measurement of iodate

The amount of iodate produced during oxidation was determined spectrophotometrically. The oxidised sample (10 µl) was added to 1 ml of water, in order to stop the reaction (Keleti and Lederer, 1974). Sodium molybdate (1 ml, 0.02 M) was added to samples in order to mask any periodate present. After thorough agitation, 0.5 M chloroacetic acid (200 µl) was added followed by water (1.5 ml) and potassium iodate (0.6 M, 250 µl). The solution was made up to 4 ml by adding water (40 µl). The mixture was allowed to stand at room temperature for 10 minutes before the absorbance at 350 nm was measured.

2.18.3 Measurement of formic acid (Keleti and Lederer, 1974)

Standard solutions of formate and samples were dispensed into clean stoppered test tubes. Ethylene glycol (10%, 50 µl) was added and the mixture left to stand for 5 minutes in order to stop the reaction in the sample. Sodium borohydride (5%, 100 µl) that had been dissolved in 0.05 M sodium tetraborate-HCl buffer, pH 8, was added with thorough agitation and the mixture left to stand for 5 minutes. After addition of 4 M H_2SO_4 (50 µl) in order to destroy excess borohydride, 2-thiobarbituric acid reagent (1 ml) was added to develop the characteristic chromophore. The solutions were incubated in a boiling water bath for 20 minutes and allowed to cool to room temperature. Butanol, acidified with HCl so that the final concentration of HCl in the mixture was 5%, was added (1.5 ml) and the solutions clarified by centrifuging for 3 minutes before measuring absorbance at 450 nm.

2.18.4 Measurement of formaldehyde (Keleti and Lederer, 1974)

To samples and standards (24 µl), HCl (4 M, 5 µl) was added, followed by sodium arsenite reagent (0.4 M, 20 µl). Water was added to make up to 100 µl and mixtures were allowed to stand at room temperature for 8 minutes. Acetylacetone reagent (1 ml) was added and the tubes placed in a boiling water bath for 3 minutes, cooled to room temperature and the absorbance at 415 nm measured. The amount of formaldehyde produced was determined from a standard curve.

2.19 Rheological properties of the polysaccharide of *C. abyssinica*

Viscosity was determined using both the capillary and rotational viscometers.

2.19.1 Measurement of viscosity

Measurements of relative viscosity of solutions of polysaccharide were done at room temperature using a Cannon Fenske Routine viscometer, size 350.

The pH of solutions was measured at room temperature using a Jenway pH meter.

2.19.2 Phenol-sulphuric acid assay (Chaplin and Kennedy, 1986)

The total carbohydrate content of solutions was determined by the phenol-sulphuric acid assay using glucose as the standard. The polysaccharide or standards solutions (containing up to 100 μg carbohydrate, 200μl) were mixed with 200 μl of phenol (5%). Concentrated sulphuric acid (1 ml) was added rapidly and directly to the solution surface taking care not to touch the sides of the tube. The solutions were left undisturbed for 10 minutes after which they were shaken vigorously. After a further 30 minutes, the absorbance was determined at 490 nm.

2.19.3 Determination of a suitable solvent for *C. abyssinica* polysaccharide

The polysaccharide (0.1 g) that had been precipitated with acid was suspended in solutions (20 ml) of $NaHCO_3$, Na_2CO_3, NH_3, NaOH and KOH at concentrations up to 1 M and the mixtures were agitated at room temperature for up to 6 hours. The time taken for the polymer preparations to dissolve and other changes that would occur during the dissolution were noted.

The viscosity of solutions of the polymer (0.5%) in the different solvents was measured using a Cannon Fenske routine viscometer, size 350.

2.19.4 Effect of pH on solubility and viscosity of polysaccharide.

The polysaccharide (0.5 g) was dissolved in a solution of Na_2CO_3 (0.1 M, 100 ml) or Tris-HCl buffer (pH 8.9) to make a 0.5% solution and portions of the solution (20 ml) dispensed into 100 ml conical flasks. Up to 30 ml of HCl (0.2 M) was added and the volume in each flask made up to 50 ml with distilled water. The pH of the mixtures was determined. After agitation, the mixtures were allowed to stand for one hour before centrifugation at 12000 rpm for 30 minutes in a BHG Hemle ZK 401 centrifuge. Viscosity of the supernatants was determined at room temperature. The amount of carbohydrate remaining in the supernatant, assessed by the phenol-sulphuric acid method, was used as a measure of the solubility of the polysaccharide at a given pH. The pH at which maximum precipitation occurred was used for all subsequent precipitation of the polysaccharide.

2.19.5 Effect of polysaccharide concentration on viscosity of solutions of polysaccharide

The relative viscosity of solutions of polysaccharide in Na_2CO_3 (0.1 M), at concentrations up to 1%, was determined at room temperature (Isobe *et al*., 1992).

2.19.6 Effect of temperature on viscosity of solutions of polysaccharide (Isobe *et al*., 1992).

The polymer (0.2 g), dissolved in 0.1 M Na_2CO_3 (20 ml), was dispensed into 50 ml conical flasks and incubated for 1 hour in water baths at temperatures up to 80 ^{o}C. When measuring viscosity, the viscometer was immersed in a water bath maintained at the desired temperature.

2.19.7 Effect of salts on viscosity of solutions of polysaccharide.

The polysaccharide (1 g)was dissolved in solutions (100 ml) of Na_2CO_3 (0.1 M) or NH_3 (0.5 M) and 20 ml aliquots dispensed into conical flasks. Solid NaCl, LiCl or KCl were added to the flasks to make

up to 1 M concentration of salt. The mixtures were agitated in order to allow complete dissolution of the salts and allowed to stand for 1 hour at room temperature before measurement of viscosity.

When studying the effects of the divalent ions Ca^{2+} and Mg^{2+} on viscosity, the polysaccharide (1 g) was dissolved in a 0.5 M solution (100 ml) of NH_3. Calcium chloride or $MgCl_2$ was added to flasks containing 20 ml of polysaccharide solution (1%) to make up to 10 mM concentration of salt and agitated to dissolve the salts. The mixtures were left to stand for 1 hour before measurement of viscosity and pH.

2.19.8 Measurements with rotational viscometer

Aqueous suspensions (1%) of the polysaccharide that had been precipitated with ethanol, were prepared by blending necessary amounts of polysaccharide in water at least 24 hours before rheological measurements were made. The suspensions were allowed to disperse at room temperature while being agitated. All determinations were conducted at room temperature, which ranged from 21^{o}C to 25 ^{o}C, unless stated otherwise.

Rheological measurements were performed using a Schott Visco-Easy rotational viscometer fitted with an R2 spindle. Steady shear viscosity was obtained at shear rates up to 100 rpm. The viscometer spindle was allowed to stand in the solution for about
3 minutes before measurements were made. Rotational speed and corresponding torque or viscosity were recorded in triplicate periodically (Guizani *et al*., 2001).

2.19.9 Measurement of viscosity at various shear rates and polysaccharide concentrations

Solutions of polysaccharide (350 ml) at concentrations up to 1% and contained in 600 ml beakers were equilibrated at room temperature before measurement of viscosity at shear rates up to 100 rpm.

2.19.10 Measurement of viscosity at various shear rates and temperatures

The viscosity of solutions of the polysaccharide (1%, 350 ml) was measured in a 600 ml beaker at temperatures up to 80 ^{o}C. For measurement at 5 ^{o}C solutions were equilibrated overnight in a refridgerator and the temperature measured before measuring the viscosity of the solutions at various shear rates or rotational speeds. For temperatures above 30 ^{o}C the solutions were equilibrated in a water bath at the respective temperatures.

2.19.11 Measurement of viscosity at various shear rates and pH

When solutions (1%) of polysaccharide were prepared the pH of the solution was pH 6.8. Solutions of higher pH were prepared by adding concentrated sodium hydroxide and solutions of pH lower than 6.8 were prepared by addition of 5M HCl from a dropper pipette. The change in volume of the solution at 0.02% or less was considered negligible. The pH and temperature were measured just before measuring viscosity of the solutions.

2.19.12 Measurement of viscosity at various shear rates and concentrations of salt

To 1% solutions of *C. abyssinica* polysaccharide (350 ml), solid sodium chloride was added to make concentrations up to 1 M. For calcium chloride, up to 5 mM concentration of solutions were prepared. The solutions were stirred continuously on addition of the salts in order to avoid the formation of lumps. The solutions were left to stand at room temperature for at least 45 minutes after which viscosity was measured at various shear rates.

2.20 Preparation of gels using the polysaccharide of *C. abyssinica*

The dry polysaccharide that had been precipitated from solution with ethanol (1.5 g), was suspended in 80 ml of water and the mixture heated to dissolve the polysaccharide. The volume was made up to 100 ml and the solutions left overnight at 4 ^{o}C. Alternatively, flakes of dry gum were suspended in 100 ml of water and left overnight at 4 ^{o}C. The formation of gels and their quality was assessed by visual inspection and a photographic record kept.

2.20.1 Determination of the minimum concentration of polysaccharide required for gelation

To determine the minimum concentration of polysaccharide that was required for gelation, 0 g to 0.2 g of the polymer was mixed with 10 ml of water. The mixture was allowed to stand overnight at 4 ^{o}C before assessment of gel formation.

2.20.2 Effect of sucrose on formation of gel

Sucrose was dissolved in distilled water (10 ml) to make solutions of concentrations from 0% to 10%. The gum (0.15 g) was suspended in the solutions of sucrose and the suspensions were allowed to stand overnight at 4 ^{o}C before assessment of gel formation.

Alternatively sucrose (1 g) was mixed with up to 0.2 g of polysaccharide and the mixture was ground to a fine powder. The ground mixture was placed into vials and water (10 ml) was added. After agitation the mixtures were allowed to stand overnight at 4 ^{o}C before assessment of gel formation.

2.20.3 Effect of metal ions and EDTA

The polymer (0.15 g) was suspended in solutions containing calcium, potassium or sodium chloride at concentrations up to 50 mM. To determine the effect of EDTA, the polymer was suspended in a 100 mM solution of EDTA. The suspensions were allowed to stand overnight at 4 ^{o}C before assessment of gel formation.

2.20.4 Effect of pH

Citrate buffer was used between pH 3 and 6 and phosphate buffer was used at pH 7 and 8. The dry polysaccharide (0.2 g) was suspended in 10 ml of buffer and the mixtures were allowed to stand overnight at 4 ^{o}C before assessment of gel formation.

2.20.5 Effect of temperature

The polymer (0.15%) was suspended in water (10 ml) in vials that were incubated at the temperatures ranging from 4 ^{o}C to 96 ^{o}C. The vials were allowed to stand overnight at the appropriate temperatures before assessment of gel formation.

In order to determine reversibility of gel formation after heating, gels were, heated in a boiling water bath for about 20 minutes. On cooling, water was added as required, to the gels to restore water that was lost during heating and the mixtures were left to stand overnight at 4 ^{o}C. The integrity of reformedgels was assessed by visual inspection.

2.21 Preparation of emulsions and assessment of emulsification properties

Solutions of *Cordia abyssinica* polysaccharide or gum Arabic, obtained from Sigma, UK, at concentrations up to 1%, were prepared in 0.067 M sodium phosphate buffer, pH 7.4 and used to

investigate the dependence of emulsification properties of solutions of the polysaccharide, on the concentration of polysaccharide and presence of sodium chloride.

Solutions of *Cordia* polysaccharide, prepared in 0.067 M sodium citrate buffer, pH 3 and 5, 0.067 M sodium phosphate buffer, pH 7, 0.067 M sodium carbonate buffer, pH 8.5 and 11, and 0.067 M KCl-NaOH buffer, pH 13 were used to study the effect of pH on the emulsifying properties of the polysaccharide.

Emulsions were prepared following the method described by Kato et *al.*, (1989). Vegetable oil obtained from a local supermarket (1 ml) was added to solutions of polysaccharide or water (3 ml) in a hand operated laboratory piston type homogeniser from Wheaton, USA, and the mixtures homogenised by making 100 successive strokes at room temperature. Aliquots of emulsion (50 µl) were removed from the bottom of the container and diluted with a solution of sodium dodecyl sulphate (0.1%, 5 ml) (Chove *et al.*, 2001). The absorbance of the diluted emulsion was measured at 500 nm in a UV 6601 Shimadzu UV-Visible spectrophotometer. Emulsifying ability was determined by measuring the absorbance immediately after formation of the emulsion and the stability of emulsions was assessed by allowing the emulsions to stand at room temperature during which time the absorbance was measured at one minute intervals.

The turbidity T, of the emulsions was calculated as T= 2.303A/l, where A was the observed absorbance and l the path length of the cuvette (Kato *et al.* 1989).

2.21.1 Microscopic examination of emulsions

Small quantities of undiluted emulsions were placed directly onto a microscope slide, covered with a glass slip and examined immediately after preparation and 30 minutes later, at magnifications of 40 and 200 times on an Olympus light microscope from Japan. The size of the oil globules was measured

using an eye piece graticule for calibration of the microscope. Photomicrographs of some of the emulsions were taken using an Ilford automatic microscope camera fitted onto a Leitz Vario Orthomat 2 microscope from Ernst Leitz Wetzler, Germany, at X 200 magnification.

2.22 Preparation of films using *C. abyssinica* polysaccharide

Aqueous solutions of the polysaccharide of *C. abyssinica* (3% w/v) were prepared by suspending the polysaccharide in water and heating the mixture in a boiling water bath. The solutions were allowed to cool and degassed under vacuum before casting the film onto flat glass plates that had been framed with masking tape in order to prevent the film formulation from flowing off the glass plate.

In order to determine the minimum amount of glycerol required for the formation of flexible films, glycerol at concentrations up to 2% (v/v) was added to 10 ml of degassed solutions of polysaccharide in different containers. Solutions were mixed and further degassed before being poured onto flat glass plates that had been framed with masking tape. The plates were left for 48 hours in order to allow the films to dry at room temperature. The dry films were peeled off the plates and their properties assessed.

2.22.1 Preparation of composite films of the polysaccharide of *C. abyssinica* and starch

Formulations of the various films were prepared by adding 1.5% (w/v) *C. abyssinica* polysaccharide, 1.5% (v/v) glycerol and starch at concentrations up to 2% (w/v). The polysaccharide was dissolved by heating suspensions of the polymer in a boiling water bath and cooled before adding solid starch. The mixture was heated further to dissolve the starch. After cooling to room temperature, solutions were degassed under vacuum and glycerol was added and mixed well before further degassing the solutions. The formulations were poured onto clean flat glass plates that had been framed with masking tape. The

plates were left to stand for 48 hours in order to allow the films to dry before peeling off the films from the glass plates.

2.22.2 Determination of adsorption isotherms of films prepared from the polysaccharide of *C. abyssinica*.

Samples of film (10 x 10 mm), in duplicate, were dried in a dessicator placed in an oven at 40 ^{o}C for 10 days after which, it was assumed, equilibrium would have been established. The dried samples were weighed to the nearest 0.0001 g into pre-weighed bottles which were then equilibrated in air tight 1L Kilner jars containing saturated solutions of different salts, at 25^{o}C for 10 days. The system of saturated salt solutions covered a range of equilibrium relative humidity from 8.2% to 97%. The moisture content of the films at equilibrium was obtained from the gain in weight.

2.22.3 Assessment of solubility and swelling of films

To determine the tendency of films to dissolve or swell, films of different formulations were prepared. Pieces of film (17 x 20 mm) were cut in triplicate from respective films and dried in the oven at 70 ^{o}C for 24 hours to obtain the initial dry mass of the film. The pieces of film were placed into test tubes containing 10 ml of water and the tubes were shaken slowly on a shaker platform at room temperature, for 24 hours. The remaining film was dried in the oven at 70 ^{o}C for 24 hours and the final dry weight of the film determined. The total amount of soluble matter (TSM) was calculated using the relationship,

$$TSM = \frac{(M_i - M_f)100}{M_i}$$

where M_i is the initial mass of dry film and M_f the final mass of dry film.

To assess the swelling of films pieces of film were cut and carefully measured using a ruler with millimetre graduations. The cut pieces were soaked in water at room temperature for 24 hours after which the dimensions were measured again. Extent of swelling was determined by comparing the initial and final areas of the films as shown in the relationship

$$\% \text{ Swelling} = \frac{(A_f - A_i)100}{A_i}$$

where A_i is the initial film area and A_f is the final film area.

2.22.4 Coating of apples with films from the polysaccharide of *C. abyssinica*

Red apples, obtained from the local supermarket, were washed, checked for lack of physical damage and weighed. The fruits were randomly divided into groups of three for different treatments. The first group contained the uncoated fruits, which were dipped in distilled water and served as the control. The second group of apples was dipped in a solution containing *Cordia* polysaccharide (3%) and glycerol (1%). The third and fourth groups were dipped in composite solutions that both contained polysaccharide (1.5%), glycerol (1.5%), but different concentrations of 0.5 and 1% (w/w) starch respectively. The fruits, which were allowed to dry at room temperature, were turned occasionally to prevent uneven distribution of coating and sticking of the apples to the resting surface. After the coating had dried, the apples were re-weighed and allowed to stand at room temperature for several weeks. During the storage period changes in fruit appearance and weight were monitored. The percent loss of mass of the apples during storage was calculated using the relationship

$$\% M_L = \frac{(M_i - M_f)100}{M_i}$$

where M_L is the loss in weight, M_i is the mass at the start of storage and M_f is the mass after storage (Wu *et al*., 2001).

3. RESULTS AND DISCUSSION

3.1 Precipitation of the polysaccharide of *C.abyssinica* using sodium chloride and ethanol

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 further addition of more than three volumes of ethanol, a particulate precipitate that settled at the bottom of the container was observed. When the fibrous and particulate precipitates were redissolved in water and freeze dried, a soft, flaky, white solid was obtained in both cases.

On performing stepwise precipitation of the polysaccharide using ethanol, four fractions of which the first three fractions consisted mainly of the fibrous precipitate and the fourth fraction consisted mainly of the particulate precipitate were collected. Before analysis of the fibrous and particulate precipitates by high performance size exclusion chromatography, the precipitates were assumed to be different polysaccharide preparations. On analysis by HPSEC of the precipitates obtained after stepwise precipitation with ethanol, similar elution patterns were observed for both the fibrous and particulate precipitates, as shown in figure 15. Because the HPLC elution patterns were similar, it was concluded that the fibrous and particulate materials were essentially the same polysaccharide preparation.

Figure 15: HPSEC profiles for the four fractions obtained from precipitation of the polysaccharide of *C. abyssinica* using sodium chloride and ethanol. The precipitates were analysed by HPSEC using water as the mobile phase at a flow rate of 2ml/min, a Zorbax GF 250 column and a refractive index detector. Fractions (A), (B) and (C) contained a fibrous precipitate and fraction (D) contained a particulate precipitate.

3.2 Fractionation of the polysaccharide using the ionic detergent CTAB

When the preparation obtained by precipitation with ethanol was treated with CTAB, a detergent that is expected to precipitate only acidic polymers, a fibrous and a particulate precipitate were collected. The detergent cations react with polyions on the polysaccharide to form salts, which are insoluble in water (Morrison, 1996). Neutral polysaccharides do not react with CTAB and acidic polysaccharides are, therefore, easily separated from the neutral polysaccharides by precipitating them directly with the detergent. It was difficult to regenerate the polysaccharide of *C. abyssinica* after precipitation with CTAB as the precipitates obtained were insoluble in water, 4M NaCl, ethanol or propanol. Eventually it was possible to regenerate the polymer by exchanging the quaternary ammonium salt with the inorganic cation Na^+ in the organic solvent ethanol.

When the fibrous and particulate samples that were obtained from precipitation with CTAB were analysed by HPSEC, similar elution profiles were obtained as shown in figure 16. The elution patterns of the samples precipitated with CTAB were similar to those obtained for samples precipitated with ethanol.

Elution profiles of the pectins isolated from carrots, onion, leek, potato fibre, pear and apple were similar to the profiles obtained from *C. abyssinica* consisting of two major peaks with minor regions in between. The pectins were considered to be a mixture of similar polysaccharides (Schols and Voragen, 1994).

Figure 16: HPSEC profiles for the two fractions obtained on precipitation of *C. abyssinica* polysaccharide using hexadecyltrimethylammonium bromide (CTAB). A Zorbax GF 250 column was used for HPSEC with water as the mobile phase at a flow rate of 2 ml/min. Samples A and B were the fibrous and particulate precipitates respectively.

3.3 Precipitation of the polysaccharide of *C. abyssinica* using 0.2 M HCl

On addition of acid to extracts of *C. abyssinica* fruit, a white particulate precipitate was obtained. On analysis of the 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, as shown in figure 17. 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.

The results for structural studies, therefore, apply for a family of polysaccharides from *C. abyssinica* that were isolated in a particular manner rather than to a single discrete polymer.

3.4 Analysis of the polysaccharide by HPSEC

In HPSEC, water was a more effective eluant than solutions of sodium bicarbonate, sodium carbonate, sodium chloride or acetate buffer. Elution with 0.5 M sodium bicarbonate resulted in rather poor resolution of the polysaccharide into two broad peaks.

When 0.5 M sodium carbonate was used as a solvent and eluant, only a single peak was observed with the baseline disturbed as shown in figure 18. When 0.5 M sodium chloride and acetate buffer were used as eluants the baseline was disturbed and some peaks appeared below the baseline. The Zorbax column used has acidic diol groups, attached to a silica base, that confer some ion exchange properties on the stationery phase. In buffer or electrolyte solutions, the hydronium ions of silanol groups would be exchangeable by cations of the solution or mobile phase, a process, which could have resulted in the disturbance of the baseline and appearance of some peaks below the baseline (Berthod, 1991).

Water was considered to be the most suitable elution solvent for the silica diol column that was used as there was no interference with the column on elution.

Figure 17: HPSEC profile of *C. abyssinica* polysaccharide that had been precipitated using 0.2 M HCl. A Zorbax GF 250 column was used for analysis with water as the mobile phase at a flow rate of 2 ml/min.

Figure 18: HPSEC profile of the polysaccharide of the fruit of *C. abyssinica* that was precipitated using acid. HPSEC was done using a Zorbax GF 250 column and 0.5 M Na_2CO_3 as the mobile phase at a flow rate 1.5 ml.

3.5 Characterisation of some physical and chemical properties of the polysaccharide of *C. abyssinica*

The handling properties of three polysaccharides that have been studied in our laboratory are shown in table 3. In contrast to the polysaccharide isolated from *Azanza garkeana*, which was sticky, hygroscopic and difficult to store dry (Benhura and Machirori, 1999), the polysaccharide preparation from *C. abyssinica* was easy to store in a dry state. It would be interesting to study the relationship between molecular structure and the stickiness, hygroscopic properties and adhesive qualities of the three polymers. The comparison should give an insight to the origin of adhesiveness in polysaccharides.

Table 3: Dry state and solution properties of some polysaccharide preparations. The relative difficulty of precipitating polymers out of solution with absolute ethanol is also shown.

Source of polysaccharide	Form of polymer at room temperature	Property of solution
Dicerocaryum zanguebarium	Dry powder or fibres Very easily precipitated out	Viscous but not sticky
Cordia abyssinica	Dry powder or flakes Easily precipitated out	Viscous and sticky
Azanza garkeana	Sticky mass, difficulty to precipitate out. Decomposes to sticky brown mass on drying	Viscous and sticky

Some of the chemical properties of the polysaccharide from *C. abyssinica* are summarised in table 4.

Table 4: 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**	
	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^{o} ± 0.0	-50.0^{o} ± 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

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 4, 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, together with the observation that the polysaccharide was precipitated with the ionic detergent CTAB and that viscosity of its solutions decreased when salt was added 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).

3.6.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 19. 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 copurified 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 HPLC profile for the native and protease treated samples was practically unchanged with the 4.822 peak showing only a slight shift to low molecular weight, as shown in figure 20. The lack of change in profile is consistent with the 15 % change observed in viscosity, as shown in figure 19. 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.

Figure 20: 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.

3.6.1 Determination of hydroxyproline residues in the polysaccharide chains

The hydroxyproline content of 0.29%, obtained for both the acid and ethanol 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*., 1988). The gum exudate from *Acacia robusta* has been found to contain protein (18%) bound to arabinogalactan (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).

3.7 Determination of intrinsic viscosity in various solvents.

Table 5: Intrinsic viscosity of solutions of *C. abyssinica* preparation in water and other 0.1 M aqueous solvents at room temperature.

Solvent	Intrinsic viscosity (dl/g)
Water	26.4
NH_3	20.9
Na_2CO_3	3.9
NaCl	3.6
NaOH	1.4

The highest intrinsic viscosity of the polysaccharide of *C. abyssinica* was observed when the polymer was dissolved in water, as shown in table 5. The intrinsic viscosity of a given polymer is dependent upon the nature of the solvent and temperature (Hokputsa *et al*., 2003). In a good solvent, one that shows a zero heat of mixing with the polymer, the polymer molecule is loosely extended approximating the spatial arrangement of a random coil that results in high viscosity. The polysaccharide of *C. abyssinica* could be adopting a random coil spatial arrangement in both water and ammonia solutions resulting in the high intrinsic viscosity of the polysaccharide in these solvents.

In a poor solvent, one in which the polymer dissolves with the absorption of heat of mixing, the polymer molecules attract each other when in solution, more strongly than they attract solvent molecules. Consequently, the molecules of the polymer will assume a more compact configuration resulting in a solution of lower intrinsic viscosity. It would appear that the polymer from *C. abyssinica*

adopted a compact configuration in sodium carbonate, sodium chloride and sodium hydroxide resulting in the low intrinsic viscosity.

3.7.1 Effect of salt on intrinsic viscosity

As shown in figure 21, a decrease in intrinsic viscosity was observed in the presence of salt. At high degrees of ionisation, the molecules of *C. abyssinica* polysaccharide would be stretched out as the equally charged segments repel each other (Nishida *et al.*, 2002). At low degrees of ionisation, the fibrous molecules coil up. In common with other polysaccharides, added electrolytes would be expected to decrease electrostatic repulsion between segments of the polymer thereby causing the polysaccharide molecules to contract, resulting in the decreased intrinsic viscosity.

3.7.2 Estimation of viscosity average molecular weight of the polysaccharide of *C. abyssinica* from intrinsic viscosity measurements.

Using the Mark-Houwink- Sakurada equation, the molecular weight of *C. abyssinica* polysaccharide was crudely estimated to be 1 800 000 Dalton as shown in appendix A. This estimate may be of limited value because the constants *k* and *a* were not properly evaluated but guessed at on the basis of constants for other acidic polysaccharides. It had been hoped that values of *k* and *a* would be available from other laboratories, where molecular weight would be determined by light scattering, before the writing up of the thesis was complete.

3.8 Hydrolysis with sulphuric acid

The polymer from *C. abyssinica* dissolved to only a limited extent in 2.5 % sulphuric acid. When the polysaccharide was refluxed in 2.5% H_2SO_4, a substantial proportion of the polysaccharide material was resistant to hydrolysis with a significant solid material remaining in the reaction vessel after twenty-four hours of treatment. After 7 hours of refluxing with concentrated H_2SO_4, a single spot corresponding to rhamnose was observed during TLC analysis, as shown in table 6. Poor solubility of the polymer partially contributed to poor hydrolysis in the acid. As with other acidic polysaccharides, incomplete hydrolysis probably arose from the stability of the glycosyl uronic acid linkage which hinders depolymerisation under normal acid hydrolysis conditions (Jung *et al.*, 2000). On increasing duration of hydrolysis to 15 hours, a smear that started from the origin to the position corresponding to rhamnose standard was observed. The smear that was observed after 15 hours of hydrolysis most likely arose from the production of fragments comprising of a spectrum of different sizes. Hydrolysis with sulphuric acid resulted in low recoveries of sugars and uronic acids, with most of the sugars being poorly resolved in the TLC chromatograms. It appears that, during prolonged treatment with sulphuric

acid, not only was there poor release of monosaccharide residues, but that released monomers were degraded. Decomposition of uronic acids would occur leading to under estimation possibly by decarboxylation (Yu lp *et al*., 1992). After 24 hours of hydrolysis the smear only started from the position of the xylose standard to the rhamnose, which indicated possible degradation of sugars and oligomers originally released when hydrolysis was only done for 15 hours. When samples that had been treated using 2 M sulphuric acid for up to 7 hours were analysed by HPLC, only rhamnose was detected.

When the polysaccharide was hydrolysed following the Saeman procedure, a two step procedure that includes solubilisation of the polymer material in 72% H_2SO_4 followed by secondary hydrolysis in dilute H_2SO_4 at boiling point or in the autoclave, and TLC, only two spots were observed, one corresponding to rhamnose and a faint spot corresponding to galactose. The galactose was not detectable when the samples were analysed by HPLC. It was concluded that treatment with sulphuric acid was not effective in releasing monomers from *C. abyssinica* polysaccharide.

Table 6: Thin layer chromatography on Whatman linear K silica gel plates of monosaccharide standards and samples that had been hydrolysed with sulphuric acid following the method of Adams (1965) for 7(A), 15 (B) and 24 (C) hours. The smears observed in B and C extended to the position of rhamnose. Uncertainties shown are standard deviations for at least three measurements.

Sample	R_f value
Xylose	0.53 ± 0.03
Galactose	0.19 ± 0.02
Arabinose	0.37 ± 0.01
Rhamnose	0.72 ± 0.04
A	0.74 ± 0.05
B	Smear
C	Smear

3.8.1 Hydrolysis using 2 M TFA

As shown in figure 22, hydrolysis with TFA was more effective than hydrolysis with sulphuric acid, in degrading the polysaccharide of *C. abyssinica* into component monosaccharides. When the polysaccharide was hydrolysed with TFA, galacturonic acid, galactose, glucose, arabinose, mannose, xylose, fucose and rhamnose were observed upon TLC analysis. The smears observed between the origin and the spot corresponding to galactose was assumed to correspond to oligosaccharides of various degrees of polymerisation. An unidentified spot that moved with the solvent front was observed. As the duration of hydrolysis was increased from 30 minutes to 2 hours, there was a

corresponding increase in the intensity of the spots on the TLC chromatograms, as shown in figure 22. Extending the duration of hydrolysis beyond 2 hours resulted in decreased intensity of spots reflecting the degradation of the sugars that had been released. Hydrolysis of the polysaccharide with trifluoroacetic acid for between one and two hours would be a suitable balance between maximising release of monosaccharides and minimising degradation of monosaccharides already released.

HPLC analyses of the samples that had been treated with trifluoroacetic acid for 2 hours showed that the polysaccharide contained, galactose, glucose, mannose, arabinose, xylose, fucose and rhamnose, as shown in table 7. Galacturonic acid was not detected in the HPLC chromatograms. Separation on the amino column used was based on normal phase partitioning which would result in the separation of simple sugars and not uronic acid components. Uronic acids would only be separated using a silica based strong anion exchange column. There was a peak that did not correspond to any of the available monosaccharides that were used as standards. In the TLC analysis, mannose and glucose, and xylose and fucose, were not distinguishable but it was possible to distinguish these in the HPLC analyses. HPLC was effective for the separation of sugars that had similar R_f values and colours when separated by TLC.

Figure 22: Thin layer chromatography of sugar standards and samples that had been hydrolysed for 0.5 hours (D), 1 hour (E), 2 hr (F) and 3 hr (G) using 2 M TFA. Lanes A, B, C, H and I are standards xylose, glucose, galactose, arabinose and mannose respectively. Analysis was done on Whatman LK 5 silica gel plate 20x20, developed with ethyl acetate-pyridine-water (20: 7: 5). Dried plates were sprayed with diphenylamine-aniline- phosphoric acid (5:5:1).

Table 7: HPLC of sugar standards and samples hydrolysed using 2 M TFA on a Zorbax-NH_2 column. Uncertainties shown are standard deviations for at least five measurements.

Sample	Retention time	Identity
Glucose	7.00 ± 0.13	
Galactose	7.70 ± 0.01	
Arabinose	4.86 ± 0.09	
Xylose	4.48 ± 0.05	
Fucose	4.29 ± 0.06	
Rhamnose	3.54 ± 0.16	
Mannose	6.36 ± 0.09	
Sample hydrolysate	3.10 ± 0.02	Unidentified
	3.54 ± 0.16	Rhamnose
	6.38 ± 0.07	Mannose
	4.30 ± 0.05	Fucose
	4.52 ± 0.05	Xylose
	4.78 ± 0.20	Arabinose
	7.01 ± 0.13	Glucose
	7.72 ± 0.18	Galactose

3.9. Treatment of *C. abyssinica* polysaccharide with Pectinases

When solutions of polysaccharide were treated with Pectinex Smash the drop in viscosity, was initially rapid but slowed after 25 minutes of hydrolysis with practically no change in viscosity being observed after 50 minutes, as shown in figure 23. The decrease in viscosity increased as the volume of enzyme was increased. At low enzyme concentration the viscosity remained relatively high and did not approach the viscosity attained at high enzyme concentration. The decrease in viscosity would be consistent with the action of endopolygalacturonase, which hydrolyses α 1,4 linkages between galacturonic acid residues (Matsuura *et al*., 2000).

An initial increase in reducing groups, which levelled off after 60 minutes of hydrolysis was observed, as shown in figure 24. A further increase in reducing groups was observed after 180 minutes of hydrolysis. An increase in reducing groups was observed for the Pectinase obtained from Sigma, UK. It appears that the pectinases initially attacked the polysaccharide chain molecules randomly resulting in the initial rapid decrease in viscosity, then progressed with hydrolysis along the polysaccharide chain, acting on the polysaccharide sequentially until it reaches a point, such as a branch point in the chain, where its action was stopped (Margarino and Jose, 2001).

Upon TLC of enzyme hydrolysates, galacturonic acid, galactose, glucose, arabinose and an unidentified spot were observed as spots whose R_f values are shown in table 8. Release of arabinose and glucose that was observed upon hydrolysis with the pectinases could be the result of the action of contaminating hemicellulases. Although a problem in analytical work, the minor contaminating enzyme activities would not normally be a problem in the industrial application of the pectinases.

Table 8: Thin layer chromatography of the polysaccharide of *C. abyssinica* that had been treated with Pectinase from Sigma and Pectinex smash from Novo, on Whatman linear K5 TLC plates 10 X 5 cm, developed 3X in a solvent of acetonitrile -water (85:15). Dried plates were dipped into a methanolic solution of 0.3% N-(1- naphthyl)-ethylenediamine and 5% H_2SO_4. The plates were dried and heated for 10 minutes to visualise the spots. Uncertainties shown are standard deviations for at least four measurements.

Standards and Samples	R_f value	Colour of spot	Identity
Galacturonic acid	0.43 ± 0.01	Purple	
Galactose	0.56 ± 0.02	Brown	
Glucose	0.65 ±0.04	Black	
Arabinose	0.73 ± 0.05	Pink brown	
Mannose	0.82 ± 0.03	Black	
Xylose	0.87 ± 0.03	Purple	
Fucose	0.87 ± 0.03	Pink	
Rhamnose	0.95 ± 0.02	Pink	
Methyl-α-D-glucoyranoside	0.91 ± 0.01	Black	
Methyl-α-D-mannopyranoside	0.93 ± 0.01	Pink brown	
Samples			
Pectinex smash hydrolysate	0.43 ± 0.01	Faint purple	Galacturonic acid
	0.56 ± 0.02	Brown	Galactose
	0.65 ± 0.05	Faint black	Glucose
	0.73 ± 0.06	Pink brown	Arabinose
	0.94 ± 0.01	Yellow	Unidentified sugar
Pectinase hydrolysate	0.43 ± 0.01	Faint purple	Galacturonic acid
	0.65 ± 0.05	Black	Glucose
	0.94 ± 0.01	Yellow	Unidentified sugar

The yellow unidentified spot that moved close to the solvent front on TLC plates of pectinase hydrolysates could be a methyl sugar. The polysaccharide of *C. abyssinica* contained pectic like regions that were susceptible to pectinase action to give galacturonic acid, galactose, glucose, arabinose, and the unidentified methyl sugar.

3.9.1 Treatment with Gamanase

When solutions of *C. abyssinica* polysaccharide were treated with Gamanase, a rapid decrease in viscosity was observed in the first 25 minutes. After 25 minutes of treatment the decrease was gradual and levelled off after 90 minutes of hydrolysis, as shown in figure 25. The decrease in viscosity got larger as the volume of enzyme was increased. At low enzyme the viscosity remained relatively high and did not approach the viscosity attained at high enzyme concentration.

An increase in reducing groups was observed as shown in figure 26. The increase in reducing groups would be expected to result from the release of various components during treatment with enzyme. Although the viscosity, as shown in figure 25, remained practically constant after 90 minutes of hydrolysis, the amount of reducing groups in the polysaccharide chain increased up to 300 minutes of hydrolysis. It was concluded that further cleavage of generated fragments continued to generate reducing groups, but resulted in a less marked drop in viscosity. A rapid change in viscosity relative to gradual change in reducing groups is consistent with random cleavage of β 1,4 bonds of the mannan regions of the polymer by Gamanase (Schmelter *et al*., 2002).

Galactose, glucose, arabinose, mannose, xylose, fucose, rhamnose and galacturonic acid were observed in the hydrolysates of the polysaccharide, as shown in table 9, on analysis of the hydrolysates by threefold development with acetonitrile-water on activated silica gel plates, using N-(1-naphthyl)-ethylenediamine- sulphuric acid for detection (Kim *et al.*, 2002). The three fold development of spots

with acetonitrile- water resulted in effective separation of monosaccharide components of the polymer. Improved identification of spots resulted from the varied bright colours of the spots that resulted from the detection solvent that was used. The reaction between the detection reagent and the sugars involves the formation of furfural which is readily formed by the reaction of sulphuric acid with aldoses resulting in brightly coloured spots, the colours of which made it possible to distinguish between different sugars even those sugars that may have the same R_f values.

From both acid and enzyme hydrolysis studies it was concluded that the polysaccharide from *C. abyssinica* is a pectin-like polymer that contained rhamnose, fucose, xylose, arabinose, mannose, galactose, glucose, and galacturonic acid and presumed but unidentified methyl sugar.

Table 9: Thin layer chromatography of the polysaccharide of *C. abyssinica* that had been treated with Gamanase, on Whatman linear K5 TLC plates 10 X 5 cm, in a solvent of acetonitrile -water (85:15) and developed three times. Dry plates were dipped into a methanolic solution of 0.3% N-(1-naphthyl)-ethylenediamine and 5% H_2SO_4. After drying, the plates were heated at 110 ^oC for 10 minutes to develop the spots. Uncertainties shown are standard deviations for at least four measurements.

Standards and Samples	**R_f value**	**Colour of spot**	**Identity**
Galactose	0.56 ± 0.02	Brown	
Glucose	0.65 ± 0.04	Black	
Arabinose	0.73 ± 0.05	Pink brown	
Mannose	0.82 ± 0.03	Black	
Xylose	0.87 ± 0.03	*Purple*	
Fucose	0.87 ± 0.03	Pink	
Rhamnose	0.95 ± 0.02	Pink	
Methyl-α-D-glucopyranoside	0.91 ± 0.01	Black	
Methyl-α-D-mannopyranoside	0.93 ± 0.01	Pink brown	
Galacturonic acid	0.43 ± 0.01	Purple	
Samples			
Gamanase hydrolysate	0.42 ± 0.02	Purple	Galacturonic acid
	0.56 ± 0.02	Brown	Galactose
	0.73 ± 0.06	Pink brown	Arabinose
	0.82 ± 0.03	black	Mannose
	0.87 ± 0.04	Purple/pink	Xylose and fucose
	0.95 ± 0.02	Pink	Rhamnose
	0.65 ± 0.05	Faint black	Glucose

3.10 Effect of concentration of periodate on periodate oxidation

As shown in figure 27, the amount of periodate that was consumed after 108 hours, when the polysaccharide was oxidised with periodate at various concentrations depended on the concentration of periodate used in the oxidation. When oxidation was done using 0.04 M and 0.05M periodate, the maximum amount of periodate consumed was in both cases about 0.8 moles. When concentrations of periodate less than 0.04 M were used for oxidation, the amount of periodate consumed after 108 hours was less.

3.10.1 Determination of periodate consumption of borohydride reduced polysaccharide.

The amount of periodate that was consumed on oxidation of the borohydride reduced polysaccharide was 1.22 moles, a 34% increase from the 0.8 moles that was observed on oxidation of untreated polysaccharide, as shown in figure 28. Pre-treatment with borohydride would be expected to result in the conversion of any carbonyl groups along the polymer chain into their corresponding hydroxyl groups. In some cases vicinal hydroxyl groups would be created thereby increasing the amount of periodate consumed during oxidation.

3.10.2 Oxidation under conditions of unusually high concentration (0.25 M) periodate

In an attempt to determine whether the use of more concentrated periodate would increase the amount of periodate consumed to the theoretical oxidation limit of one mole, the polymer was oxidised in 0.25 M periodate. When oxidation was done in periodate whose concentration was unusually high, the amount of periodate consumed was 1.24 moles compared to 0.8 moles, observed on oxidation under normal conditions, as shown in figure 28.

The amount of periodate consumed during oxidation in unusually concentrated periodate of the native polysaccharide was similar to the amount of periodate (1.22 moles) consumed on oxidation of polysaccharide that had been reduced with sodium borohydride before oxidation, as shown in figure 28. The prolonged or forced conditions of oxidation using unusually concentrated periodate favour more rapid oxidation of unoxidised residues that may have otherwise been shielded from oxidation by their involvement in the formation of hemiacetal or hemialdal residues during the first stage of oxidation (Christensen *et al*., 2001)

The prolonged oxidation using unusually concentrated periodate, of the galactan from lupin seeds and of amylose resulted in both polysaccharides ultimately giving their expected oxidation limits when lower oxidation limits had been observed during normal conditions of oxidation (Smidsrod *et al.*, 1970; Painter and Larsen, 1970).

3.10.3 Formation of formic acid and formaldehyde

As shown in figure 29, the production of formic acid during the oxidation of the polysaccharide from *C. abyssinica* using 0.05 M metaperiodate increased up to 60 hours after which no more formic acid was produced. Because formic acid arises from oxidation that involves three vicinal groups, we would expect that 2,3,4 triol systems occur in the polysaccharide of *C. abyssinica*. In polysaccharides 2,3,4 triol systems occur in non- reducing terminal units or 1,6 linked non- terminal units having three adjacent hydroxyl groups. The production of formic acid from the non-reducing terminal of polysaccharides is likely to be quantitatively insignificant. The production of formic acid, therefore, was taken to be an indication of branching along polymer chains. Many single monosaccharide residues attached to the main chain through 1, 6 linkages would give rise to substantial amounts of formic acid during periodate oxidation.

Up to 0.17 mmols of formaldehyde was produced on oxidation of the polysaccharide of *C. abyssinica*. The production of formaldehyde was rapid and levelled off after the first few hours of hydrolysis. Much less formaldehyde than formic acid was produced during oxidation of the polysaccharide. Monosaccharide residues may be oxidised to yield intermediates with primary alcohol groups, further oxidation of which gives rise to formaldehyde.

3.10.4 Thin layer chromatography of hydrolysates of the periodate oxidised and borohydride reduced polymer

When the polysaccharide that had been oxidised with periodate and reduced with borohydride was hydrolysed with acid and the products analysed by TLC, galactose, glucose, arabinose, mannose, xylose, galacturonic acid and three presumed oligomers were observed. Two of the oligomers had not been observed in hydrolysates of the native

unoxidised sample, as shown in table 10. The sugars along the chain of *C. abyssinica* could be shielded from oxidation by involvement in linkages at branch points. The protection restricts oxidation to the carbon atoms or prevents it altogether, and these sugars would be detected in the TLC chromatograms after hydrolysis of the periodate oxidised polymers.

Table 10: Sugars produced during hydrolysis with 2M trifluoroacetic acid of periodate oxidised and borohydride reduced polysaccharide of *C. abyssinica*. TLC plates were developed using ethylacetate-pyridine- water (20:7:5) and sprayed with diphenylamine-aniline-phosphoric acid (5:5:1). Uncertainties shown are standard deviations for at least three measurements.

Sample	R_f Value	Identity
Galacturonic acid	0.00 ± 0.00	
Galactose	0.25 ± 0.02	
Glucose	0.32 ± 0.03	
Arabinose	0.36 ± 0.02	
Mannose	0.40 ± 0.01	
Xylose	0.52 ± 0.02	
Rhamnose	0.67 ± 0.01	
Periodate oxidised hydrolysate	Origin	Galacturonic acid
	0.01 ± 0.01	Oligosaccharide
	0.16 ± 0.01	Oligosaccharide
	0.22 ± 0.01	Oligosaccharide
	0.28 ± 0.02	Galactose
	0.32 ± 0.01	Glucose
	0.36 ± 0.02	Arabinose
	0.40 ± 0.01	Mannose
	0.50 ± 0.02	Xylose

3.11 Solubility of the polysaccharide of *C. abyssinica*

The polysaccharide from *C. abyssinica* dissolved in solutions of Na_2CO_3, NaOH, KOH and NH_3 at concentrations of 0.1 M and above. When dissolution of polymer was done at base concentrations below 1 M, it took more than 6 hours to dissolve the polysaccharide completely in solutions of NH_3 and Na_2CO_3, with less time being taken to dissolve the polysaccharide in solutions of NaOH and KOH. The time taken by the polysaccharide to dissolve increased when the concentration of base was decreased.

In solutions of Na_2HCO_3, at a concentration of 0.1 M the polysaccharide did not dissolve after 6 hours. In 0.1 M NH_3, a gel was formed. In alkaline solution, the polymer would be expected to be negatively charged, a situation that discourages interaction between chains and consequent precipitation. It is not clear why a gelled mass was produced in 0.1 M ammonia.

Less viscous solutions were formed in NaOH and KOH than when NH_3 and Na_2CO_3 were used as solvents, as shown in figure 30. The ability of the polymer molecules to increase viscosity by interfering with solvent flow depends predominantly on the volume which the molecules occupy in solution (Lauray, 1986). The relevant volume for a polymer coil in solution is not simply the space occupied by the component residues but the larger hydrodynamic volume that is swept out by the coil as it tumbles around in the flowing liquid. Hydrodynamic volume can vary enormously from one polymer solvent system to another, even if molecular weight and concentration are held constant. Sodium carbonate (0.1 M) was chosen for routine dissolution of *C. abyssinica* polysaccharide.

3.11.1 Effect of pH on solubility of *C. abyssinica* polysaccharide

As shown in figure 31, the polysaccharide from *C. abyssinica* was most soluble above pH 6 when dissolved in Na_2CO_3, a condition that promoted ionisation of the carboxyl groups along the polysaccharide chain. In the ionised form the negative charge developed along the polysaccharide

chains would cause repulsion of the chains and the formation of stable solutions (Whistler and BeMiller, 1997).

Decreasing the pH below 6 resulted in reduced solubility with most of the polysaccharide being precipitated out at pH 4. At low pH ionisation of carboxyl groups along the polysaccharide chain is expected to be repressed. The unionised molecules would associate through the formation of hydrogen bonds between carboxyl groups and hydroxyl groups in neighbouring chains (Whistler and BeMiller, 1997). Such intermolecular interactions are difficult to break even when the particles are placed in excess water and would eventually result in precipitation. In subsequent procedures, adjusting the pH to 4 was used to effect precipitation of the polysaccharide.

When dissolved in Tris-HCl buffer, the polysaccharide remained soluble down to pH 4 below which solubility decreased rapidly with the polysaccharide being practically insoluble at pH 2.5, as shown in figure 32.

3.11.2 Effect of polysaccharide concentration on viscosity of solutions of the polysaccharide of *C. abyssinica*

As shown in figure 33, the viscosity of *C. abyssinica* polysaccharide, dissolved in 0.1 M Na_2CO_3, increased with increase in concentration of the polymer. For *C. abyssinica* polysaccharide, at concentrations below, 0.5%, viscosity would result from disruption of the flow of solvent by isolated polymer chains. The individual polymer chains would be free to move independently through the solvent and the dependency of viscosity on concentration would be less marked (Kar and Arslan, 1999). At concentrations above 0.5 %, viscosity behaviour would largely be determined by polymer-polymer interactions where individual polymers would be brought into physical contact with each other and would be accompanied by the increase in concentration dependency on viscosity, as shown in figure 33.

3.11.3 Effect of pH on viscosity of solutions of the polysaccharide of *C. abyssinica*

Below pH 9, decrease in pH resulted in a decrease in the viscosity, as shown in figure 34. Maximum viscosity of solutions of *C. abyssinica* was observed at pH 9. At low pH, the ionisation of carboxyl groups along the polysaccharide chain is repressed, a situation that promotes interaction between chains, aggregation and consequent decrease in viscosity. At the lowest pH, a decrease in viscosity would arise from the precipitation of the polymer out of solution. When molecules occupy a smaller volume in space through interaction, as illustrated in figure 12, a reduction in viscosity would be observed. The viscosity of polymer solutions is a function of the size and shape of its molecules and the conformations they adopt in the solvent (Whistler and BeMiller, 1997). The high viscosity observed at pH 9 would be a result of coulombic repulsions that prevent chains from approaching each other resulting in an overall extended conformation of the polymer and to high viscosity. The decrease in viscosity beyond pH 9 may be due to degradation of the polymer due to β- elimination. At high pH,

increased ionisation of carboxyl or sulphate half ester groups would be expected to promote extended configuration of the polysaccharide chains and, therefore, increased viscosity.

3.11.4 Effect of temperature on viscosity of solutions of *C. abyssinica* polysaccharide.

The decrease of viscosity with increase in temperature was nearly linear as shown in figure 35 and reflects the expected decrease in chain overlap and entanglement as temperature is increased. A similar decrease in temperature was observed in other polysaccharides such as pectin and starch (Kar and Arslan, 1999, Abu-Jdayil *et al*., 2004).

3.11.5 Effect of salts on viscosity of solutions of the polysaccharide of *C. abyssinica*

When *C. abyssinica* polysaccharide was dissolved in 0.1 M Na_2CO_3 or 0.5 M NH_3, the viscosity decreased to varying extents, with the increase in concentration of KCl, LiCl and NaCl, as shown in figure 36. At the high degrees of ionisation that is expected in base, the polysaccharide molecules are stretched out as the equally charged segments repel each other. Added electrolytes decrease the electrostatic repulsion between segments thereby causing the polysaccharide molecules to contract resulting in a decrease in viscosity (Nishida, *et al*., 2002). The different effects on viscosity of various salts would be a result of different precipitating ability of different ions ranked according to the lyotropic or Hofmeister series, in which, ions are arranged in order of decreasing precipitation effectiveness (Zapsalls and Berck, 1985). The lyotropic series for cations has the order, $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Li^{+} > Na^{+} > K^{+} > NH_4^{+}$.

The effect of NaCl was greater on the polymer that was dissolved in a solution of ammonia than on the polymer dissolved in Na_2CO_3 as shown in figure 36. The greater decrease of viscosity when NaCl was added to polymer dissolved in a solution of ammonia could be due to the additive effect of

the Na^+ and NH_4^+ ions in solution. When more than one electrolyte is present in solution the ions could have an additive effect resulting in a greater decrease in viscosity (Zapsalls and Berck, 1985).

A greater decrease in viscosity was observed for divalent cations than for monovalent cations. Viscosity decreased as the concentration of the divalent ions Ca^{2+} and Mg^{2+} was increased, with magnesium having a greater effect, as shown in figure 37. Because of their ability to interact with oppositely charged groups on different polymer chains, divalent ions, such as calcium, would be expected to be more effective in reducing viscosity than monovalent ones.

3.11.6 Variation of viscosity with shear rate at various concentrations of polysaccharide

At 1% concentration solutions showed pronounced pseudoplastic behaviour with the decrease of viscosity at high shear rate being less for more dilute solutions as shown in figure 38. At high concentrations of polymer, interpenetration of polymer coils in concentrated solutions rise to give an entangled network structure (Hokputsa *et al*., 2003). Shear thinning, as was observed for solutions of *C. abyssinica* polysaccharide, occurs when the rate of disentanglement by shearing forces becomes greater than the rate of formation of new entanglements and thus the polymer molecules become disentangled, the network is depleted and viscosity is reduced. At lower concentrations of polysaccharide shear thinning was less pronounced. When shear rate is increased, the polymer molecules would be expected to become disentangled and align themselves in the direction of flow thus offering less resistance to flow leading to a decrease in viscosity. The behaviour of solutions at 0.25% or less was nearly Newtonian. The degree of pseudoplasticity of solutions of polysaccharide is dependent upon the concentration of the gum, its salt form if it is ionic, and its molecular weight (Whistler and BeMiller, 1997). Thus, a gum solution may have almost Newtonian behaviour at low concentration and pseudoplastic flow after the 'break point' in concentration is reached. In general, solutions of high molecular weight polysaccharides are more pseudoplastic and are, therefore, more affected by shear than low molecular weight gums.

For solutions of concentrations 0.5% and 1%, there was a nearly linear relationship between the log of the apparent viscosity (η_{ap}) and the log of the shear rate (γ) at low shear rates as shown in figure 39. The slope of the graph was high initially and decreased with increasing shear rate, thus the name shear thinning. The linear shape of the log graphs were consistent with those observed for most high molecular weight polysaccharides and characterises the pseudoplastic behaviour of the solutions (Guizani *et al*., 2001). Similarly, linear plots were observed when double logarithmic plots relating viscosity to shear rates were made when studying the effect of temperature, pH, and salts on viscosity of *C. abyssinica* polysaccharide.

3.11.7 Variation of viscosity with shear rate at different temperatures

As shown in figure 40, the viscosity of solutions of the polysaccharide decreased with increase in shear rate at all the temperatures included in the study. As temperature was increased exhibition of pseudoplastic behaviour decreased with behaviour at 80 ^{o}C being almost Newtonian. When solutions are heated the viscosity decreases as the thermal energy of molecules increases and the intermolecular distances increase.

3.11.8 Variation of viscosity with shear rate at different pH

At pH 6.8 the viscosity of solutions of *C. abyssinica* polysaccharide decreased with shear rate, as shown in figure 41. To a lesser extent, a similar decrease in viscosity was observed at pH 12.3. At pH 4.8, viscosity of the solutions decreased and the solutions behaved in a Newtonian manner perhaps, at least partly because most of the polymer would have precipitated out of solution. The observed behaviour was consistent with the behaviour of an acidic polysaccharide. At low pH, neutralisation of

negative charges would lead to association of uncharged polymer chains leading to reduction in viscosity. In the extreme, the polysaccharide would precipitate out of solution.

3.11.9 Variation of viscosity with shear rate at various concentrations of salt

As shown in figure 42, viscosity decreased with shear rate at all salt concentrations indicating pseudoplastic behaviour at various salt concentrations. Pseudoplastic behaviour decreased with increase in concentration of salt with solutions becoming nearly Newtonian in 30 mM sodium chloride.

At the same concentration calcium chloride reduced viscosity to a greater extent than sodium chloride. Pseudoplastic behaviour decreased with $CaCl_2$ concentration, being practically absent at 4 mM $CaCl_2$, as shown in figure 43. Calcium ions, because of their double positive charge, are able to interact with negative charges carried by groups on separate polymer chains. When the polymer chains are brought together, their combined hydrodynamic volume is usually less than that of the free separate polymer chains, a situation that would lead to reduced viscosity.

3.12 Determination of the minimum concentration of polysaccharide required for formation of gels

As shown in table 11, the minimum concentration of *Cordia abyssinica* polysaccharide that was required in order to form a firm translucent gel was 1.5%. The concentration of polymer at which gel formation occurred was similar to the concentrations of 0.5% to 2% at which most polysaccharides gels form (Walkenstrom *et al*., 2003; Yuguchi *et al*., 2003). At low concentrations of polysaccharide, small gel particles were suspended in bulk liquid. As the concentration increased, the size of the gelled masses increased until, at about 0.5%, a continuous firm gelled mass was observed. Because there was no facility for assessing the firmness of gels, assessments of firmness were made subjectively. Increasing the concentration of polysaccharide resulted in increased firmness of the gels. A picture of the gel formed from the polysaccharide of
C. abyssinica is shown in figure 44.

Formation of gel occurred in polysaccharide that had been precipitated using 0. 25 M sodium chloride and ethanol and dried by heating in an oven at 100 ^{o}C. Gels were not formed when the polymer was precipitated using acid with resulting suspensions not soluble in water, as shown in figure 44. In acid the polysaccharide material would occur in an unionised form which favours precipitation of the polymer, hence the polysaccharide would not be soluble in water.

3.12.1 Effect of sucrose in the formation of gels

Addition of sucrose at concentrations up to 10% had no observable effect on the formation of the gels. The result that the addition of sugar was not required for the formation of a gel by the polysaccharide from *C. abyssinica* would be an advantage in the preparation of gels containing small amounts of sugar.

Table 11: Effect of polysaccharide concentration on the formation of gels

Polysaccharide concentration (%)	Subjective rating of gelling	Comments
0.05	+	Small gelled areas in bulk liquid
0.2	+	Small gelled areas in bulk liquid
0.4	++	Large gelled areas in a small amount of liquid
0.5	+++	Loose gel extending throughout the container
1.0	++++	Loose gel extending throughout the container
1.5	+++++	Firm gel extending throughout the container
2.0	++++++	Firmer gel

Figure 44: Example of the gel formed from the polysaccharide of *C. abyssinica* in water at 4 ^{o}C, vial D. Vials A to C contained aqueous suspensions of the polysaccharide precipitated using HCl which, were not soluble in water and vial D contained the gel formed from the NaCl -ethanol precipitated polysaccharide. Picture was taken by Professor Benhura, Department of Biochemistry, University of Zimbabwe.

3.12.2 Effect of calcium, potassium, sodium and EDTA on gel formation

At concentrations of calcium chloride above 5 mM, a precipitate which was not clear, rather than a gel was formed. Although without observable effect at concentrations below 1 mM, at concentrations above 1 mM, calcium ions resulted in decreased firmness of gels as shown in table 12. Addition of calcium ions at increasing concentrations to the gel forming suspensions of the polysaccharide of *C. abyssinica*, could have resulted in aggregation of the polymer molecules, giving rise to the formation of precipitates with poor water binding capacity (Bowers, 1992).

Inclusion of the monovalent ions, sodium and potassium, at concentrations up to 50 mM had no effect on the formation of gel.

When EDTA was added to the gel forming suspensions, the firmness of the gels formed decreased as shown in table 13. When EDTA was present at 50 mM or higher concentrations, no gel was formed at all with the material appearing as a hydrated mass. It is not clear why the addition of both metal ions and EDTA to the gel forming mixtures had similar effects on gel formation.

3.12.3 Effect of pH on gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

In aqueous, gel forming suspensions of the polysaccharide of *C. abyssinica* at pH 3, a hard insoluble material remained in the suspending medium. Raising the pH to 4 resulted in the formation of a compact hydrated material. Firm gels were formed at pH 5.6, as shown in table 14. Raising the pH above 7 resulted in small pieces of gel floating in bulk of liquid.

Table 12: Effect of calcium ions on the gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

Concentration of calcium ions (mM)	Subjective rating of gelation	Comments
0.0	+++++	Firm gel
0.5	+++++	Firm gel
1.0	+++++	Firm gel
5.0	+++	Loose gel
10.0	++	Gelled masses and liquid
20.0	No gel	Hydrated material but no gel
50.0	No gel	Unhydrated lumps

At pH 3 and 4, the polysaccharide molecules would be uncharged and extensive aggregation of the polysaccharide chains would be promoted. Aggregation of the polysaccharide molecules would be such that the formation of a precipitate rather than a gel is promoted. A gel is an intermediate state between a precipitate and a solution (Bowers, 1992). At pH 5.6, the formation of firm gels was promoted but loose gels were formed at pH 7 and above. Above pH 7, polysaccharide molecules in solution would be negatively charged and experiencing repulsive forces against each other, such that aggregation of polymer chains to form gels would not be promoted, resulting in the formation of loose gels.

Table 13: Effect of EDTA on the gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

Concentration of EDTA (mM)	Subjective rating of gelation	Comments
0.0	+++++	Firm gel
10	++++	Less firm gel
20	+++	Loose gel
30	++	Loose gel
40	+	Gelled masses and a little liquid
50	+	Gelled masses and bulk liquid
100	No gel	Hydrated lumps with no gel

3.12.4 Effect of temperature on gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

When assessed visually the gels formed by 1% polysaccharide at room temperature were nearly as firm as those formed at 4 ^{o}C. It is possible that differences could have been detected if a more objective method of assessment had been used. At temperatures above 30 ^{o}C, the firmness of the gels formed decreased as shown in table 15.

Upon heating the gels formed at 4 ^{o}C, at 100 ^{o}C for about 20 minutes, the gels broke up to form viscous masses. Upon cooling of the degraded gels to 4 ^{o}C and maintaining that temperature for 5 hours, individual lumps of gel formed but no continuous gel mass was formed. Similarly, gels formed in the presence of sucrose were not reformed after heating at 100 ^{o}C and cooling to 4 ^{o}C. It appears that gels formed by the polysaccharide of *C. abyssinica* do not reform easily after being destroyed by heat. Temperature changes influence the bonds responsible for maintaining the gel structure. As temperature rises bonds become increasingly labile until the semi- solid state of the gel assumes viscous liquid properties. In thermoreversible gels the conversion can be reversed upon subsequent cooling (Zapsalls and Berck, 1985).

Table 14: Effect of pH on the gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

PH	Subjective rating of gelation	Comments
3	No gel	Hard insoluble material
4	No gel	Hydrated material but no gel
5.6	+++++	Firm gel
7	++++	Less firm gel
8	++	Hydrated lumps

Table 15: Effect of temperature on the gelation of aqueous suspensions of the polysaccharide of *C. abyssinica*

Temperature (^oC)	Subjective rating of gelation	Comments
4	+++++	Firm gel
25	++++	Gel less firm than at 4^oC
30	+++	Flowing weak gel
40	++	Flowing gel
60	Viscous mass	No gel formed at all
96	Viscous mass	No gel formed at all

3.13 Emulsifying properties of the polysaccharide of *C. abyssinica*

The emulsifying properties of the polysaccharide from *C. abyssinica* were investigated and compared to those of gum arabic, a standard commercial emulsifying agent.

3.13.1 Microscopic examination of emulsions

When emulsions were not diluted with SDS, clumping or flocculation of oil globules to form aggregates of various sizes was observed. Coalescence of flocculated particles were observed under the microscope for both the polysaccharide from *C. abyssinica* and gum arabic. Dilution of the emulsion with 0.1% sodium dodecyl sulphate solution was effective in disrupting flocculation to produce a more dispersed system. Sodium dodecyl

sulphate is a surfactant which is normally used to stabilise oil in water emulsions by forming a protective layer around the droplets (McClements and Dercker, 2000).

A photomicrograph of the emulsion that was prepared in the presence of 1% *C. abyssinica* polysaccharide at pH 7.4 is shown in Figure 45. When emulsions were prepared in the presence of polysaccharide, improved uniformity in the size of the oil droplets was observed, with the smallest droplets being less than 2 μm in diameter. The average size of droplet was much larger when no polymer was included. The polysaccharide would be surrounding the oil globules and would help in keeping the oil globules dispersed by acting as barriers that hinder movement between the dispersed oil globules.

Figure 45: Photomicrograph of a 25% oil in water emulsion prepared using 1% solution of *C. abyssinica* polysaccharide at 25 ^{o}C, pH 7.4 showing droplets of oil suspended in an aqueous phase. Magnification 200 X.

3.13.2 Effect of concentration of polysaccharide on emulsification properties

As shown in Figure 46, the emulsifying activity of both the polysaccharide from *C abyssinica* and gum arabic increased practically linearly with concentration. Increasing the concentration of polysaccharide resulted in finer dispersions. When no polysaccharide was added in preparing the emulsions, the oil droplets quickly coalesced and, after 30 minutes, the aqueous and oil layers were completely separated. For samples prepared with *C. abyssinica* polysaccharide and gum arabic, coalescence of the oil droplets

became less and less pronounced with increase in polymer concentration. Increasing the concentration of *C. abyssinica* polysaccharide resulted in increased viscosity of solutions. Polysaccharides are often added to oil in water emulsions to enhance viscosity of the aqueous phase, which imparts desirable textural attributes and stabilises the droplets against coalescence by slowing down the movement of reactants (McClements and Dercker, 2000).

At polysaccharide concentration of 0.25%, emulsions prepared with *C. abyssinica* polysaccharide were unstable and the measured turbidity after 10 minutes was only slightly greater than that for samples in which no polysaccharide had been added, as shown in Figure 47. At polysaccharide concentrations of 0.5%, the emulsions formed, retained nearly 70% of the original emulsifying capacity after 30 minutes. At a polysaccharide concentration of 1%, the size of the oil droplets in the emulsions had not changed 30 minutes after preparation of the emulsions. It is expected that the polysaccharide from *C. abyssinica* act as an emulsifier by forming a physical barrier, which prevents close contact between the emulsified droplets. Hydrogen bonding and

polar interactions would occur between water and the polar end of the emulsifier as illustrated in figure 48 (Chanamai and McClements, 2002).

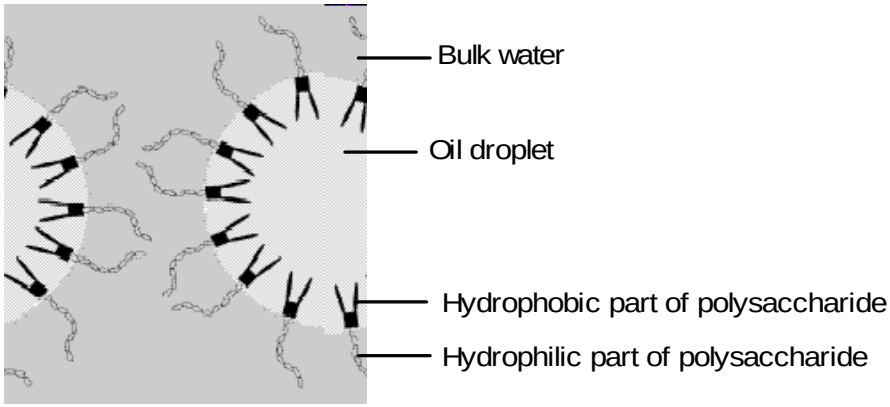


Figure 48: Possible mechanism of the stabilisation of an oil in water emulsion by a polysaccharide. Hydrophobic positions of the polysaccharide interact with the oil droplet, with the hydrophilic position extending into the aqueous environment. By: Clyde E. Stauffer, Emulsions, http:/www.foodesource.org/content/pdf/emulsifiers/ch1.pdf, 16 July 2004.

In contrast to emulsions prepared with the polysaccharide from *C. abyssinica*, emulsions prepared with gum arabic were more stable, as shown in Figure 49. As a result of its globular, greatly branched molecular structure, gum arabic forms a continuous flexible film round insoluble particles. In emulsions, gum arabic being acidic, forms a negatively charged coat around each oil globule causing globules to repel one another and thus preventing their recombination.

3.13.3 Effect of salt on emulsifying properties of *C. abyssinica* polysaccharide

Emulsions formed using *C. abyssinica* were much less sensitive to salt than those prepared with gum arabic, as shown in Figure 50. For gum arabic up to 40% increase in turbidity was observed with increase in concentration of salt, whereas practically no increase was observed for *Cordia abyssinica* polysaccharide.

In contrast to the mucilage prepared from *Ruredzo* (*Dicerocaryum zanguebarium*), which showed dimished emulsifying capacity in the presence of salt (Benhura & Marume, 1993), the polysaccharide from *C. abyssinica* could act as an emulsifying agent in systems with relatively high salt concentrations.

3.13.4 Effect of pH on emulsification properties of *C. abyssinica* polysaccharide

As shown in Figure 51, *C. abyssinica* polysaccharide had maximum emulsifying capacity at pH 11. Below pH 3, the polysaccharide precipitated out of solution and would, therefore, not be useful in emulsification under conditions of low pH. From microscopic examination, the droplet sizes of emulsions formed at pH 11 were much smaller compared to those that were formed under the lower pH conditions. Emulsions formed at pH 11 were the most stable, as shown in figure 52, retaining about 73% of their original emulsifying capacity after 30 minutes. Emulsions formed at neutral pH with a 1% concentration of polysaccharide would be suitable for food formulations, but, because the polymer has poor emulsifying properties at low pH, the polymer would be of limited value in many food applications such as salad dressings and sauces. The much finer emulsions formed at alkaline pH would be suitable for shampoo formulations, as these formulations usually have pH requirements above pH 7.

3.14 Films formed from the polysaccharide of *C. abyssinica*

Films that were formed using the polysaccharide of *C. abyssinica* on its own were transparent but brittle and could not be peeled off the glass plate without breaking. The brittleness would be expected to arise from extensive intermolecular forces involving chain to chain interaction through hydrogen bonding between polysaccharide chains (Sorthonvit and Krochta, 2000). The large number of hydroxyl and carboxyl groups along the polysaccharide molecules as well the presence of cations would be responsible for the numerous hydrogen bonds and cation crosslinks between the polysaccharide molecular chains. Extensive interchain interactions would contribute to the high mechanical strength and low flexibility or brittleness of the polysaccharide films. Because an effective coating should be durable, maintain its structure during storage and provide an effective barrier against moisture and gas (Ball, 1999), *C. abyssinica* polysaccharide would not be suitable, on its own, for preparing edible coatings.

3.14.1 Effects of glycerol content

When glycerol was added to film formulations, transparent, shiny and flexible films that could be easily peeled off from the glass plate intact were formed. Increasing the concentration of glycerol in the film formulations resulted in increased flexibility of the films. When glycerol was added at concentrations below 1%, the films formed were brittle and would not peel off from the glass plate without breaking. At least 1% glycerol was needed to reduce brittleness and obtain films that peeled off intact. When glycerol was included at levels of 1.5% and above, films were soft, sticky and difficult to work with. Examples of films that were formed from the polysaccharide of *C. abyssinica* with glycerol included at concentrations up to 1.5% are shown in figure 53. In the presence of glycerol or with glycerol present at levels greater than 1.5%, films that were unacceptably soft and sticky were formed. Addition of glycerol to the film formulations that were prepared from pure polysaccharide was

effective in decreasing brittleness of films and enhancing flexibility. The addition of glycerol as a plasticiser is expected to lead to a decrease in intermolecular forces along the polymer chains improving flexibility and facilitating the peeling off of films from the glass plate (Sorthonvit and Krochta, 2000). The effectiveness of glycerol as a plasticiser is most likely due to its small size that allows it to be more readily inserted between the polymer chains enabling the establishment of polymer-plasticiser hydrogen bonds at the expense of polymer- polymer interactions. Reduction of direct interactions between the polysaccharide would result in increased chain mobility and film expensibility.

Figure 53: Films formed by 3% polysaccharide of *C. abyssinica* in the presence of various levels of glycerol. The films contained no glycerol (A), 1% glycerol (B) and 1.5% glycerol (C).

3.14.2 Polysaccharide- starch composite films

Upon addition of starch to films of *C. abyssinica* polymer in the absence of glycerol, the films that were formed were brittle and broke easily. Addition of starch to film formulations in the presence of 1.5% glycerol, resulted in flexible films that peeled off the glass plate relatively easily. When the concentration of starch was increased in the film formulations, films formed became translucent

compared to the transparent films formed using *Cordia* polysaccharide only. Films containing starch in addition to glycerol were softer but more elastic than the polysaccharide-glycerol films.

3.14.3 Adsorption isotherms of edible films prepared from the polysaccharide of *C. abyssinica*

As shown in figure 54, the sorption curve for films made from the polysaccharide of *C. abyssinica* was continuously concurve upward, behaviour that is typical of water sensitive films of biopolymers (Yang and Paulson, 2000). The pattern of the adsorption isotherms would result from the formation of water clusters in the polymer matrix as the water activity was increased, as well as swelling of the matrix that may cause exposure of more binding sites for water sorption.

3.14.4 Solubility and swelling of the films

Films made with *C. abyssinica* polysaccharide and glycerol only were completely soluble in water. Composite films containing starch at concentrations up to 0.75% showed an average % total soluble matter of 45% whereas the 1% starch composite film had an average % total soluble matter of 25%, which was lower than at lower starch concentrations. Inclusion of starch in the film formulations would improve effectiveness under conditions in which the films are exposed to water, by making the films less soluble in water. Film solubility affects film use, for example, films on high moisture foods must be insoluble, while films for water soluble pouches must be readily soluble (Sorthonvit and Krochta, 2000).

The degree of swelling of *C. abyssinica* films was determined only for films that kept their integrity when in contact with water, which meant films that had starch at concentration above 1%. Composite films of polysaccharide-glycerol containing 0.5 to 0.75% starch did not keep their integrity when in contact with water, but broke up into gel- like segments. Composite films containing 1% content of starch showed an average degree of swelling of 565% whereas films containing 1.5% content of starch had a degree of swelling of 1035%, nearly double that for films containing 1% starch. The resulting hydrated films were gel like and slippery. The polysaccharide of *C. abyssinica* and starch are hydrophilic polysaccharides and thus films formed from the polysaccharide and starch would be expected to progressively hydrate and swell when exposed to water or in environments of high humidity as observed from the shape of the moisture sorption isotherms of the films. Water absorbed on the films would be expected to behave as a plasticiser along with glycerol (Yang and Paulson, 2000). During hydration of the films, newly added water molecules may have developed water-polymer hydrogen bonds reducing the interchain interactions between polymer chains and enhancing the chain mobility. Due to their swelling properties and solubility in water, edible films made from the

polysaccharide of *C. abyssinica* would not be suitable for use in high moisture environments but would be appropriate for water soluble pouches which may be used for packaging food.

3.14.5 Coating of apples with films

The use of edible films for coating fruit, in some ways, mimics controlled atmosphere storage which, is labour intensive and expensive. Controlled atmosphere storage can be done with an environmentally friendly and biodegradable edible coating. Fruits that were coated with polysaccharide - glycerol film were shiny and attractive, as can be seen in figure 55. Fruits coated with composite films that contained 0.5% and 1% starch were also attractive but less shiny, possibly because of the translucent nature of the polysaccharide-glycerol-starch composite films. On continued storage the control uncoated fruits lost their luster as they continued to lose moisture. After two weeks of storage, the control fruits became dull and the fruits began to develop wrinkles that became more pronounced with storage time. The coated fruits maintained their firmness for six weeks of storage. The colour of the fruits remained vibrant in contrast to the control fruits. As shown in figure 56, the loss of moisture was about the same for the coated apples irrespective of the type of film used for coating. After 30 days of storage, the control fruits had lost 12% moisture whereas the coated fruits had lost 8%. Although the edible films from *C. abyssinica* only provided minimum moisture barriers because of their hydrophilicity, they were effective in delaying the loss of moisture in the apples and in prolonging the shelf life of the apples.

Films made from polysaccharides are expected to be effective oxygen barriers because of their tightly packed ordered hydrogen bonded network structure. The ability of water soluble polysaccharides to reduce oxygen and increase carbon dioxide levels in internal atmospheres of coated fruits reduces respiration rates thereby extending the shelf life of fresh produce in a manner that may be similar to controlled atmosphere storage, where there are controlled high levels of carbon dioxide and

low levels of oxygen in the internal atmosphere (Diab *et al*., 2001). The reduction of gaseous transfer could, however, promote fermentation as respiration is reduced within the coated fruit.

Figure 55: Apples coated using films from the polysaccharide preparation of *C. abyssinica* after 30 days of storage. The apples were (A) uncoated, and coated with polysaccharide -glycerol (B), polysaccharide-glycerol-starch (1.5:1.5:0.5 w/w) (C) and polysaccharide -glycerol -starch (1.5:1.5:1.0 w/w) (D).

5. CONCLUSIONS

The polysaccharide of *C. abyssinica*, composed of galactose, glucose, arabinose, mannose, fucose, xylose, rhamnose, galacturonic acid and an unidentified methyl sugar, had a molecular weight, crudely estimated, of 1.8 million daltons. Upon HPSEC of solutions of the polysaccharide after isolation using various methods, it was concluded that the polysaccharide material was made up of a family of closely related polymers. The acidic polysaccharide, had a uronic acid content of 9% with some of the uronic acid residues in the polymer being methyl esterified . The methoxyl content was 38%. Upon oxidation of the polysaccharide by periodate, a thousand times more formic acid than formaldehyde was produced from which it was concluded that the polysaccharide was branched. The polysaccharide was associated with between 2.6% and 4.6% protein, some of which appeared to be covalently bound.

Although the polysaccharide was extremely resistant to hydrolysis by acids, some neutral sugar components of the polysaccharide were released by hydrolysis with 2 M TFA. Prolonged treatment with TFA resulted in degradation of released monosaccharides. Hydrolysis of the polysaccharide with pectinases released galacturonic acid, galactose, arabinose, glucose and an unidentified methyl sugar. The polysaccharide from *C. abyssinica* appears to contain pectin- like regions in the polysaccharide that contained a galacturonic acid core. It was speculated that to the galacturonic acid core was attached the sugars galactose, glucose and arabinose in a manner that is still unclear.

The pseudoplastic flow behaviour of solutions of polysaccharide resembled the flow properties of other high molecular weight polysaccharides that are currently used commercially in food systems. Viscosity of the solutions increased with increase in polysaccharide concentration but decreased upon

addition of salts to the polymer solutions. The decrease in viscosity upon addition of salt to polysaccharide solutions is consistent with the polyelectrolyte nature of the polysaccharide.

Although relatively effective at neutral and alkaline pH, the polysaccharide of *C. abyssinica* would not be useful as an emulsion stabiliser under conditions of pH lower than pH 5. Stable emulsions were formed at relatively low polysaccharide concentrations. The basis of the interfacial activity of the polysaccharide in not clear but the activity could arise from the presence of protein or the esterified uronic acid groups.

The polysaccharide from *C.abyssinica* has a potential for use as a gelling agent in products where low sugar gels are required. Further work would be needed to determine the optimum conditions for the formation of gels. It appears that gelation of solution of *C. abyssinica* polysaccharide is similar to that observed for pectin and may be due to the formation of egg- box type junction zones.

The polysaccharide of *C. abyssinica* has potential for use in the preparation of films for coating fruits in order to prolong their shelf life. The polymer would be used in the presence of glycerol and in combination with other polysaccharides to produce films of different properties that would be suitable for specific applications.

6. Further Work

It would be useful to determine the nature and types of linkages between the monosaccharide constituents of the polysaccharide using techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy. It would be interesting to investigate the nature of association between protein and the polysaccharide.

A structural comparison of the polysaccharides from *Dicerocaryum zanguebarium*, *Azanza garkeana* and *Cordia abyssinica* should provide an insight into the molecular basis of adhesive properties in polysaccharides. The polysaccharides isolated from the three plants produce solutions that are sticky and a comparison of their structures could give an insight into the molecular basis of the stickiness.

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8. APPENDICES

8.1 Appendix A

Calculations of molecular weight of polysaccharide isolated from *C. abyssinica* from viscosity measurements.

Ratio of the viscosity of polymer η_p to that of solvent η_s gives the relative viscosity η_r. η_r-1 is called the specific viscosity η_{sp}. The reduced viscosity, η_{red} is obtained by dividing η_{sp} by concentration in g/ml. The intrinsic viscosity [η] is obtained by an extrapolation of η_{sp}/c and ln η_{red}/c to zero concentration.

Calculations

The Mark-Houwink equation was used to determine viscosity average molecular weight:

$$[\eta] = KM^a$$

where [η] is the intrinsic viscosity, M is the viscosity average molecular weight and *K* and *a* are constants that are dependent on the nature of the polymer, solvent and temperature of viscosity measurements (Seymour and Carraher, 1988).

Taking logarithms:

Log [η] = alog M+ log K

Log M = (log [η] -log K)/a

= log 26.4 - log (2.73×10^{-4})*/ 0.80

= 1.422-(-3.5751)/ 0.80

=4.997/ 0.80

= 6.2463975

M = 1 763 589

The values for *K* and *a* were average values calculated using *K* and *a* values for other acidic polymers, such as pectin, gum arabic and alginic acid assuming a random coil conformation of the polysaccharide in water (Schmelter *et al*., 2002; Kar and Arslan, 1999, Sanche *et al*., 2002, Larsen *et al*., 2003).

8.2 Appendix B

PUBLICATIONS ARISING FROM THE WORK DESCRIBED IN THIS THESIS

1. Benhura, M. A. N. and **Chidewe, C.K.** (2000). Preliminary study of the gelling properties of polysaccharide isolated from the fruit of *Cordia abyssinica*. *Gums and Stabilisers for the Food Industry,* **10**, 69-75

2. Benhura, M. A. N. and **Chidewe, C. K**. (2000). Viscosity and solubility properties of the polysaccharide isolated from the fruit of *Cordia abyssinica*. *Advances in Food Science (CTML),* **22 (5,6),** 165-169

3. Benhura, M. A. N. and **Chidewe., C. K**. (2002). Some properties of a polysaccharide preparation that is isolated from the fruit of *Cordia abyssinica.* *Food Chemistry*, **76**, 343-347

4. Benhura, M. A. N. and **Chidewe, C. K**. (2004). Emulsifying properties of a polysaccharide isolated from the fruit of *Cordia abyssinica.* *International Journal of Food Science and Technology*, **39,** 579-583.