CHARACTERIZATION OF THE STRUCTURE AND PHYSICAL PROPERTIES OF YELLOW MUSTARD (*Sinapis alba* L.) POLYSACCHARIDES

By
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BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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FOREWORD

This thesis is presented in a paper-style format. The first chapter gives the general introduction while the second chapter covers the literature review. The next five chapters (Chapter 3-7) are presented according to the sequence of research conducted as five papers or manuscripts. The final chapter includes a general discussion of the overall work with several conclusions based on this study. Chapters 3 and 4 were published in Food Chemistry and Carbohydrate Polymers, respectively. Chapter 5 is submitted to Journal of Agriculture and Food Chemistry while Chapter 6 and Chapter 7 will be submitted to Carbohydrate Research, the latter as a research note. All manuscripts and/or papers in this thesis are presented in a uniform format for consistency.

ABSTRACT

Mucilage was extracted from yellow mustard (*Sinapis alba* L.) seeds by an improved extraction procedure followed by precipitation with 95% ethanol. The yield obtained was approximately 5% of seed weight compared to 2% reported by previous researchers. Chemical analysis of crude mucilage (CM) and its fractions revealed they were composed mainly of polysaccharides (80%-94%) together with ash (4.7%-5.0%) and proteins (1.6%-4.0%). Glucose (22%-35%) was the major neutral monosaccharide present followed by galactose (11%-15%), mannose (6.0%-6.4%), rhamnose (1.6%-4.0%), arabinose (2.8%-3.2%) and xylose (1.8%-2.0%) together with 14.6% uronic acids. Functional properties of the extracted CM and its fractions were examined in terms of viscosity, flow behavior, surface tension, emulsion and foaming capacity and stability. The CM prior to dialysis exhibited superior emulsion capacity and stability compare to the commercial gums, xanthan, guar and gum arabic. The flow behavior of CM and its water-soluble fraction was similar to xanthan gum in terms of shear thinning and viscoelasticity.

The water-soluble fraction (WS) of yellow mustard mucilage was the major fraction (55.6%) exhibiting pronounced shear thinning behavior. It was separated into a CTAB-precipitated fraction (WSCP) and a CTAB-soluble fraction (WSCS) by precipitation with 5% CTAB (hexadecyltrimethylammonium bromide) under optimum conditions of complexation of CTAB with acidic polysaccharides. The major fraction WSCP (52%) was a mixture of a pectic polysaccharide and 1,4-linked β-D-glucan while the minor fraction WSCS (34.0%) was composed of two polysaccharide fractions differing in molecular size and containing 1,4-linked β-D-glucan and another polysaccharide containing a non-reducing end glucuronic acid. Both WSCP and WSCS contributed to the rheological properties of WS although they differed significantly in chemical structure and molecular size distribution. WSCP exhibited similar shear thinning and "weak gel" properties to WS as determined by

steady shear and dynamic rheological measurements.

Both WSCP and WSCS were further separated by ion exchange chromatography on high capacity DEAE cellulose.Ten sub-fractions were obtained (5 from each of WSCP and WSCS), of which only two neutral fractions (WSCP-I and WSCS-I) and one acidic fraction (WSCP-III) exhibited shear thinning behavior at 0.5% concentrations. The remaining fractions showed Newtonian flow behavior under the same conditions. Methylation analysis and/or ^{13}C NMR spectroscopy revealed that both WSCP-I and WSCS-I were composed mainly of 1,4-linked β-D-glucan; WSCP-I appeared to be more heterogeneous. WSCP-III was a relatively homogenous pectic polysaccharide containing a non-reducing end glucuronic acid (13.3%) 1,4-linked galacturonic acid (13.6%), 1,6-linked galactose (22.9%), 1,2-linked (11%) and 1,2,4-linked (18%) rhamnose.

Of the three fractions exhibiting shear thinning behavior, WSCP-III and WSCS-I were subjected to further structural analysis but was not done with WSCP-I due to its poor water solubility. WSCP-III was partially hydrolyzed, and the resulting oligosaccharide mixtures isolated by ion exchange chromatography on DEAE-Sephadex A 25 column and further purified by gel filtration chromatography on Bio-Gel P-2 column. Purified oligosaccharides were characterized by FAB-MS and one and two-dimensional NMR spectroscopy and a average structure of WSCP-III was proposed . WSCS-I was subjected directly to one and two dimensional NMR spectroscopy as it had a rather simple structure. In addition to a major 1,4-linked β-D-glucan backbone chain, methyl or ethyl ether groups were found associated with the cellulose-like structure.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Denotes
G'	Storage modulus (Pa)
G''	Loss modulus (Pa)
η	Apparent viscosity (Pa.s)
η_0	Zero shear-rate viscosity (Pa.s)
η'	Dynamic viscosity (Pa.s)
η^*	Complex viscosity (Pa.s)
δ	Phase angle (degree)
	Chemical shift (ppm)
$\dot{\gamma}$	Shear rate (s^{-1})
ω	Oscillatory frequency ($rads^{-1}$)
f	Oscillatory frequency (Hz)
n	Flow index (dimensionless)
K	Consistency index (Pa s)
CM	Crude mucilage
WS	Water-soluble fraction of CM
WI	Water-insoluble fraction of CM
WSCP	Water-soluble CTAB-precipitated fraction
WSCS	Water-soluble CTAB-soluble fraction
CTAB	Hexadecyltrimethylammonium bromide
FAB-MS	Fast-atom-bombardment mass spectrometry
NMR	Nuclear magnetic resonance
Rf	Relative retention time
M	Mole concentration
TFA	Trifluoroacetic acid

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CHAPTER 1

INTRODUCTION

Natural hydrocolloids or gums are accepted worldwide as food additives because they have many functional properties that make them useful in food applications. Controlled water management during food processing through the use of hydrocolloids allows for the production of many interesting food textures. Important parameters such as viscosity, surface/interfacial tension, emulsification, foaming capacity and stability play a major role in food processing. Carbohydrate hydrocolloids or polysaccharide gums of plant origin play a prominent role in food preparation and processing because of their functional properties as listed in Table 1.1.

Yellow mustard (*Sinapis alba* L.) has been cultivated as a condiment for 2000 years. Bailey and Norris (1932) reported that white or yellow mustard seeds, *Brassica alba* L., *Brassica hirta*, currently referred to as *Sinapis alba* L., were rich in mucilage. Weber and co-workers (1974) suggested this mucilage was important for the consistency of prepared mustard products. In Canada approximately 400,000 acres of yellow mustard are grown, and most of the seeds is used as condiments in the food industry. Mucilage from yellow mustard seeds could be a potentially important indigenous gum material for use by the Canadian food industry.

Table 1.1: Functional properties of polysaccharide gums.

Function	Example
Adhesive	Glazes, icings, frostings
Binding agent	Pet food
Bodying agent	Dietetic beverages
Crystallization inhibitor	Ice cream, sugar syrups, frozen foods
Clarifying agent (fining)	Beer, wine
Cloud agent	Fruit drinks
Coating agent	Confectionery, fabricated onion rings
Dietary fiber	Cereals, bread
Emulsifier	Salad dressings
Encapsulating agent	Powdered flavors
Film former	Sausage casings, protective coatings
Flocculating agent	Wine
Foam stabilizer	Whipped topings, beer
Gelling agent	Puddings,desserts, confectionery
Molding	Gum drops, jelly candies
Protective colloid	Flavor emulsions
Stabilizer	Salad dressing, ice cream
Suspending agent	Chocolate milk
Swelling agent	Processed meat product
Syneresis inhibitor	Cheese, frozen foods
Thickening agent	Jams, pie fillings, sauces
Whipping agent	Toppings, marshmallows

Adapted from Glicksman, 1982a

A number of studies have been conducted in Canada on yellow mustard seed mucilage, but the methods were of limited commercial value because of poor yields (0.3 to 2% of seed weight). Recent studies conducted in our laboratory, however, using a different extraction procedure, resulted in much higher yields of yellow mustard mucilage (5% of seed weight). Rheological tests performed on solutions/dispersions of this material showed that at equivalent concentration, it had similar shear thinning properties to xanthan gum dispersions, a microbially produced and widely used food gum. Very few data are available on the chemical structure and physical properties of mucilage from yellow mustard seeds. An understanding of the chemical structure and functional properties of yellow mustard mucilage polysaccharides is essential for developing this material as a valuable adjunct for the food industry. The main objectives of this study were to:

1). maximize the yield of mucilage from yellow mustard seeds;

2). determine the functional properties of yellow mustard mucilage;

3). separate yellow mustard mucilage into different fractions and evaluate their rheological properties;

4). characterize the linkage patterns and structural features of the polysaccharides exhibiting the most pronounced shear thinning effect in their dispersions, and relate the structures to the functional properties of these polymers.

CHAPTER 2

LITERATURE REVIEW

2.1 EXTRACTION, YIELD AND MONOSACCHARIDE COMPOSISTION OF MUCILAGE FROM YELLOW MUSTARD SEEDS

2.1.1 Extraction

Weber and co-workers (1974) and Vose (1974) used similar methods to extract polysaccharides from the hulls of yellow mustard seed. In Weber's method, the hulls were defatted with a mixture of hexane, ethanol and water. The defatted and dried bran was then water extracted (1:20 seed:water ratio) and separated using a basket centrifuge. The mucilage was precipitated from water by adding 2 volumes of 95% ethanol. In Vose's method, hull samples (100g) were extracted sequentially in a Soxhlet apparatus with the following solvents: acetone (8 h), hexane (24h), and ethanol : water, 4 : 1 v/v (48 h). The defatted hulls were then milled and extracted with water and ammonium oxalate at different temperatures. The remaining hulls were subjected to four additional stepwise treatments to produce seven major fractions.

Theander and co-workers (1977) extracted hulls of rape and mustard seeds with 80% ethanol to remove low molecular weight substances. The extracts were divided into a chloroform soluble fraction (A) and water-soluble extracts which were further fractionated into neutral (B), basic (C) and acidic (D) fractions. In a study conducted by Woods and Downey (1980), whole mustard seeds (5 g) were extracted overnight (16 h) with 90 ml of chloroform water (2.5 ml chloroform/L water) using a slow reciprocating shaker. Chloroform

was used to prevent undesirable fermentation. The whole seeds were removed by filtering through cheese cloth and the mucilage precipitated with acidified acetone (2.5 ml conc. HCl/L). Siddiqui and co-workers (1986) extracted mucilage from yellow mustard seed hulls with boiling water (1 : 16 w/v) for 35 min. and then precipitated the mucilage with isopropanol.

Different methods have also been reported to extract mucilages from other plant seeds. El-Mahdy and El-Sebaiy (1984) heated ground Fenugreek seeds at 70°C for 15 min. to inactivate enzymes prior to soaking in water (1 : 6 seed : water ratio). Trichloroacetic acid (TCA, 10%, 2L) extraction at 4°C for 6 h was used to extract polysaccharides from black gram and cowpea by Muralikrishna *et al.* (1987) and Susheelamma and Rao (1978) respectively. The extracts were pooled and added to 3 volumes of acetone to precipitate the mucilage. In a more recent study, Mazza and Biliaderis (1989) extracted flax seed mucilage by mixing the seed with water (1 : 20 w/v) and shaking the seed-water mixture for 0.5 to 8 h at 25 - 100°C. The mucilage extract was then separated by filtration through a 40 mesh screen and concentrated on a rotary vacuum evaporator at 40°C. The concentrated mucilage was precipitated with 80% ethanol in water, freeze-dried and milled to a powder. In studies conducted in our laboratory mucilage was extracted from Canola and mustard seeds using the procedure decribed by Sharafabadi (1987): 100 g of seeds were placed in 1 litre flask with 250 ml of boiling water and the mixture kept in a water bath at 75°C for 25 min. Cold water was then added to the flask to adjust the seed: water ratio to 1:7. The mixture was stirred with a magnetic stirrer for 16 hr at room temperature and 2.5 ml chloroform/L water was added during the extraction to prevent microbial growth. The seeds were removed by passing the mixture through three-layers of thick cheese cloth. The mucilage was precipitated by adding 4 volumes of 95% ethanol to the mixture and then freeze-dried.

2.1.2 Yield and Monosaccharide Composition

The levels of mucilage in four cultivars of yellow mustard seeds grown at four different locations in Western Canada were found to range from 0.34-2.05% with an overall average of 1.28% (Woods and Downey, 1980). This compared to 2% mucilage reported in white mustard seeds by Bailey and Norris (1932). Vose (1974) extracted polysaccharides from defatted hulls of several rapeseed and mustard seed varieties. Of these, only yellow mustard was found to yield mainly glucose on hydrolysis. The remaining monosaccharides were galacturonic acid (30%), arabinose (30%), glucose (20%), xylose (6%), fructose (6%), rhamnose (6%) with traces of galactose and mannose. Theander and co-workers (1977) examined the neutral water- soluble polysaccharides from white mustard (*S. alba*) and reported the presence of glucose (39.3%), arabinose (25.4%), galactose (17.9%), xylose (7.5%), mannose (5.4%), rhamnose (4%) and fructose (1%). Siddiqui and co-workers (1986) extracted mucilage from the seed coats (hulls) of yellow mustard seeds with boiling water for 35 min. and monitored its release using optical and scanning electron microscopy. The isolated mucilage contained galactose, glucose and galacturonic acid and smaller amounts of mannose, arabinose, xylose, and rhamnose. In studies conducted in our laboratory mucilage was isolated from yellow mustard seeds (*S. alba* L.) using a modified method in yields of approximately 5% mucilagenous material (unpublished data). This material was cotton-like in appearance and its chemical composition was significantly different from the material extracted by Theander *et al.* (1977).

2.2 FUNCTIONAL PROPERTIES OF POLYSACCHARIDE GUMS

2.2.1 Rheological Properties

Polysaccharide gums are long-chain polymers which on dissolving or dispersing in water thicken or increase the viscosity of the system because of their large hydrodynamic volume or the ability to form gel network by physical cross-linking (Glicksman, 1982b). The increase in viscosity of an aqueous solution or dispersion is one of the most common and important properties of polysaccharide gums from which all other functional properties are derived. Knowledge of the flow behaviour or rheological properties of polysaccharide solutions or dispersions is of practical significance to processing (pumping, extrusion and compression molding), product development and perceived organoleptic properties (appearance, texture and mouthfeel) of these materials (Glicksman, 1982b; Whitcomb *et al.*, 1980). The flow behaviour or the rheological properties of polysaccharide solutions or dispersions can be studied by either steady shear tests or oscillatory small strain deformation testing. Steady shear tests performed at different shear rate ranges have been useful in relating the performance of a polysaccharide under different processing conditions (Whitcomb *et al.*, 1980) while the dynamic rheological measurements (oscillatory tests) frequently reveal the molecular basis of the thickening process .

2.2.1.1 Steady Shear Rheological Tests

In a steady shear rheological test, polymer solutions or dispersions are usually subjected to a shear sweep from shear rate 10^{-3} s^{-1} to 10^{3} s^{-1} which covers most of the shear rate range encounntered in food processing operations (Table 2.1) (Whitcomb *et al.*, 1980). The response of the apparent viscosity to the shear rate sweep can be used to evaluate

polysaccharide solutions or dispersions. Above quite low concentrations most food gum solutions are non-Newtonian in flow behaviour with the apparent viscosity decreasing with increase in shear rate; this is referred to as shear thinning or viscoelastic behaviour. In contrast, some solutions or dispersions of polysaccharide gums exhibit Newtonian flow behaviour in which the apparent viscosity remains constant as the shear rate increases, thereby, making it independent of shear rate (Glicksman, 1982b). The flow behaviour of polysaccharide solutions or dispersions is affected by such factors as, shear rate range, polymer concentration, chemical structure, molecular size, as well as interactions between polymer-solvent and polymer-polymer (Morris *et al.*, 1981;Whitcomb *et al.*, 1980; Hales *et al.*, 1982).

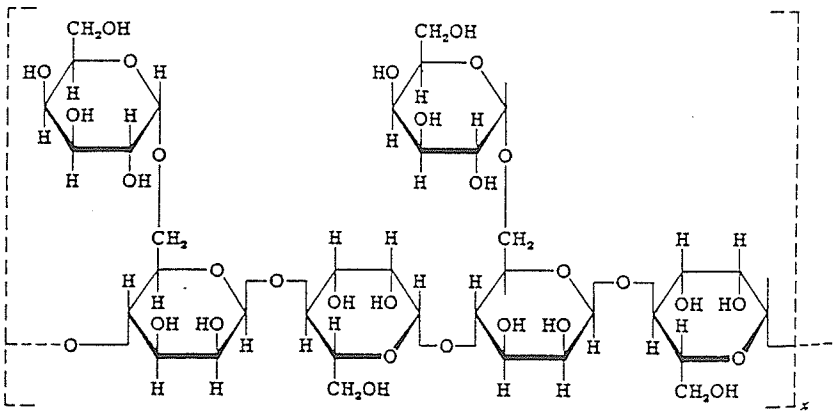
Table 2.1. Typical shear rates for processings

$\dot{\gamma}$ s^{-1}	Phenomina
< 0.1	Film sag or film flow on a vertical plate
0.1-10	Compression molding Normal range for Brookfield readings
10-100	Tumbling or pouring Calendering Shear rate encountered in the mouth during
100-1000	Home mixers Extrusion
1000-10,000	Waring Blendor Injection molding
>10,000	Colloid mill Drilling

Adapted from Whitcomb *et al..*, 1980.

2.2.1.2 Shear Rate Dependence of Viscosity

Whitcomb and co-workers showed that guar gum solutions exhibited Newtonian behaviour at sufficiently low and high shear rates, but exhibited non-Newtonian behaviour (shear thinning or viscoelastic) over an intermediate shear rate range (Whitcomb *et al.*, 1980). This can be explained by the guar gum solutions giving an entangled "random-coil" polysaccharide solution, (i.e. at concentration above the critical concentration C*) (Morris *et al.*, 1981). At low shear rates, viscosity remained constant at the maximum "zero-shear" value. There was no net change in the "cross-link density" of the entangled network because of sufficient time for the entanglements pulled apart by the flow of the solution to be replaced by new entanglements formed between different chain partners . With increasing shear rate, however, a point is eventually reached beyond which re-entanglement can no longer keep up with the rate of destruction of the existing entanglements, so that the overall entanglement density is reduced, and viscosity falls. Morris *et al.* (1981) concluded that concentrated polysaccharide solutions above C* showed similar shear thinning profiles irrespective of chemical structure, molecular weight, solvent environment or concentration .



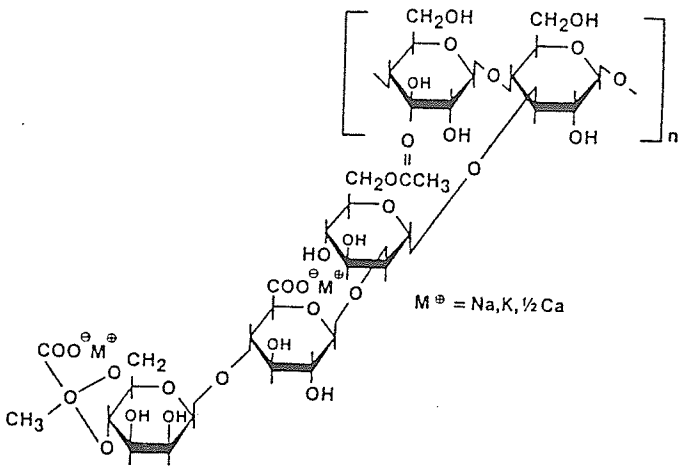
Primary structure of guar gum

2.2.1.3 Effect of Polymer Concentration on Viscosity

Mazza and Biliaderis (1989) showed that flaxseed mucilage exhibited Newtonian-like-flow behaviour at lower concentrations and shear thinning behaviour at higher concentrations over a wide range of shear rates. Similar observations were also reported for guar gum solutions (Whitcomb *et al.*, 1980) which suggested that the concentration of gum solutions not only affect the apparent viscosity but also the flow behaviour of the system. For most polysaccharide solutions or dispersions, there is a specific concentration (C*) which marks the onset of significant coil overlap and interpenetration (Morris *et al.*, 1981). A solution at a concentration below C* is considered as a dilute solution in which further increase in concentration results in only a slight increase in viscosity since no effective molecular overlap occurs. A dilute gum solution usually exhibits Newtonian flow behaviour due to the absence of formation and destruction of chain entanglements when the shear rate is increased. At concentrations above C*, viscosity increases substantially due to the occurrence of effective molecular overlapping (Morris *et al.*, 1981).

2.2.1.4 Effect of Chemical Structure and Molecular Weight on Viscosity

The chemical structure of a polysaccharide determines its flow behaviour and viscosity. For example, xanthan gum has a 1,4-linked β-D glucose backbone with a trisaccharide side chain on the alternate glucose. The stiff 1,4-linked β-D glucopyranose backbone chain makes xanthan gum unique among commercial gums exhibiting typical shear thinning behaviour even at low concentrations (0.05%) (Pettitt, 1982). Another example is gum arabic which has a 1,3-linked β-galactopyranose backbone chain with numerous branches containing 1,6-linked β-galactose, arabinose, rhamnose and glucuronic acid (Williams *et al.*, 1990). At relatively low concentrations, gum arabic yields solutions



Primary structure of xanthan gum

which are Newtonian-like in flow behaviour and exhibit very low viscosity compared to other polysaccharides of similar molecular size. Non-Newtonian solutions are only obtained at very high concentrations (>30%), above the so-called "interactive volume" , where effective molecular overlap occurs. The highly branched compact structure of gum arabic was involved to explain the Newtonian flow behaviour and low viscosity of this hydrocolloid (Anderson and Andon, 1988).

In general, the higher the molecular weight of the polysaccharide, the more viscous is its solution. Izydorczyk and Biliaderis (1992) examined the rheological properties of wheat arabinoxylans differing in molecular size and found that the higher molecular size fractions exhibited more pronounced shear thinning behaviour than low molecular size fractions which were having an almost Newtonian flow behaviour. A quantitative description of the relationship between viscosity and molecular weight is based on the Mark-Houwink

relationship (Eq. 2.1).

$$[\eta]=K'Mr^{\alpha} \qquad (2.1)$$

where $[\eta]$ represents the intrinsic viscosity; Mr is the average molecular weight. K' and α are two parameters related to the "stiffness" of the polymer. The α value for guar galactomannan in dilute solutions was 0.723 which is within the typical range of 0.5-0.8 for flexible coils whereas, α is ~1.8 for a rigid rod-like structure. A structure leading to a very compact conformation usually has a small α of 0.1 to 0.3 (Robinson *et al.*, 1982).

The solvent enviroments, including pH and the presence of salts and sugars, also affect the rheological properties of polysaccharides (Mazza and Biliaderis, 1989; Bhat, 1987). Salts influence the rheological properties of polysaccharide solutions or dispersions either through specific interaction or cooperative binding, or simply by altering the solvent quality of the water via a lyotropic effect (Norton *et al.*, 1984; Morris *et al.*, 1978 and Searle *et al.*, 1982). Yalpani *et al.*(1983) showed the apparent viscosity of aqueous solutions of a branched chitosan derivative increased two-fold in the presence of sodium chloride where as the viscosity of xanthan gum at low concentrations (<0.1%) was decreased by the addition of salt (0.1 M KCl) (Dentini *et al.*, 1984). Anguilar and Ziegler (1990) found that the addition of salts to yellow mustard mucilage solutions or dispersions to have a negative effect on viscosity. The effect of pH on the rheological properties depends on the structure of the polysaccharides. The apparent viscosity of flaxseed mucilage was reduced with decreasing pH (Mazza and Biliaderis, 1989). In the case of yellow mustard mucilage solutions, higher apparent viscosities were found in both the low and the high pH regions with the lowest viscosity occurring between pH 4 and 7 (Weber *et al.*, 1974).

2.2.1.5 Dynamic Rheological Properties of Polysaccharide Gums

Knowledge of dynamic rheological properties of polysaccharide gums in solutions or dispersions is essential for understanding the molecular origin of the thickening phenomena. The dynamic rheological properties of gums can be assessed by small deformation oscillatory tests which provide estimates of two important parameters: G' and G''. G', the storage modulus, represents the elastic component of a system, while G'', the loss modulus, characterizes the viscous properties of the system (Bohlin *et al.*, 1984). The spectrum of G' and G'' vs oscillatory frequency is used to evaluate the viscoelastic properties of a polysaccharide aqueous solution or dispersion. For example, a guar gum solution (1 to 2%) is a typical viscoelastic system, in which, the loss modulus G'' is greater than the storage modulus G' at lower frequencies indicating a more prominent viscous character. However, at higher frequencies the reverse is observed indicating a more elastic response (Robinson *et al.* 1982). The dynamic rheological moduli were also used by Tako and Nakamura (1984 and 1985) to monitor the synergistic interactions between different polysaccharide gums, as well as to evaluate the viscoelastic properties of linseed mucilages extracted from different varieties (Wannerberger *et al.*, 1991).

2.2.2 Interfacial Properties of Gums and Mucilages

Interfacial properties of a hydrocolloid include the ability to reduce water-air surface tension and emulsifying and foaming capacity and stability. Weber and co-workers (1974) reported that mustard mucilage reduced the surface tension of water from 69.2 dynes/cm to 57.1 and 55.2 dynes/cm (0.25% and 0.5% polymer concentrations).The reduction of water surface tension was also occurred with wheat arabinoxylan and arabinogalactan preparations (Izydorczyk *et al.*, 1991). Gaonkar (1991) showed that different cellulose derivatives

exhibited different surface and interfacial properties at similar molecular weights.

Polysaccharides are usually not true emulsifiers, but they can be used as emulsion stabilizers by thickening or increasing the viscosity of the aqueous phase. However, Weber and co-workers (1974) attributed the emulsification capacity of mustard mucilage to its ability to lower the interfacial tension rather than through its contribution to viscosity. A study by Gaonkar (1991) confirmed that some gums adsorb at the oil/water interface to form an interfacial film which functions as an emulsifier in addition to acting as thickening and/or gelling agents in the continuous phase of a dispersed syatem. Many methods can be used to determine the emulsion capacity and stability of gums, including a light scattering technique which measures droplet size (Gaonkar, 1991), a mixing and centrifugation method to measure the percentage of the emulsion layer (Yasumatsu *et al.*, 1972) and a spectrophotometric method to determine the droplet size after emulsification (Gaonker, 1991). Perhaps the simplest method is that described by Yasumatsu and co-workers which is a mixing and centrifugation method. In this procedure gum samples are suspended in 40 ml of distilled water and 40 ml corn oil, emulsified using a polytron homogenizer at 10,000 rpm for 1 min. and centrifuged at 1,300 g for 5 min. The emulsion capacity is calculated as:

$$\frac{\text{Height of emulsion layer}}{\text{Height of whole liquid}} \times 100\% \qquad (2.2)$$

Emulsion stability is determined by heating the emulsion at 80°C for 30 min., cooling with tap water for 15 min, and then centrifuging at 1,300 g for 5 min. Emulsion stability is calculated as:

$$\frac{\text{Height of remained emulsion layer}}{\text{Height of whole liquid}} \times 100\% \qquad (2.3)$$

Foams are also two-phase systems, consisting of a mass of gas bubbles dispersed in a liquid or solid continuous phase. Foam stability is another important interfacial property of polysaccharide gums which has important implications in foods. A number of methods have been described to determine foaming stability. Bhat and co-workers (1987) used a Steipel-Type foam meter to study the foam capacity and stability of okra mucilage. A shaking method was employed by Yasumatsu and co-workers (1972) to evaluate foam stability of soya proteins and by Muralikrishna and co-workers (1987) to determine the foam stability of okra mucilage. In the shaking method (Yasumatsu *et al.*, 1972), foam capacity is expressed as the initial foam volume after shaking while foam stability is evaluated by the volume remaining as a function of time. A study conducted by Muralikrishna et al. (1987) observed that black gram polysaccharides exhibited higher foam stability compared to the corresponding cowpea polysaccharides. Mazza and Biliaderis (1989) demonstrated that the foam capacity and stability of flax seed mucilage in ovalbumin solutions decreased with decreasing concentration.

2.3 ISOLATION AND PURIFICATION OF POLYSACCHARIDES

2.3.1 Separation with Chemical Treatment

Classical carbohydrate chemists generally used chemical methods to separate and purify plant polysaccharides by partial precipitation with ethanol, copper salts, quaternary ammonium salts, and barium hydroxide (Whistler and Sannela, 1965; Adams, 1965; Jones and Stoodley, 1965; Scott, 1965 and Meier, 1965). Most of these methods are still used to separate and purify plant polysaccharides in combination with chromatographic methods. For example, in a recent study by Rodriguez and co-workers (1990) a polysaccharide

preparation was obtained using three cycles of precipitation of crude extracts with an aqueous solution of ethanol (to 80% final concentration), and this followed by repeated extractions with cold aqueous 80% phenol (buffered to pH 6.5 with sodium acetate) to remove contaminating proteins. The residue was further purified by elution through Sephadex G-50 column. An earlier study by Rao et al. (1986) described the isolation of an acid-extractable polysaccharide (5 g) from a green seaweed by dissolving in H_2O (200 ml) and then precipitation upon dropwise addition of 20 ml of 10% $Cu(OAc)_2$ solution. EtOH (100 ml) was finally added until precipitation was completed. The precipitate (A, 4.1g) formed was collected by centrifugation and a second volume of the reagent solution was added to the supernatant and this was followed by addition of sufficient EtOH (200 ml) to obtain a precipitate (B, 10 mg). Fractions A and B were washed separately with cold EtOH containing 5% (v/v) concentrated HCl, dialysed after redissolving in minimal amount of H_2O and then freeze-dried. Gupta and co-workers (1990) using a similar copper complex combined with ion-exchange resins isolated and purified a polysaccharide from the seeds of *Nekukitus ubduxa ALL.*

Acidic polysaccharides can be separated readly from neutral polysaccharides by precipitation with detergent cations which form insoluble salts with polyanions (Scott, 1965). Using this method Adams (1965) successfully purified an arabinogalactan from Western Larch (*Larir occidentalis*) Heartwood. This method was also recently employed by Rodriquez et al.(1990) where the polysaccharide extract was treated with 1 volume of aqueous 2% cetyltrimethylammonium bromide (CTAB) prior to the ethanol treatment. Chemical fractionations however are generally incomplete; ion exchange and gel filtration chromatography may improve separations.

2.3.2 Chromatographic Methods

Ion-exchange and gel filtration chromatography are widely used powerful methods for separating polysaccharides. Gel chromatography (gel filtration or gel permeation) is a liquid chromatographic method which separates molecules according to differences in molecular sizes and shapes. Since polysaccharides differ considerably in size, gel chromatography is a particularly effective tool for separating complex mixtures of these substances. The principle of ion exchange chromatography is based on the ionic interaction of charged polysaccharides with the opposite charge on the resin. Under appropriate buffer conditions (pH and ionic strength), neutral polysaccharides pass through the column while acidic polysaccharides interact and bind to the resin. Subsequently, the acidic polysaccharides can be separated into several fractions by elution with an ionic strength gradient, or by stepwise increase of the ionic strength (Kato and Vennis, 1991).

Considerable information is available on the application of gel chromatography for separation of polysaccharides. Sequential ion-exchange chromatography on DEAE-Sephadex G-25 and gel filtration chromatography enabled Gonda and co-workers (1990) to fractionate an acidic polysaccharide from the leaves of *Malva sylvestris* var mauritiana.They also purified crude mucilage extracts from *Hibiscus syriacus* leaves using DEAE-Sephadex A-25 (Pharmacia Co), Sephadex G-15 and Sephacryl S-300 columns. York and co-workers (1990) isolated a xyloglucan (XG) from rapeseed hulls by Sephadex column chromatography while Doco and co-workers (1990) used ion-exchange chromatography (Dowex 50-X8, 20-50 mesh, H^+; Dowex 1-X8, 20-50 mesh) and gel filtration (Sephacryl S-1000, Sepharose 4B) to successfully isolate an exocellular polysaccharide from S. *thermophilus* and established its molecular weight by calibrating against several dextran standards. Gupta and BeMiller (1990) separated a neutral galactomannan from *Crotalaria medic* aginea seeds by successive

elution on columns of Dowex 50W-X8(H^+) and Dowex 3(OH^-) ion-exchange resins combined with chemical treatment.

2.4 STRUCTURAL ANALYSIS OF POLYSACCHARIDES

2.4.1 Complete Acid Hydrolysis

Complete acid hydrolysis reveals the monosaccharide composition of polysaccharides which are composed of different monosaccharides linked by glycosidic bonds, each differing in acid lability. For example, furanoside linkages are much more labile than pyranoside linkages, α-glycosidic bonds are usually more labile than ß-form while pentoglycans in the pyranoside form are more readily hydrolysed than pyranoside hexoglycans. The presence of uronic acid groups or amino sugars increase the resistance of adjacent glycosidic bonds to acid hydrolysis (Adams, 1965). In a study conducted by Adams (1965), α-linked D-hexoglycans were refluxed with hydrochloric acid and ß-linked-D-hexoglycans with sulfuric acid. For pyranoside pentoglycans (such as xylans) and furanoside pentoglycans (arabans), mild acid conditions (e.g. 0.01N sulfuric acid at 90°C) were found to be adequate (Adams, 1965). Polysaccharides containing uronic acids only hydrolyse to aldobiouronic stage (Adams, 1965). Gupta and Bose (1986) hydrolysed a purified polysaccharide from the seeds of *Melilotus india* with sulfuric acid and trifluoroacetic acid. Similar hydrolysis procedures were carried out by other researchers in which monosaccharides were confirmed by PC (paper chromatography), GLC or GC-MS (Bhattacharyya *et al.*, 1983; Englyst *et al.*, 1984; Matruura and Hatanaka, 1988; Gupta and BeMiller, 1990; Oxley and Wilkinson, 1990; Kiefer *et al.*, 1990; Dutton *et al.*, 1990; Gonda *et al.*, 1990 and Jackson *et al.*, 1990).

2.4.2 Partial Acid Hydrolysis

Partial acid hydrolysis of polysaccharides releases oligosaccharides together with monosaccharides and makes it possible to determine linkages and or sequences within the polysaccharides. Dilute sulfuric acid is generally employed in which polysaccharides are partially hydrolysed in 0.025 M sulfuric acid at 85°C for 13 hr (Gupta and Bose, 1986; Bhatacharyya *et al*, 1983). Dilute HCl was used by Susheelamma and Rao (1978) in studying the structure of arabinogalactan, while oxalic acid was used by Bhattacharyya *et al*. (1983) in the examination of a galactomannan from *Seebania aegyptica*. In a recent study conducted by O'Neil *et al* (1990) an acidic polysaccharide was partially hydrolysed using 0.4 M trifluoroacetic acid (TFA) at 100°C for 2.5 h. The advantage of using TFA is that it is easy to remove by evaporation after hydrolysis. The partially hydrolysed polysaccharide yielded oligosaccharides and monosaccharides which were then isolated by ion exchange and gel filtration chromatography on Bio-Gel P-2 (O'Neil *et al.*, 1990).

2.4.3 Enzymatic Hydrolysis

Enzymes have been used to identify the major linkage-types in polysaccharides. Enzymes can release oligosaccharides in high yield and cleave acid resistant linkages. Characterization of resultant fragments provides details of the glycan structure. Polysaccharides hydrolyzates may have an endo or exo action pattern. Endo-enzymes normslly act by random cleavage of susceptible bonds in the glycan chain yielding a series of oligosaccharide fragments, which may undergo further cleavage. In contrast, exo-enzymes sequentially release monosaccharides or oligosaccharide repeating-units from one end of the glycan chain, most commonly from the non-reducing end (McCleary, 1986). York et al. (1990) and Kiefer et al. (1990), used an endo-(1->4)-ß-D-glucanase (EC 3.2.1.4) to

digest purified xyloglucans (XGs) from different sources. The endo-(1->4)-ß-D-glucanase appeared to specifically hydrolyze unbranched (1->4)-ß-D glucose residues in the XGs (York et al., 1990). Gao and co-workers (1990) reported an endo-α-(1->4)-polygalacturonase which digested a heteroglycan by base-catalysed ß-elimination. Another application of polygalacturonase was reported by Matsuura and Hatanaka for the degradation of pectic substances (Matsuura and Hatanaka, 1988). Her *et al.*(1990) prepared oligosaccharide fragments from a novel exopolysaccharide using a crude enzyme preparation obtained from lysates of *R. meliloti* during transducing phase. Isolation of the oligosaccharide products of enzymatic hydrolysis were achieved using gel chromatography on Sephadex or Bio-Gel columns (Matsuura and Hatanaka, 1988; York *et al.*, 1990; Gao etr al., 1990; Her *et al.*, 1990 and Kiefer *et al.*, 1990). The availabability of size-exclusion gels allowed the isolation of reasonable quantities of oligosaccharide fractions. The cleavage products can then be examined by further enzymic degradation, methylation analysis, NMR spectroscopy and mass spectrometry to reveal the detail structure of the polymer.

2.4.4 Methylation Analysis of Oligosaccharides and Polysaccharides

Characterization of the individual methylated sugars in a hydrolyzate of methylated polysaccharides or oligosaccharides reveals the presence of any unmethylated hydroxyl groups. These free hydroxyl groups are those involved in linking that particular unit in the polysaccharides or oligosaccharides. Carbohydrates are usually methylated in methyl sulphoxide with methyl iodide and a strong base such as sodium methanesulphinylmethanide (Hakomori, 1964), sodium hydride, potassium tert-butoxide or alkali-metal hydroxide (Ciucanu and Kerek, 1984). Gonda et al. (1990) performed methylation on an acidic polysaccharide from *M. sylvestn's* var *mauritiana* with methylsulfinyl carbanion and methyl

iodide in dimethyl sulfoxide. The final product was checked for the existence of unmethylated hydroxyl groups using IR (infrared) spectroscopy and was then hydrolysed with dilute H_2SO_4 in CH_3COOH, reduced and acetylated. The partially methylated alditol acetates were analyzed by GC-MS to reveal individual sugars and their connecting carbons (Gonda *et al.*, 1990). Using the so-called Hakomori method, Gao *et al.* (1988) and Moreau *et al.* (1990) successfully methylated polysaccharides or oligosaccharides in dimethyl sulfoxide solution. In a study conducted by Gupta and Bemiller (1990), a galactomannan (100 mg) was methylated successively with dimethyl sulfate and alkali followed by treatments with methyl iodide and Ag_2O to yield a syrup which showed no hydroxyl absorption in the IR spectrum (Gupta and BeMiller, 1990). A simple methylation procedure was reported by Ciucanu and Kerek (1984) in which the sample was dissolved in methyl sulphoxide, powder sodium hydroxide was added followed by methyl iodide. By reversing the order of addition of sodium hydroxide and methyl iodine the degradation of uronic acid, due to β-elimination during methylation could be avoided (Ciucanu and Luca, 1990). The solubilization of the polysaccharide in the solvent is very critical to obtain a complete methylation (Aspinall, 1991, personal communication).

2.4.5 Periodate Oxidation and Smith Degradation of Polysaccharides

When vicinal-glycols are treated with periodic acid or salts, the carbon chain will cleave and form two aldehydic groups. In the case of α, ß, γ triols, two aldehyde groups and one mole of formic acid are produced with a double cleavage of the carbon chain. Oxidation of a polysaccharide as measured by the periodate consumption, formic acid production, and the determination of the proportion of surviving sugar units provide important information concerning the nature and proportion of the glycosidic linkages present in the

polysaccharide. Periodate oxidation can also be used to calculate the degree of polymerization (DP) of a linear (1->4) linked polymer since three molecular proportions of formic acid are liberated per linear chain, one from the non-reducing end and two from the reducing end. In the case of branched polysaccharides, formic acid may provide a measure of the ratio of terminal to non-terminal sugar residues (Goldstein, 1965).

When a sugar residue of a polysaccharide is cleaved by periodate and then reduced, the resulting alcoholic derivative, being a true acetal, is sensitive to acid. When a sugar unit which survives cleavage because of its substitution pattern is joined to a unit which is cleaved, the unoxidised units appear as glycosides which are relatively stable to acid. Because of the marked difference in stability between true acetals and glycosides, it is possible to obtain, from a wide variety of polysaccharides, glycosides of mono, di, and oligosaccharides, which may reflect the relative fine structure of the parent polysaccharide. This controlled periodate degradation is well known as Smith degradation (Goldstein, 1965). In the study conducted by Rao and Rao (1986), periodate oxidation of a sulphated polysaccharide obtained from a green seaweed resulted in the reduction of 0.4 mol of the oxidant, producing 0.54 mol of formic acid per anhydrosugar unit in 72 hr. The oxo-polysaccharide, isolated in 60% yield, on Smith degradation yielded glycerol, erythritol and unoxidized galactose in the mole ratio 11.6 : 5.1 : 4.9. The glycerol was derived from hexose units which were 1,6-linked and /or non-reducing end units and from 1,4-linked pentopyranose units. The formation of erythritol and its mole yield indicated the presence of 1,4-linked mannopyranose units and the completion of periodate oxidation (Rao and Rao, 1986). Bhattacharyya and co-workers (1986) studied a water-soluble galactomannan using periodate oxidation and found the glycerol- erythritol ratio was 3 : 5. Similar results were also reported by Gupta and Bose (1986) on a purified green seaweed polysaccharide.

2.4.6 FAB MS of Oligosaccharides

FAB MS (Fast Atom Bombardment Mass Spectrometry) was introduced in 1981 and it has since become very importmant to carbohydrates analysis. Egge and co-workers (1983) used FAB MS to characterize neutral oligosaccharides from human milk. Dell *et al.* (1983, 1987) found the FAB technique could be used to study unmodified oligosaccharides and their derivatives. The current workable mass range of carbohydrates in FAB MS is about 4000 daltons for underivatized samples. Reduction in hydrogen bonding capability, e.g., by derivatization, extended the mass range to about 6000 daltons, and by using special "mapping" technique, this mass range can be extended to 20,000 daltons (Dell, 1987). The sequence of monosaccharides linkages as well as the cleavage mechanism of oligosaccharides could also be determined (Dell, 1987).

FAB MS of native samples, if successful, defines the molecular weight and provides valuable information on the sugar composition. Native oligosaccharides can also be analyzed by FAB MS, preferably in the negative ion mode which provides more information regarding the sugar sequence (Dell, 1987). FAB MS spectra of unmodified carbohydrates are limited because of their poor signal-to-noise ratio; this drawback can be overcome by derivatization including permethylation and peracetylation. The formation of derivatives is essential in almost all FAB MS studies of carbohydrates since they facilitate interpretation of spectra, improve sensitivity, permit analysis of salty samples, allow unambiguous sequencing, confirm the presence of cyclic structures, enable spectra to be obtained from very large molecules and help in the location of O-acylated residues in oligosaccharides (Dell, 1987).

Permethylation and peracetylation of carbohydrates have been used extensively in carbohydrate research. Permethylation gives the smallest increase in the molecular weight of the sample while most permethylated glycoconjugates fragment very selectively, resulting

in a limited number of sequence ions that are easy to assign. Peracetylation gives a large increase in molecular weight; fragmentation pathways are less specific than permethylated samples but spectra, although more difficult to interpret, may provide more structural information.

The pseudomolecular ion region may be complex if several types of ions are present. In the positive-ion mode, four species are commonly present, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$. Fig. 2.1 indicates the relationship between these ions. For example, two pseudomolecular-ion signals separated by five mass units must be $[M+NH_4]^+$ and $[M+Na]^+$ (Dell, 1987). In the negative-ion mode, $[M-H]^-$ is usually observed. Molecules that cannot lose a proton, such as permethylated saccharides, give negative spectra if anions are present in the matrix, for example, $[M+Cl]^-$ or $[M+SCN]^-$ (Dell, 1987).

Molecular weights are generally defined by analyzing underivatized samples while per-O-acetyl and per-O-methyl derivatives are used extensively for sequence analysis and for providing molecular weight information at very high sensitivity. The major ions formed from both the peracetyl and the permethyl derivatives are derived from a single, pathway A cleavage; less abundant ions arise from beta-cleavage (Pathway B) together with a Pathway A cleavage. The latter can be readily identified, because they lack an acetyl or methyl group on the non-reducing residue. The hypothetical sequence M-N-P-Q-R (where the letters refer to unspecified sugar residues that are fully methylated or acetylated,) is expected to fragment to give sequence ions of composition M^+, MN^+, MNP^+ and $MNPQ^+$, but may also give such minor ions as NP^+ and NPQ^+, in which N bears a free hydroxyl group resulting from a "double cleavage".

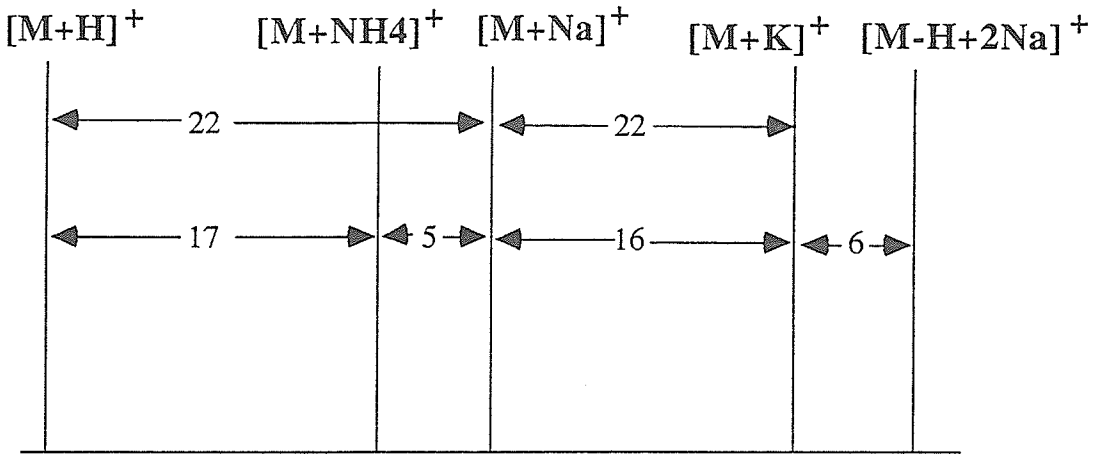


Fig. 2.1. Schematic representation of molecular-ion signals that may be formed in the positive mode, showing commonly observed mass differences (Adapted from Dell, 1987)

There are many successful applications of FAB-MS in recent carbohydrate related research. For example, in a study conducted by York and co-workers (1990) the glycosyl sequence of oligoglycosyl-alditols was determined by mainly relying on FAB MS. Kiefer and co-workers (1990) also used FAB-MS to examine an arabinose-containing heptadecasaccharide in both the positive and negative ion modes.

2.4.7 NMR Spectroscopy of Polysaccharides and Oligosaccharides

Of all the modern methods for complex carbohydrates, high field proton NMR yields

the most complete and detailed structural information. This method is non-destructive, and is recommended as the one to be carried out first on an isolated sample (Bush, 1988). A simple proton NMR spectrum can provide immediate information on the purity and some general information on the structure. The proton NMR spectrum of a typical complex oligosaccharide in D_2O solution shows some isolated resonances which are called "structural reporter resonances" (Bush, 1988). In a pyranoside, the chair conformation is fixed in which protons are oriented axially or equatorially. Since the proton-proton coupling is angular determent the coupling patterns are characteristic of the stereochemistry of the pyranose ring. The resonances of the anomeric protons, and those of various protons with distinctive chemical shifts correlated with known structures provide a powerful tool for use in conjunction with other methods of structural analysis (Bush, 1988). It is often possible to assign the structure of an oligosaccharide directly from the spectrum simply by comparison with published data on similar or identical oligosaccharides.

2.4.7.1 ^{1}H NMR Spectra of Polysaccharides and Oligosaccharides

Proton NMR is desirable for the structural analysis of oligosaccharides since protons are spaced linearly around each oligosaccharide residue. There is usually one proton only at each optically active ring carbon which serves as a structural "reporter group" at that carbon. A complete assignment of the proton NMR spectrum of an oligosaccharide would provide information regarding its sugar type, anomeric configuration, ring conformation, linkage position and sequence (Dabroski, 1987). The H-1 or anomeric proton of each residue resonates in a characteristic region (4.0-5.0ppm) that contains few other signals. The structural "reporter resonances", however, occur at a rather crowdy region (3.5-4.0ppm). It has been shown that complete proton assignments in oligosaccharides in a size range up

to 10 to 15 residues is generally practical with sufficient observation time on 300-500 MHz NMR instruments (Dua *et al.*, 1986; Homans *et al.*, 1987). Unfortunately, signal overlap in oligosaccharides spectra often provents the complete assignment of their spectra.

2.4.7.2 ^{13}C NMR Spectra of Polysaccharides and Oligosaccharides

^{13}C NMR spectroscopy has proven to be the most efficient spectroscopic method for configurational and conformational investigations in carbohydrate research (Breitmaier and Voelter, 1987). In contrast to the rather crowded proton spectrum, the ^{13}C NMR spectrum of a pentasaccharide had fewer overlapping lines even when recorded at low field at 25 MHz because its chemical shift dispersion is much greater than that of proton NMR. In higher field, superconducting spectrometers allow individual lines to be observed for essentially all the carbons of an oligosaccharide having modest size. In the proton-decoupled carbon spectrum of a milk pentasaccharide at 75 MHz, the anomeric region between 95 and 105 ppm contained the signals from reducing terminal glucose and one line for each of the other four residues (Bush, 1988). The downfield shifts were characteristic of the glycosidic substitution. The methylene carbons of the C6 were all resolved and located between 61 and 63 ppm (Bush, 1988).

Gorin (1981) reviewed the extensive application of ^{13}C-NMR in the field of polysaccharides while ^{13}C-NMR spectra data for oligosaccharides and monosaccharides were compiled by Bock and co-workers (1983). This available data simplifies the assignments of ^{13}C NMR spectra by allowing comparison with data obtained from similar or identical residues. The C-1 resonances of the pyranoid forms of glucose, xylose, galactose, arabinose, methyl glucoside, and methyl xyloside were shown to be sensitive to the anomeric configuration. This also is true for the aldofuranoid series. The C-1 signals of oligosaccharides or

polysaccharides fall into two different ranges of shifts, depending on whether they contain α or ß-linkages. Small, internal variations may occur, due to different O-substitution α-shifts, which can sometimes be diagnostically useful. The C-1 chemical shifts of furanosides are generally at lower field than those of their anomeric counterparts in the pyranose series. Sometimes, an immediate identification may be made when very low field signals of 107 ppm or more are present, for example, for ß-galactofuranoside and arabinofuranoside. Generally, the characteristic signals of furanoside-ring carbon atoms are present at low field (80-85 ppm) (Gorin, 1981). The ^{13}C-NMR spectra of polysaccharides having mixed linkages can sometimes be interpreted by reference to the spectra of homopolymers representing each type of linkage. For example, the spectrum of lichenan, which contains one ß-D-(1->3) every two ß-D-(1->4) units, may be interpreted as a composite of those of laminaran and cellulose in a 1:2 ratio. More poorly resolved spectra of glucans containing α-(1->4) unit and α-(1->6) units, α-(1->3), α-(1->4), and α-(1->6) units, and ß-(1->3) and ß-(1->6) units may be interpreted similarly (Gorin, 1981).

It is also possible to distinguish and assign ^{13}C resonances of branched-chain polysaccharide since the side chain units should exhibit more segmental motion than those of the main chain. The branched mannan from *Saccharomyles rouxii* consisted of a main chain of (1->6)-linked α-D-mannopyranosyl residues substituted at O-2 by an O-α-D-mannopyranosyl-(1->2)-O-α-D-mannopyranosyl side chains, containing C-1 atoms with T1 values at 70° of 0.2s (non-reducing end- unit), 0.13s (adjacent,side-chain unit), and 0.09s (main-chain unit). This contrasted with the linear mannan from *Han senula* capsulata, which contained a repeating sequence of α-D-(1->2), α-D-(1->6) units that each has a T1 value for C-1 atoms of 0.14s (Gorin, 1981).

2.4.7.3 Two-Dimensional NMR Spectra of Polysaccharides and Oligosaccharides

Two dimensional NMR spectroscopy is presently the most powerful technique for determination of carbohydrate structure. The 2-dimensional homonuclear ^{1}H-^{1}H shift correlated (COSY) spectrum can be used to establish the connectivities of a given sugar residue while ^{1}H-^{13}C heteronuclear shift correlated experiments allow the complete assignment of the proton resonances to the corresponding carbon of an oligosaccharide or polysaccharide. In the NOESY experiment, dipole coupling is usually observed between anomeric (H-1) and non-anomeric resonances and between pairs of non-anomeric resonances; however, only the former is essential for establishing the oligosaccharide (polysaccharide) primary structure. Two types of dipole (through-space) coupling could be observed for anomeric protons: intra-residue and inter-residue couplings. The intra-residue couplings are "cross the ring" couplings which are helpful in confirming resonance assignments while inter-residue couplings are couplings across the glycosidic linkage which are very important in identifying linkage sites and sequence (Dabrowski, 1987).

In the structural analysis of xyloglucan, York and co-workers (1990) confirmed the stereochemistry and the anomeric configurations of the glycosyl residues of 7 oligosaccharides. Well resolved resonances were assigned by evaluating 1D NMR spectra in light of the deduced glycosyl sequences. 2D NMR techniques, including double-quantum filtered COSY and homonuclear Hartmann-Hahn (HOHAHA) spectroscopy allowed nearly all of the 1H signals of the 7 oligosaccharides to be assigned. In a structural study of plant cell walls, Kiefer *et al.* (1990) deduced much of the structure of a xyloglucan oligosaccharide by comparing the ^{1}H-NMR spectrum of this oligosaccharide to the previously assigned ^{1}H NMR spectra of a similar structure. The signals from H-1 and H-2 of the L-arabinofuranosyl residue were assigned by comparing published chemical shifts and vicinal coupling constants

for methyl α-and α-L-arabinofuranosides to those obtained from the COSY spectrum of the oligosaccharide (Kiefer *et al.*, 1990). Jackson *et al.* (1990) applied a long range heteronuclear correlation technique which established the connection between H-1 and C-3 of a α-Rhamp unit. COSY spectra, enabled identification of the H-2 and H-3 signals, whereas the attached carbon assignments followed from the HETCOR plot. The long-range HETCOR experiment showed the following couplings: H-2 to C-1 and C-3 of ^{13}C shifts which confirmed the linkage assignments. The establishment of the sequence of sugar residues resulted from the inter-residue connectivities revealed by the long-range ^{1}H-^{13}C correlation experiments (Jackson, 1990). In a study conducted by Rodriquez et al.(1990), a K11 antigen gave 18 ^{13}C signals in ^{13}C NMR, three of which (104.4 ppm, 103.5 ppm and 95.9 ppm) were in the region for anomeric carbons. The anomeric configurations of the glucose residues were determined using gated decoupling . The signal at 103.5 ppm had a $J_{c\text{-}1,H\text{-}1}$ value of 165 Hz and was assigned to a ß residue, while that at 95.9 ppm had 175Hz, indicating an α configuration. A ß configuration of a D-fructofuranose residue was assigned to the signal at 104.4 ppm.

2.4.8 Summary of Experimental Approches in Structure Elucidation of Carbohydrates

Methylation analysis only yields information on positions of substitution and not the anomeric configuration or sequence of linkages. Periodate oxidation of vicinal diols is still widely used in spite of difficulties in interpreting the data. Although enzymatic degradation appears to be a valuable method, the battery of known exoglycosidases is quite small and the routine use of endoglycosidases, the "restriction enzymes" of complex carbohydrate chemistry, has only been possible in recent years. The availability of endoglycosidases

with known specificities is well documented but very limited. Although oligosaccharide mass spectrometry, especially FAB MS, seems very attractive on account of its speed and sensitivity there is no method available that can give more than a limited partial structure. Furthermore, while 2D NMR spectra can provide information related to the detailed structure and configuration of polysaccharides, poor sensitivity limits their application. Therefore, a combination of different techniques described above provides powerful tools for elucidating the structure and linkage configuration of complex polysaccharides.

CHAPTER 3

CHEMICAL AND PHYSICAL PROPERTIES OF YELLOW MUSTARD (*Sinapis alba* L.) MUCILAGE

3.1 INTRODUCTION

White or yellow mustard seeds, *Sinapis alba* L., are grown primarily for their use as condiments. The outer seed coat of mustard seeds was first reported, almost 60 years ago, to be rich in mucilaginous material (Bailey and Norris, 1932). The consistency of prepared mustard products, such as salad dressings and food pastes, was later attributed by Weber *et al.* (1974) to the presence of mucilage. The mucilage in four yellow mustard cultivars grown at four different locations in Western Canada was reported by Woods and Downey (1980) to range from 0.34 to 2.05% with an overall average of 1.28%. A number of researchers have since shown mustard seed mucilage to be composed primarily of polysaccharides containing glucose, arabinose, xylose, rhamnose, galactose, mannose and galacturonic acid (Vose, 1974; Theander *et al.*, 1977; Siddiqui *et al.*, 1986). A recent report by Anguilar and Ziegler (1990) showed both temperature and electrolytes had a negative effect on the viscosity of aqueous dispersions of mustard seed mucilage. These studies were all based on mucilage extracted using the Weber's method which only extracted 2% mucilage (Weber *et al.*, 1974; Anguilar and Ziegler, 1990). Studies conducted in our laboratory yielded higher amounts of mucilage (~5.0%) from yellow mustard seeds using a

modified extraction procedure (Sharafabadi, 1987). The material obtained was white and cotton-like in appearance, exhibiting shear-thinning behaviour similar to xanthan gum dispersions.

The initial objective of the present study was to maximize the extraction yield of mucilage from yellow mustard seeds. Since mustard mucilage was shown to contain two fractions, a water-soluble (WS) fraction and a water-insoluble (WI) fraction, further objectives included separation of these fractions to determine their chemical composition and physical properties. The potential food application of mustard mucilage was assessed by comparison with commercial gums.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Whole mustard seeds (*Sinapis alba* L. cv Tilney) were obtained from United Grain Growers (UGG), Winnipeg, Manitoba, Canada. Xanthan, guar and arabic gums were purchased from Sigma Chemical Co. (St. Louis, USA). All chemicals used were of reagent grade.

3.2.2 Extraction, Fractionation and Analysis of Yellow Mustard Seed Mucilage

Crude mucilage (CM) and its respective water-soluble (WS) and water-insoluble fractions were obtained following the procedure of Sharafabadi (1987) which was modified by successive aqueous extraction with a seed:water ratio of 1:6 (Fig. 3.1).

Moisture and ash contents were determined using the AOAC oven method (AOAC,

1980). Protein was determined by a microKjeldahl method using a Kjeltec Auto 1030 Analyzer (Tecator, Sweden). Phosphorus and sulphur were determined using the method described by McKeague (1978), while other minerals were determined by atomic absorption (Perkin Elmer 560 Atomic Absorption Spectrometer, wet-ashed). Uronic acids were measured colorimetrically according to Blumenkrantz and Asboe-Hansen (1973). Monosaccharide determination was carried out by GLC using a SP-2330 glass capillary column, 30m x 0.75mm ID, according to Englyst *et al.*(1982). ^{13}C-NMR spectrum (500 Hz) was recorded on a Bruker AMX500 FT spectrometer at 85°C; polymer conc. 2.0% (w/v) in D_2O, 40,000 pulses, pulse repetition time 1.3s and r.f. pulse angle 80.0°.

Gel permeation chromatography was conducted on a Sephacryl S- 300 (HR, 1.6x70 cm) column that was eluted with 0.1 M NaCl solution. Samples dissolved in the same buffer (1mg/ml) were applied onto the column, and fractions of 2 ml were collected. Blue dextran 2,000 and D-glucose were used to determine the void and total volumes respectively, while linear dextran T-70 (Pharmacia Ltd, Montreal, PQ) was used as a relative molecular weight marker. Carbohydrates in the fractions were determined by the anthrone method (Loewus, 1952), while uronic acids were measured according to the method of Blumenkrantz and Asboe-Hansen (1973).

3.2.3 Interfacial Properties

The interfacial activity of yellow mustard mucilage was examined following the procedure of Izydorczyk *et al.* (1991). Emulsion capacity and stability tests were performed as described by Yasumatsu *et al.* (1972) with the following modification: 0.50 gram of gum or mucilage was suspended in 40 ml of distilled water followed by the addition of 40 ml of corn oil. All mixtures were then emulsified using a polytron at 10,000 rpm for 1 min.

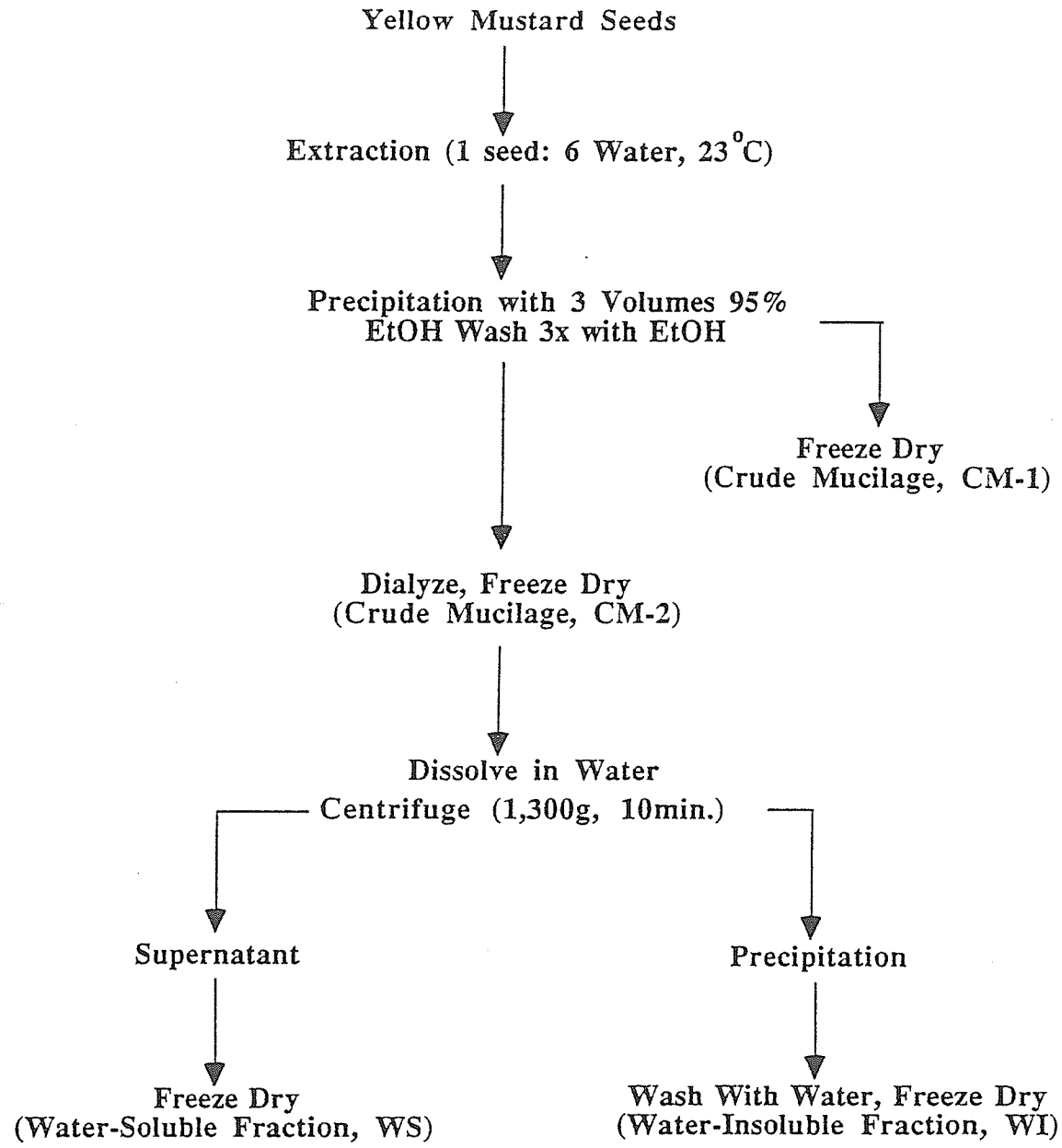


Fig. 3.1 Procedure for extraction and fractionation of yellow mustard mucilage.

and centrifuged at 1,300 g for 5 min. Emulsion capacity was calculated as :

$$\frac{\textit{height of emulsion layer}}{\textit{total height of fluid}} \times 100\%$$

Emulsion stability was determined by heating the emulsion at 80°C for 30 min., cooling with tap water for 15 min. and then centrifuging at 1,300g for 5 min. Emulsion stability was calculated as :

$$\frac{\textit{height of remaining emulsion layer}}{\textit{total height of fluid}} \times 100\%$$

Foaming capacity and stability of mustard mucilage and commercial gums were determined by the method of Yasumatsu and co-workers (1972) with a slight modification: 0.3% of mucilage (gum) solutions were made in 0.1 % Ovalbumin (Sigma Chemical Co.). The reported data represent means of triplicate measurements.

3.2.4 Rheological Measurements

All rheological properties were determined on a Bohlin VOR Rheometer (Bohlin Reologi, Sweden). A concentric cylinder geometry with a height of 63.0 mm and inner and outer radii of the containers of 12.5 and 13.75 mm, respectively, was used throughout the rheological study (Mazza and Biliaderis, 1989). The samples were subjected to shear sweeps between 3.682 to 734.3 sec^{-1}. Viscosity measurements were conducted using aqueous solutions of 0.3, 0.5 and 1.0% (w/w). The influence of pH, salt and sugar on the

viscosity of mucilage solutions was examined at 0.5% (w/w), while temperature effects were examined at 1.0% (w/w). Dynamic rheological measurements on 1.0% (w/w) solutions and dispersions of mucilage as well as of commercial gums were carried out as a function of oscillatory frequency (f: 0.5-20.0 Hz) with a maximum input strain of 4% at 22 °C. The rheological parameters used to evaluate the viscoelastic properties of these materials were the storage modulus (G'), loss modulus (G''), dynamic viscosity ($\eta'=G''/2\pi f$) and phase angle, δ ($\tan \delta = G''/G'$). Data presented are means of triplicate measurements.

3.3 RESULTS AND DISCUSSION

3.3.1 Chemical Composition

The yield of mucilage obtained upon successive extractions of yellow mustard seeds is shown in Table 3.1. The initial extraction removed 65.2% of the mucilage, with the second and third successive extractions accounting for 17.6 and 10.2% of the total mucilage, respectively. The first three fractions accounted for 93.0% of the total mucilage extracted which represented 4.9% of the total seeds. Crude mucilage (CM) was fractionated into a WS fraction and a WI fraction according to the procedure outlined in Fig. 3.1. Following extraction, the mucilage was precipitated with 3 volumes of 95% ethanol, dialysed and then freeze-dried to provide CM. CM was further fractionated into a water-soluble (WS) fraction and a water-insoluble (WI) fraction by centrifugation which accounted for 55.6 and 38.8% of the crude mucilage, respectively, with an overall recovery of 94.4%.

Table 3.1. Yield of crude mucilage (CM) from yellow mustard seeds upon sequential aqueous extraction (100 g seeds/600 g H_2O)

Extraction	1	2	3	4	5	Total
Weight (g)	3.45	0.93	0.54	0.25	0.12	5.29
Percent (%)	65.2	17.6	10.2	4.7	2.3	100

The proximate analysis of crude mucilage and its fractions is shown in Table 3.2. Crude mucilage contained 80.4% carbohydrates, 4.4% protein and 15.0% ash. Dialysis reduced the ash content to 4.8% with a corresponding increase in carbohydrates from 80.2 to 91.1%. The carbohydrate content was similar for both WS and WI fractions, while the ash content was higher in the WS fraction and protein content higher in the WI fraction. The monosaccharide composition of yellow mustard mucilage and its fractions is summarized in Table 3.3. Glucose appeared to be the predominant neutral sugar, followed inturn by galactose, mannose, rhamnose, arabinose and xylose, but for the WI fraction arabinose was higher than rhamnose. Uronic acid content was highest in the WS fraction accounting for 18.6% and lowest in the WI fraction at 10.2% with CM being in between at 14.7%.

^{13}C NMR (Fig. 3.2) confirmed the presence of uronic acid by the characteristic resonance at δ=174.93 ppm from the carboxyl group and rhamnose by the typical C-6 resonance at δ=17.99 ppm. Due to the complexity of the spectra and the heterogeneous nature and complex structure of the polysaccharide species present in the WS fraction, a complete assignment of resonances to characteristic carbons of monosaccharide residues

Table 3.2. Chemical compositions of yellow mustard seed mucilage and its fractions

Component (on dry base)		CM-1[c]	CM-2[d]	WS	WI
Water	(%)	6.9	8.6	10.2	8.1
Ash	(%)	15.0	4.8	4.3	1.7
Protein[a]	(%)	4.4	4.1	2.2	4.2
Fat	(%)	0.2	-[e]	-	-
Carbohydrate[b]	(%)	80.4	91.1	93.5	94.1
Potassium	(%)	2.1	0.04	0.01	0.02
Calcium	(%)	2.2	1.2	1.6	0.48
Magnesium	(%)	1.3	0.6	0.4	0.16
Phosphorus	(%)	2.1	0.6	0.4	0.09
Sulphur	(%)	1.4	0.29	0.16	0.02
Iron	(ppm)	212.5	260.0	223.0	358.0
Zinc	(ppm)	53.5	70.0	123.0	208.0
Manganese	(ppm)	73.0	70.0	72.0	27.0
Copper	(ppm)	13.5	12.0	34.0	18.0

CM: mucilage; WS: water-soluble fraction; WI: water-insoluble fraction.
[a]: N x 6.25.
[b]: By difference.
[c]: Crude mucilage without dialysis.
[d]: Cude mucilage after dialysis.
[e]: Not determined.

Table 3.3. Yield, monosaccharide composition and uronic acid content of yellow mustard mucilage and its fractions[a]

Mucilage fraction	Yeild (%)	Uronic acid (%)	Glucose (%)	Galactose (%)	Mannose (%)	Rhamnose (%)	Arabinose (%)	Xylose (%)
CM	-	14.64±0.62	23.54±0.77	13.83±0.30	6.07±0.20	3.15±0.16	3.02±0.17	1.80±0.09
WS	55.6±2.2	18.68±1.36	22.26±1.57	15.21±0.70	6.31±0.30	3.93±0.0	3.22±0.08	1.77±0.10
WI	38.8±1.8	10.30±1.07	34.95±1.35	11.70±0.76	6.35±0.01	1.65±0.35	2.84±0.03	2.00±0.11

CM: crude mucilage (after dialysis); WS: water-soluble fraction; WI: water-insoluble fraction.
[a]: n=3, mean±SD.

was not feasible. Nevertheless, in the anomeric carbon region (95-108 ppm) at least six resonances were present which correspond with the main sugars identified by monosaccharide analysis (Table 3.3).

Gel filtration chromatography of CM and WS fractions on the Sephacryl S-300 column (Fig.3. 3) indicated the presence of both high and low molecular weight polysaccharide species, the most prominent being the peak at the void volume. Uronic acids were detected in both high and low molecular weight regions of the eluted carbohydrates.

3.3.2 Interfacial Properties

The effect of yellow mustard mucilage fractions on the surface tension of water is shown in Fig. 3.4. Increasing the mucilage concentration up to 0.05% substantially reduced surface tension. Further additions of mucilage only decreased the surface tension slightly. WS exhibited the greatest reduction in surface tension compared to CM or WI fraction. The surface and interfacial activities of some plant hydrocolloids (guar and locust bean gums) were recently attributed to the presence of residual surface active constituents/impurities (Gaonkar, 1991). The protein present in CM and its fractions (Table 3.2) could thus contribute to the surface activity of the polysaccharides; interestingly, the WS fraction, although having the lowest protein content, was the most surface active of all mucilage fractions. The emulsion capacity and stability of yellow mustard mucilage and its fractions were compared to commercial gums as shown in Fig. 3.5. Before dialysis, the CM fraction exhibited the highest emulsion capacity and stability as compared to the other mucilage fractions or commercial gums. While dialysis reduced the emulsion capacity and stability of CM substantially, it still exhibited higher emulsion capacity and stability compared to the commercial gums.

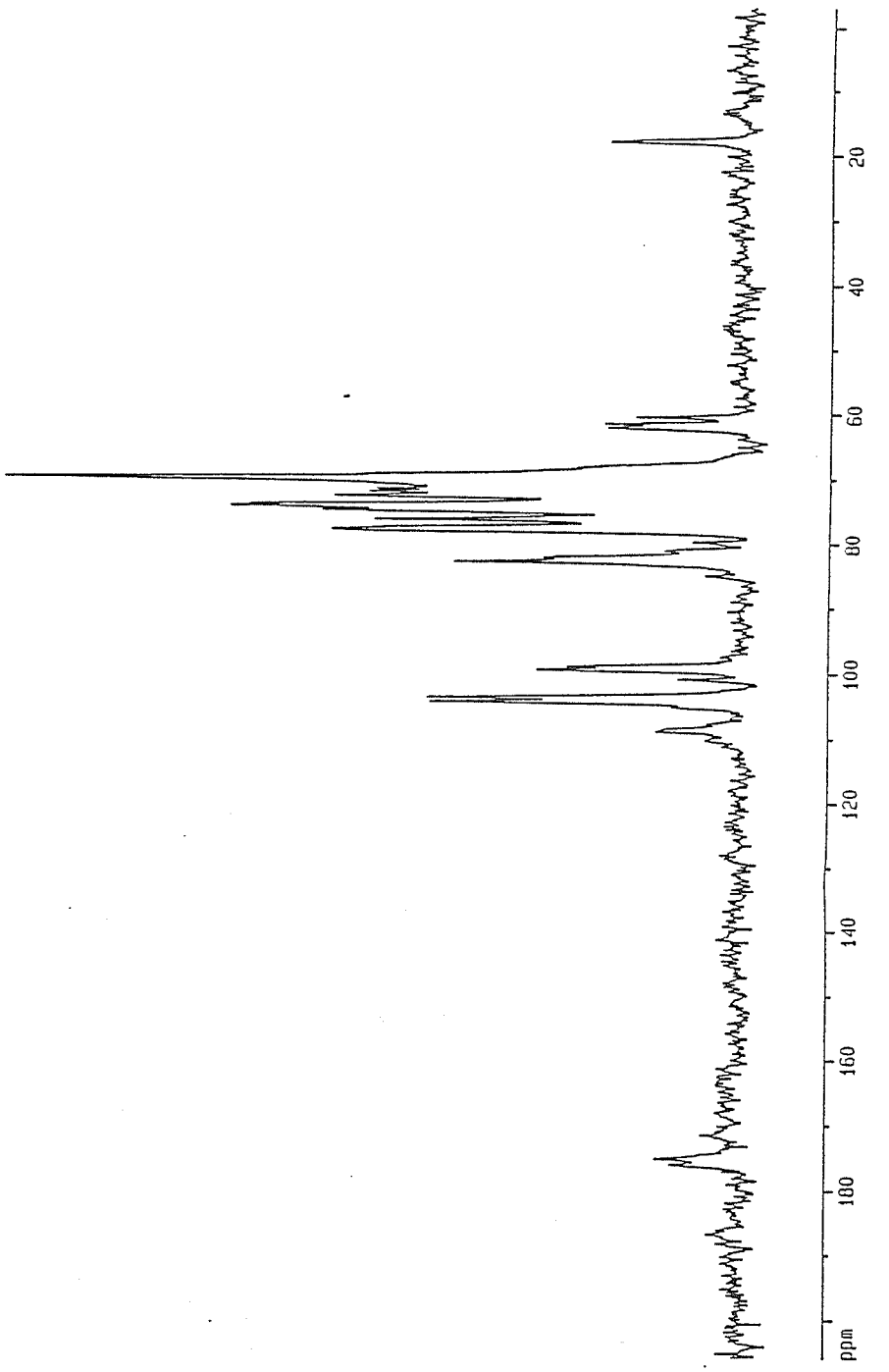


Fig. 3.2. ^{13}C NMR spectrum of the water-soluble fraction of yellow mustard mucilage (in D_2O). The chemical assigned relative to 1,4-dioxan.

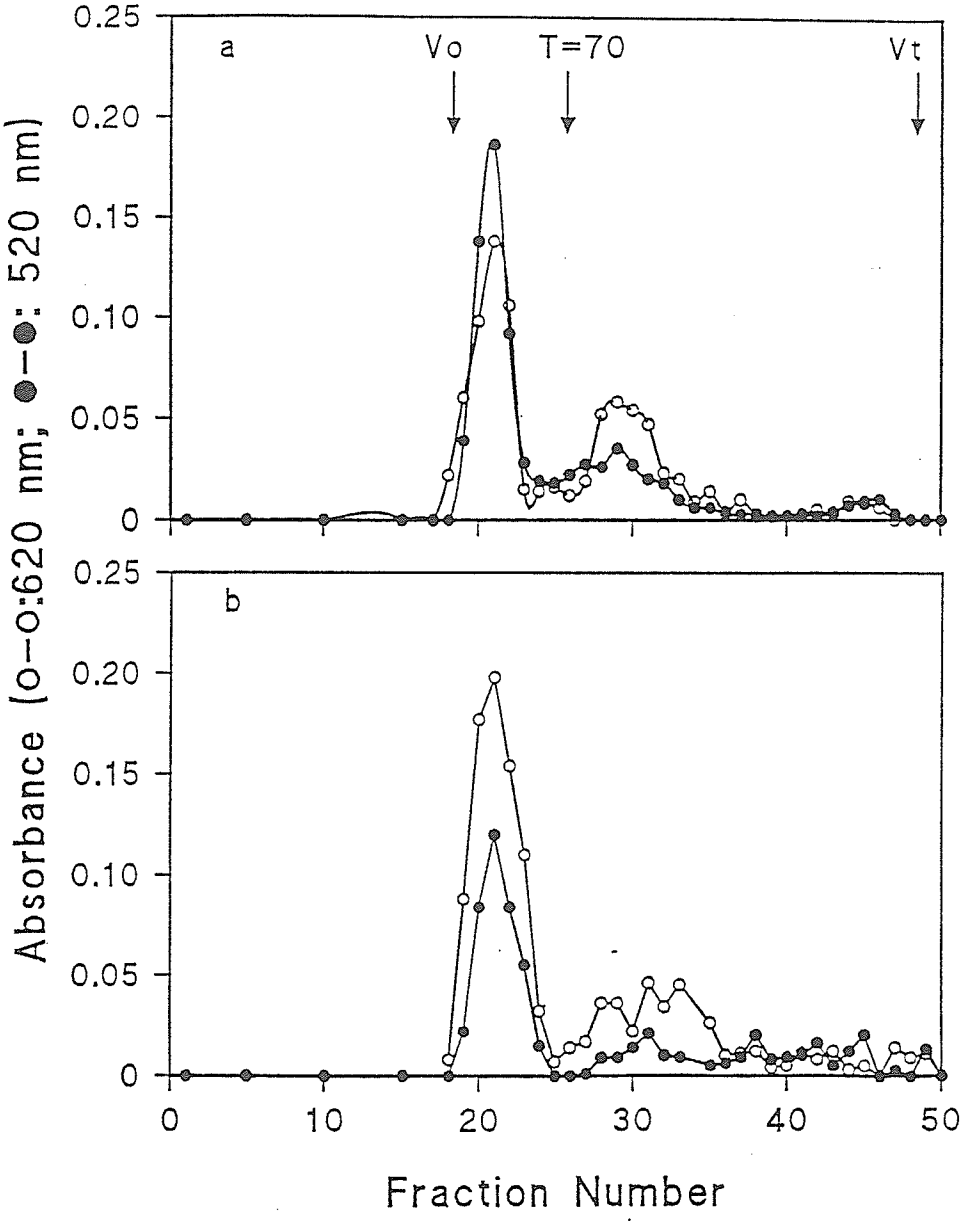


Fig. 3.3. Chromatographic profiles of (a) CM, and (b) WS fractions on a Sephacryl S-300 HR column (1.6x70 cm) eluted with 0.1N NaCl solution, flow rate 1 ml/min. temperature 23°C; arrows indicate peak elution volumes of dextran standards (Blue T-2000, Vo: T-70; Glucose, Vt) used as molecular weight markers (o-o total carbohydrate; uronic acids). (CM: crude mucilage; WS: water-soluble fraction).

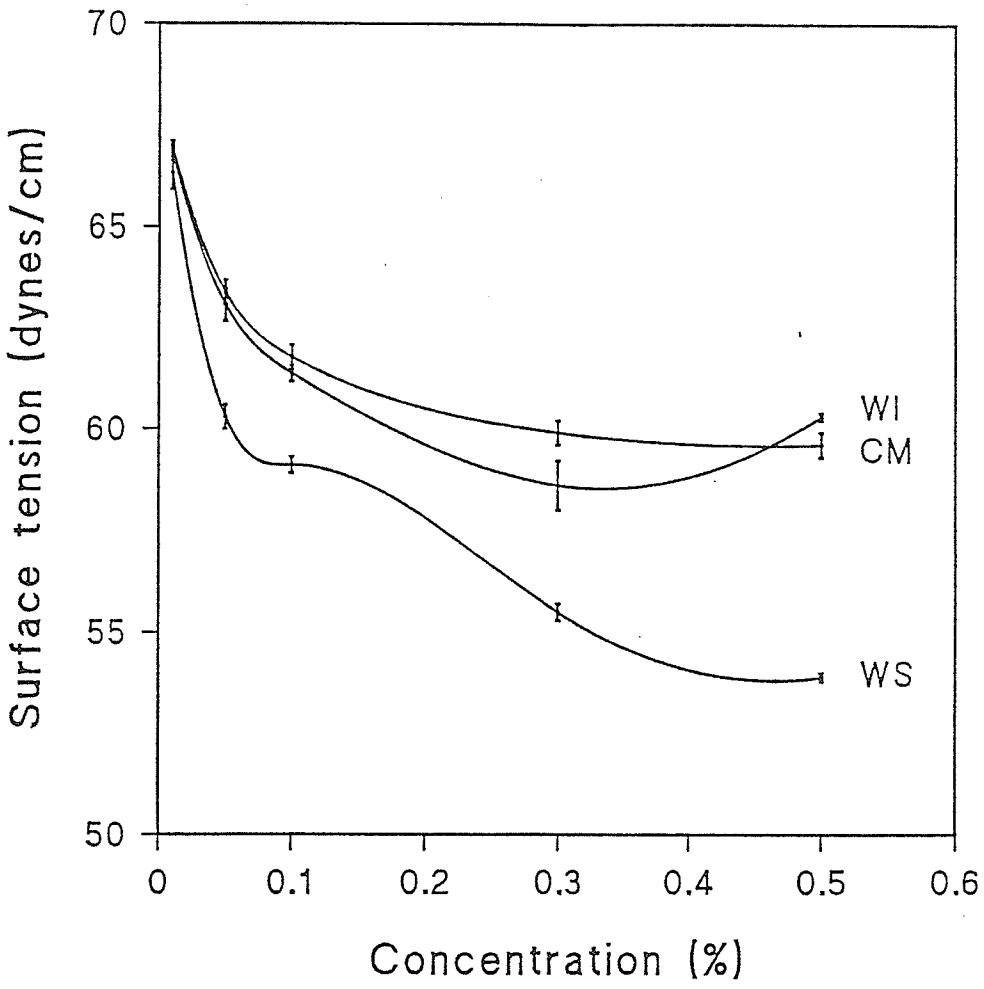


Fig. 3.4. Reduction of surface tension of water by CM, WS and WI at various concentrations; the corrected surface tension of distilled water was 69.0±0.7 dyne/cm at 23.0±0.5°C. (CM: crude mucilage; WS: water-soluble fraction; WI water-insoluble fraction.)

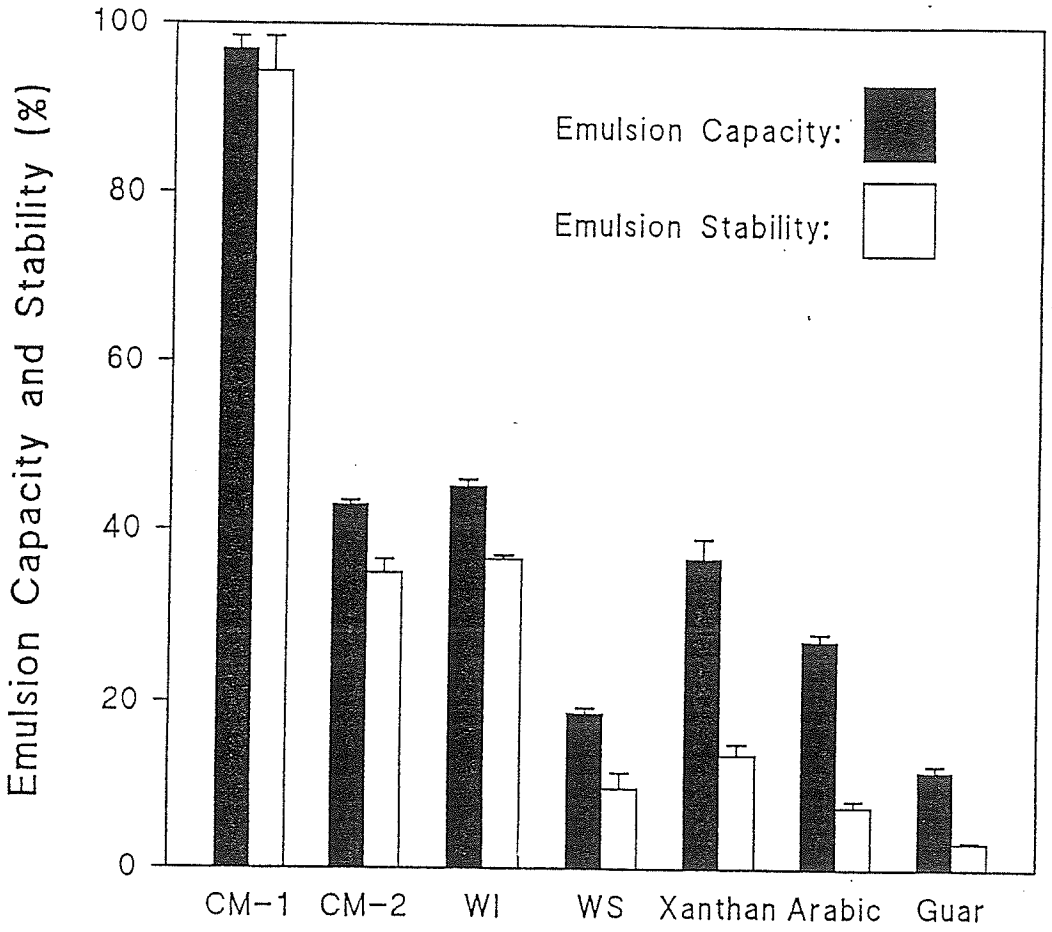


Fig. 3.5. Emulsion capacity and stability of yellow mustard mucilage fractions and other commercial gums; CM-1 and CM-2 are crude mucilage samples before and after dialysis.

The foaming capacity and stability of yellow mustard mucilage fractions and commercial gums are summarized in Table 3.4. Prior to dialysis, the CM fraction exhibited the highest foaming stability among the yellow mustard mucilage fractions. Following dialysis, the foaming capacity of CM increased, but its foam was less stable. Similar trends were observed for both WS and WI fractions which exhibited higher foaming capacity but poorer foaming stability.

Table 3.4. Effect of yellow mustard mucilage on foaming capacity and stability of 0.1% bovine serum albumin solutions

Time (h)	Foam volume (ml)						
	CM-1[a]	CM-2[b]	WS	WI	Xanthan	Arabic	Guar
0.0	23.5±0.5[c]	38.3±0.6	52.3±1.5	39.0±1.0	36.5±0.9	41.8±1.0	12.8±0.3
0.5	19.2±1.0	16.5±1.3	29.2±0.7	16.7±0.8	36.5±1.0	36.3±1.2	9.3±0.3
1.0	18.3±0.8	13.7±0.3	20.3±0.4	15.0±1.3	36.2±0.5	32.8±1.3	8.2±0.0
3.0	15.9±0.1	6.3±1.0	0.0±0.0[d]	0.0±0.0	18.7±1.5	20.6±0.6	7.3±0.6
5.0	14.8±0.3	1.0±0.0	0.0±0.0	0.0±0.0	17.5±1.3	8.0±0.9	6.6±0.5
6.0	14.7±0.3	0.0±0.0	0.0±0.0	0.0±0.0	17.2±1.0	6.6±1.3	5.9±0.1
23.0	13.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	13.2±1.3	0.5±0.5	4.0±0.0

CM: crude mucilage; WS: water-soluble fraction; WI: water-insoluble fraction.
[a]: CM-1 before dialysis.
[b]: CM-2 after dialysis.
[c]: n=3, mean±SD.
[d]: Diminished.

3.3.3 Rheological Properties

The flow profiles of yellow mustard mucilage and its two fractions vs shear rate are shown in Fig. 3.6. The shear thinning behaviour exhibited by yellow mustard mucilage and its fractions resembled those of xanthan gum dispersions at all concentrations. In contrast, guar gum solutions showed shear thinning behaviour only at higher concentration (e.g. 1.0% w/w) and almost Newtonian behaviour at lower concentration (0.3%).

The most widely used mathematical expression for pseudoplastic rheological behavior of hydrocolloid solutions/dispersions is the power law described by Ostwald (Whitcomb *et al.*,1980):

$$\eta = K\dot{\gamma}^{n-1} \qquad (3.1)$$

where η is the apparent viscosity (Pa.s), $\dot{\gamma}$ is the shear rate, K is the consistency index (Pa.s) and n is the flow index which measures the pseudoplasticity of the system. A comparison of n and K values of yellow mustard mucilages against xanthan and guar gums is given in Table 3.5. The K values increased with increasing polysaccharide concentration while the n values decreased. This is in agreement with the findings of Whitcomb *et al.* (1980) on guar gum solutions that the higher the concentration, the more pronounced the pseudoplastic behavior of a system becomes.

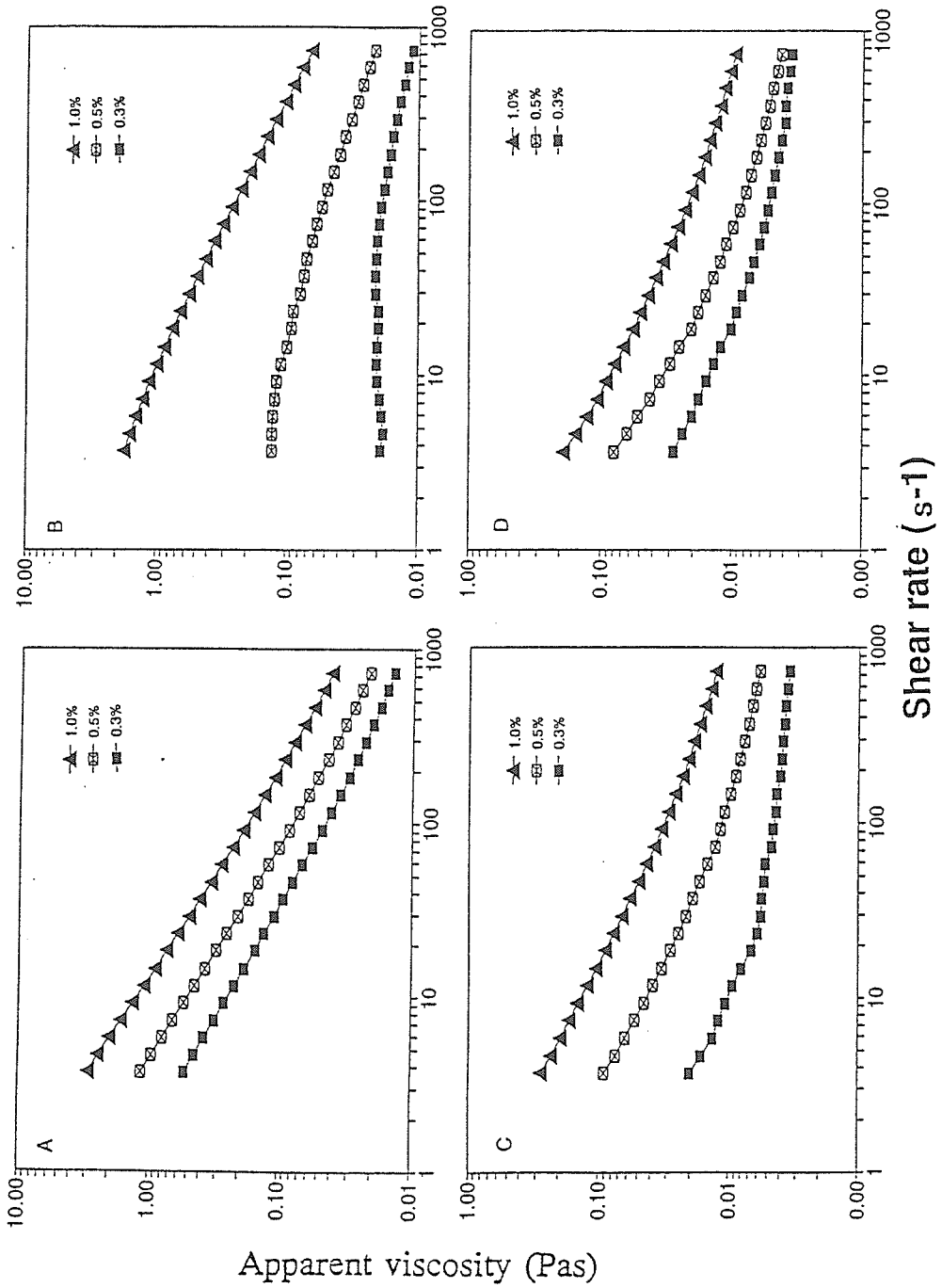


Fig. 3.6 Effect of shear rate on the apparent viscosity of (A) xanthan gum, (B) guar gum, (C) CM, and (D) WS at concentration between 0.3-1.0%, 22.0 C (CM: crude mucilage; WS: water soluble fraction)

Table 3.5. Comparison of n and K values of mustard mucilage fractions against xanthan and guar gums solutions/dispersions (at 22.0°C, shear rate range: 3.682-734.3 s^{-1})

Concentration	CM		WS		WI		Xanthan		Guar	
	n	*K* (Pa s)	n	*K* (Pa s)	n	*K* (Pa s)	n	*K* (Pa s)	n	*K* (Pa s)
1.0%	0.427	0.480	0.466	0.280	0.436	0.957	0.188	7.813	0.350	4.830
0.5%	0.507	0.123	0.492	0.099	0.557	0.163	0.243	2.767	0.629	0.271
0.3%	0.740	0.016	0.638	0.031	0.639	0.064	0.309	1.167	0.885	0.028

CM: crude mucilage; WS: water-soluble fraction; WI: water-insoluble fraction.

The small strain oscillatory rheological testing of yellow mustard mucilage provided evidence which confirmed earlier observations (Fig. 3.6) that yellow mustard mucilage in solution behaves more like xanthan gum than guar gum (Fig. 3.7). Both xanthan gum and yellow mustard mucilage solutions/dispersions exhibited a gel-like structure with G' > G'' (storage and loss moduli) over the entire frequency range examined. In contrast, guar gum solutions of identical concentration (1.0% w/w) behaved like typical viscoelastic fluids, where G''>G' at low frequencies with the reverse occurs at high frequencies. This type of rheological response was also evident by the phase angle changes vs frequency of the hydrocolloid solutions. The tangent of phase angle δ, defined as the ratio of G''/G', expresses the relative contributions of the viscous and elastic components to the visoelastic properties of a material. The constant increase of both G' and G'' vs frequency resulted in relatively constant phase angle values for both xanthan gum and yellow mustard mucilage solutions over the entire frequency range examined (Fig. 3.8). In contrast, the phase angle values of guar gum solutions were highly dependent on frequency.

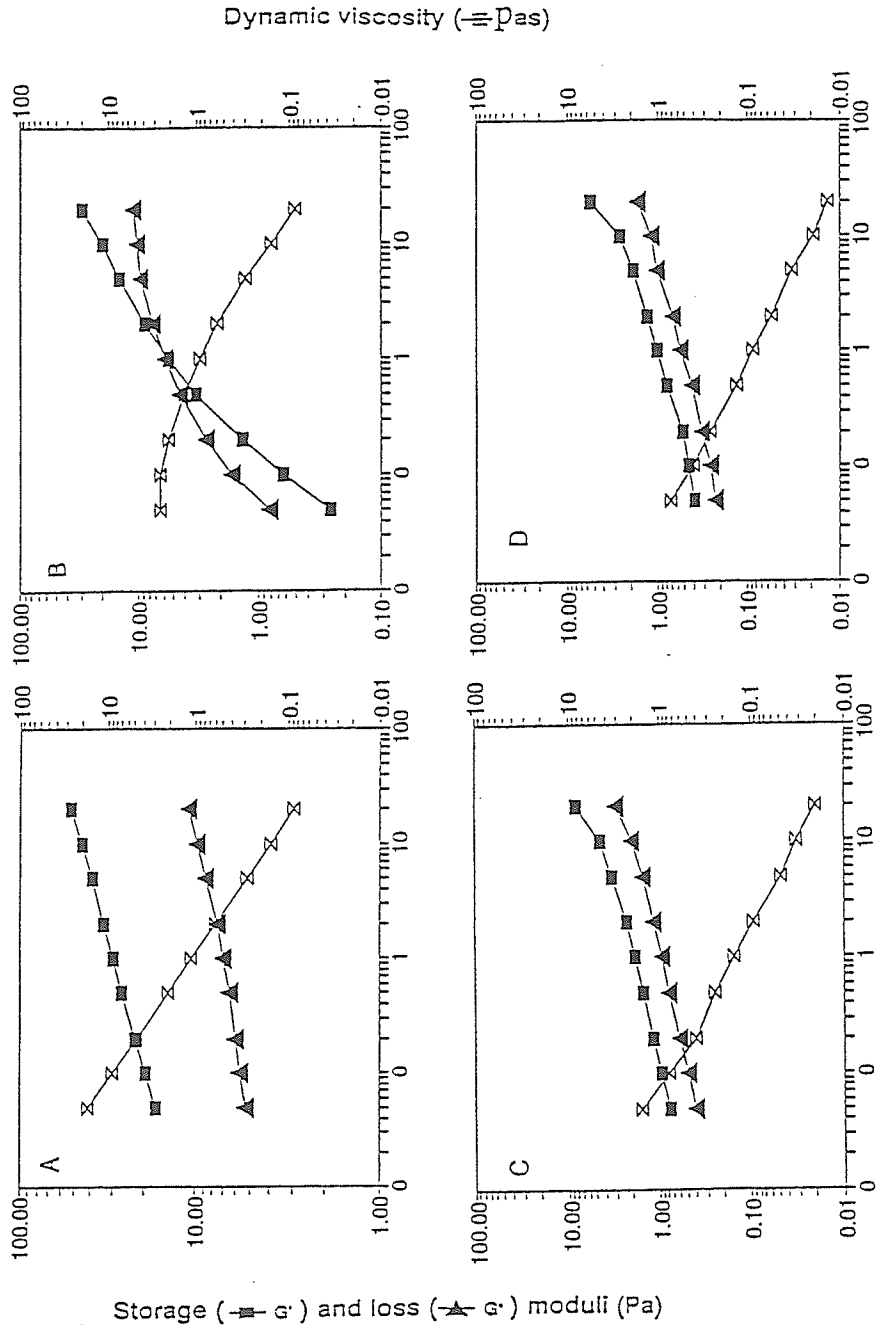


Fig. 3.7. Frequency dependence of storage (G') and loss (G") moduli, and dynamic viscosity (η') of (A) xanthan gum, (B) guar gum, (C) CM (crude mucilage) and (D) WS (water soluble fraction) for 1.0% (w/w) solutions/dispersions.

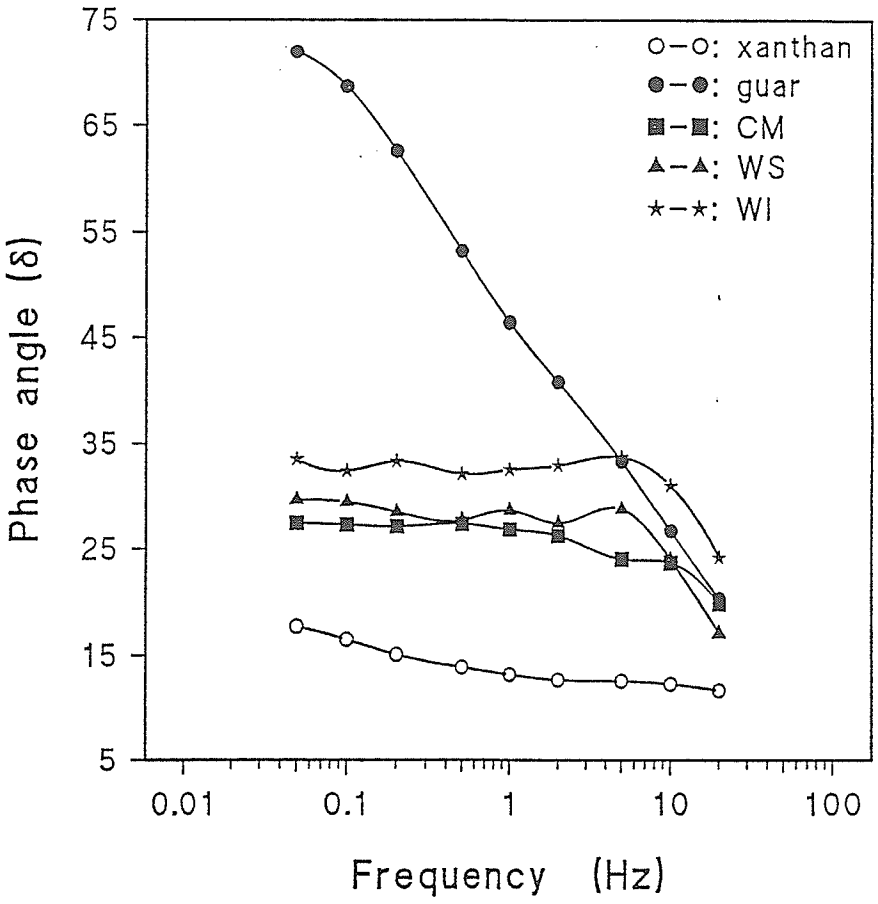


Fig. 3.8. Phase angle value profiles as a function of frequency of yellow mustard mucilage and commerical gum for 1.0% (w/w) solutions/dispersions. (CM: crude mucilage; WS: water-soluble fraction; WI: water-insoluble fraction)

The effects of pH on the rheological properties of mucilage solutions (0.5% w/w) are shown in Fig. 3.9. Viscosity at a shear rate 93.32 s^{-1}, approximating mouthfeel conditions (Sherman, 1975), increased by either addition of dilute HCl or NaOH solutions. These results are in agreement with the earlier work of Weber and co-workers (1974). The increase in viscosity at both low and high pH regions suggested that acid or alkali environments alter the conformation of the polysaccharides and most likely affect intermolecular interaction due to modification of electrostatic effects. Furthermore, the viscosity increase of WI in the alkaline region could be due to a more effective dispersion/solubilization of the insoluble mucilage fraction under these conditions. The extent of the influence of pH on the apparent viscosity of mucilage solutions (suspensions) was in the order of WI>CM>WS.

Similar trends in viscosity for CM and its fractions (0.5% w/w) were observed with the addition of either NaCl (0.4-3.5 M) or sucrose (0.15-1.2 M). Solutions of CM and WS generally exhibited higher viscosity values with increasing additive concentration. In contrast, WI dispersions exhibited an initial reduction in viscosity in the presence of low concentrations of NaCl (< 0.5 M) and sucrose (< 0.25 M). At much higher solute concentrations, the rheological responses of WI were similar to those of CM and WS fractions.

The effect of temperature on the apparent viscosity of yellow mustard mucilage solutions and dispersions is also presented in Fig. 3.9. An increase in temperature resulted in a continuous reduction in viscosity. In the case of WI, the reduction in viscosity was more pronounced compared to the other two fractions. This may suggest the presence of extended interparticle associations for this material at low temperatures.

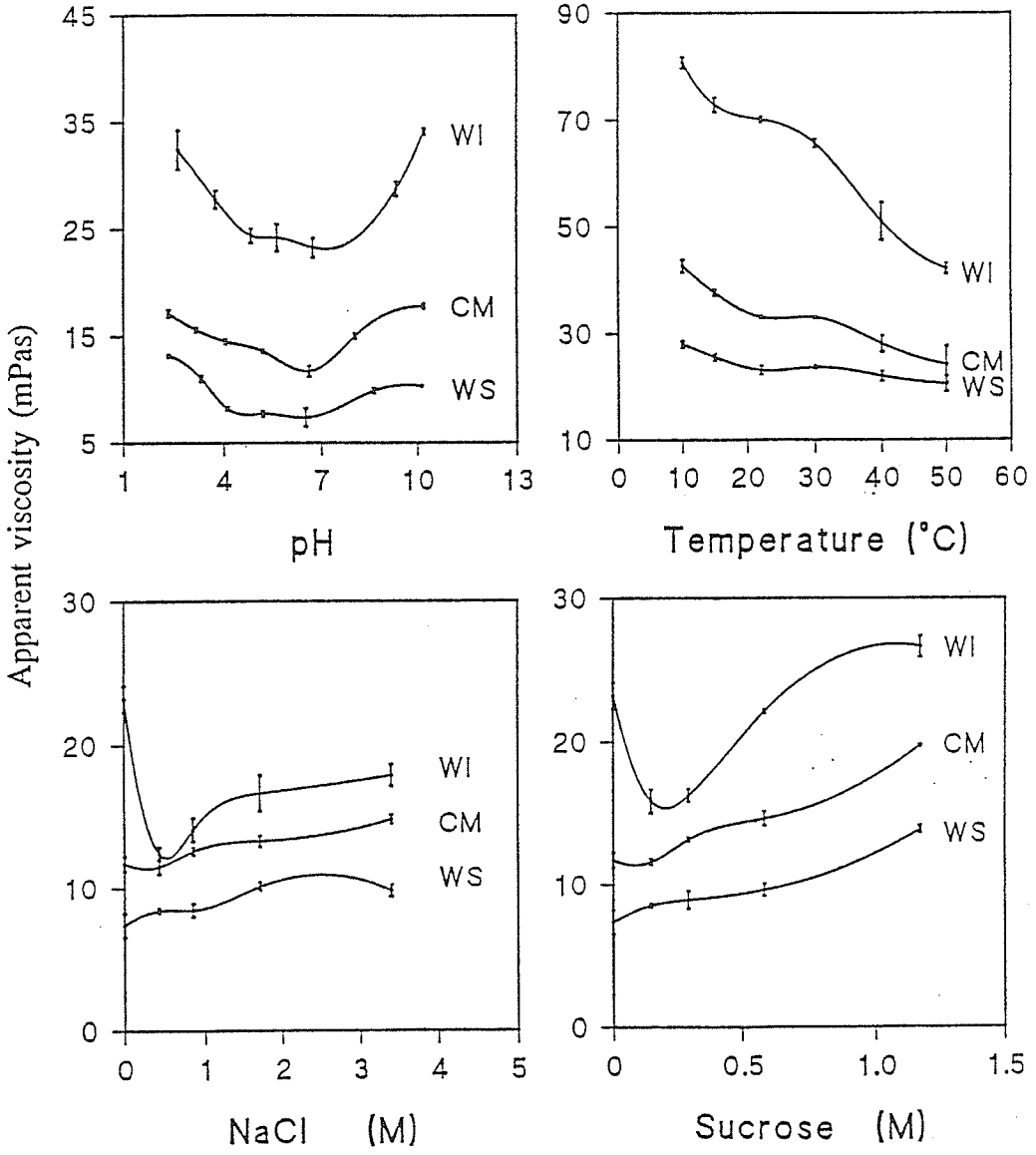


Fig. 3.9. Effect of pH, temperature, salt and sugar on the apparent viscosity of yellow mustard mucilage fractions at shear rate 92.32 s^{-1}. (Concentration for temperature effect: 1.0% w/w; concentrations for pH, salt and sugar effect: 0.5% w/w; pH for temperature, salt and sugar effects: 6.3).
WI: Water-Insoluble fraction
CM: Crude Mucilage after dialysis
WS: Water-soluble fraction

The results of this study demonstrated the similarity between the emulsion capacity and stability and rheological properties of mucilage obtained from yellow mustard seeds and xanthan gum solutions/dispersions which suggest yellow mustard mucilage could be used as a substitute for xanthan gum in food dispersions.

CHAPTER 4

WATER-SOLUBLE YELLOW MUSTARD (*Sinapis alba* L.) POLYSACCHARIDES: PARTIAL CHARACTERIZATION, MOLECULAR SIZE DISTRIBUTION AND RHEOLOGICAL PROPERTIES

4.1 INTRODUCTION

Aqueous dispersions of yellow mustard (*Sinapis alba* L.) mucilage and its fractions were previously reported to exhibit similar rheological responses to that of xanthan gum dispersions in terms of shear thinning properties and dynamic rheological patterns (Cui *et al.*, 1993a). Of the yellow mustard mucilage fractions examined, the water-soluble (WS) fraction was the major component (55.6%) which in solution also showed the shear thinning behaviour of yellow mustard mucilage dispersions (Cui *et al.*, 1993a). Substantial interfacial activity was observed for the WS fraction as assessed by its ability to reduce the surface tension of water and to stabilise water/oil emulsions and foams formed by 0.1% albumin solutions. WS appeared as a heterogeneous mixture of polysaccharides which consisted of both neutral sugars and uronic acids. The monosaccharides identified were glucose (22.3 %), galactose (15.2 %), mannose (6.3 %), rhamnose (3.9 %), xylose (1.8 %) and arabinose (3.2 %). Uronic acids (18.7%) consisted of both glucuronic and galacturonic acids (Cui *et al.*, unpublished data) which is contrary to earlier studies on mustard mucilage

(Vose, 1974 and Siddiqui *et al.*, 1986) in which only galacturonic acid was reported .

The structure and molecular weight distribution of natural hydrocolloids are important determinants of their physical properties (Dea and Clark, 1986). For example, in dilute polymer solutions or dispersions, the shear thinning behaviour is attributed to a decrease in the "cross-link" density of the existing entangled network with increasing shear rate (Morris, 1990). The "cross-link" density is in turn highly dependent on the primary structure and conformation of the polymer molecules as well as the polymer-solvent and polymer-polymer interactions (Dea *et al.*, 1977). This paper reports on the primary structure, linkage pattern and molecular size distributions of two polysaccharide fractions (a CTAB-precipitated fraction, WSCP and a CTAB-soluble fraction, WSCS) of the water-soluble (WS) yellow mustard mucilage obtained by precipitation of the acidic polysaccharides with hexadecyltrimethylammonium bromide (CTAB). The rheological properties of these materials were also examined.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The water-soluble fraction (WS) of yellow mustard mucilage was isolated according to the procedure described in Chapter 3. All chemicals used were of reagent grade unless otherwise specified.

4.2.2 Fractionation of WS Fraction

The WS fraction was fractionated by co-precipitation with 5% CTAB (hexadecyltrimethylammonium bromide) (Scott, 1965). Optimization of the precipitation

conditions was attained by stepwise addition of 5% CTAB (0.20 ml) to 0.20 g WS in 0.3% solution (Fig. 4.1). The precipitate was dissolved in 4.0 M NaCl, then precipitated in 3 volumes of 95% EtOH (x3), dialysed against running water (18°C) for 24 hr, against distilled water at 35°C for 3x24 hr, and finally freeze-dried. The supernatant was precipitated in 3 volumes of 95% EtOH (x2), dialysed against running water (18°C) for 24 hr, against distilled water for 3x24 hr at 35°C, and freeze-dried.

4.2.3 Chemical Analysis and ^{13}C-NMR Spectra

Neutral monosaccharides were determined following the procedure described by Englyst *et al.* (1982) on a SP-2330 glass capillary column, 30m X 0.75mm ID. All samples were treated with 72% H_2SO_4 at 35°C for 30 min prior to the hydrolysis with 2M H_2SO_4. Uronic acids and protein were estimated according to the procedures of Blumenkrantz and Asboe-Hansen (1973) and Lowry (Lowry *et al.*, 1951), respectively. ^{13}C-NMR spectra (500 Hz) were recorded on a Bruker AMX500 FT spectrometer at 40°C, a polymer conc. 2.0% (w/v) in 10% D_2O and over 40,000 pulses with a pulse repetition time of 1.3s and a r.f. pulse angle 80.0°. Acetone was used as an internal standard.

4.2.4 Gel Chromatography

Gel permeation chromatography was conducted on a Sephacryl S- 500 (HR, 1.6x70 cm) column and eluted with 0.1 M NaCl solution. Samples dissolved in the same buffer (1mg/ml) were applied onto the column, and 3 ml fractions were collected. D-glucose was used to determine the total volume, while linear dextrans T-500 (MW 460,000) and T- 70 (MW 69,000) (Pharmacia Ltd, Montreal, PQ) were used as relative molecular weight markers. Carbohydrates in the fractions were determined by the anthrone method (Loewus,

1952), and uronic acids were monitored by the method of Blumenkrantz & Asboe-Hansen (1973).

4.2.5 Methylation Analysis

Methylation analysis was carried out as described by Ciucanu and Kerek (1984). The reduction of carboxyl group after methylation was performed according to O'Neill *et al* (1990). GC-MS was performed on an SP 2330 capillary column (60m x 0.25mm) while quantitative measurement of partially permethylated acetyl alditols was obtained on a SP 2330 capillary column (30m x 0.75mm), programmed from 160 to 210°C at 2°C per min.; helium was used as carrier gas, at 15 psi.

4.2.6 Rheological Measurements

All rheological properties were determined on a Bohlin VOR Rheometer (Bohlin Reologi, Sweden). A concentric cylinder geometry, with a cylinder height of 63.0 mm and radii of the inner and outer containers of 12.5 and 13.75 mm respectively, was used throughout the rheological study. In steady shear tests, samples were subjected to shear rate sweeps between 0.01 to 1164 sec^{-1}. Viscosity measurements were conducted using aqueous solutions of 0.3, 0.5, 1.0 and 2.0% (w/w). The influence of temperature, pH, salt and sugar on the apparent viscosity of mucilage solutions was examined at 0.5% (w/w) polymer solutions. Dynamic rheological measurements were determined on 2.0% (w/w) mucilage solutions as a function of oscillatory frequency (f: 0.05-20.0 Hz) with a maximum input strain of 4% at 22°C. The dynamic rheological parameters used to evaluate the viscoelastic properties of these materials were the storage modulus (G'), loss modulus (G''), dynamic viscosity ($\eta'=G''/2\pi f$), complex viscosity ($\eta^*=[G'^2+G''^2]^{0.5}/\omega$) and phase

angle, δ (tan δ = G''/G'), as described previously (Cui *et al.*, 1993a). All data presented are means of triplicate measurements.

4.3 RESULTS AND DISCUSSION

4.3.1 Fractionation and Chemical Composition

The optimum precipitation conditions for the acidic polysaccharides of the WS fraction of yellow mustard mucilage were found when 0.8 ml of 5% CTAB was added to a 0.3% (w/v) solution (total weight of WS 0.20g), as shown in Fig. 4.1. Exceeding this amount of CTAB resulted eventually in the complete precipitation of all polysaccharides. Under the optimum precipitation conditions, two fractions were obtained, a CTAB-precipitated fraction (WSCP) which contained 22.7% uronic acids (including both galacturonic and glucuronic acids) and a CTAB-soluble fraction (WSCS) which still contained 12.5% glucuronic acid (Table 4.1 and 4.2).

There appeared to be a preference for CTAB to precipitate galacturonic acid containing polymers over those of glucuronic acid as shown by methylation analysis and ^{13}C NMR spectra that galacturonic acid was only present in WSCP (Table 4.2 and Fig. 4.2). Although there was no clear separation of the two uronic acids by CTAB precipitation, the two fractions varied in composition, structure and rheological behaviour. The yield of WSCP was 52.0% and consisted mainly of pectic polysaccharides, while the yield of WSCS was 34.0% and composed predominantly of a 1,4-linked ß-D-glucose polymer.

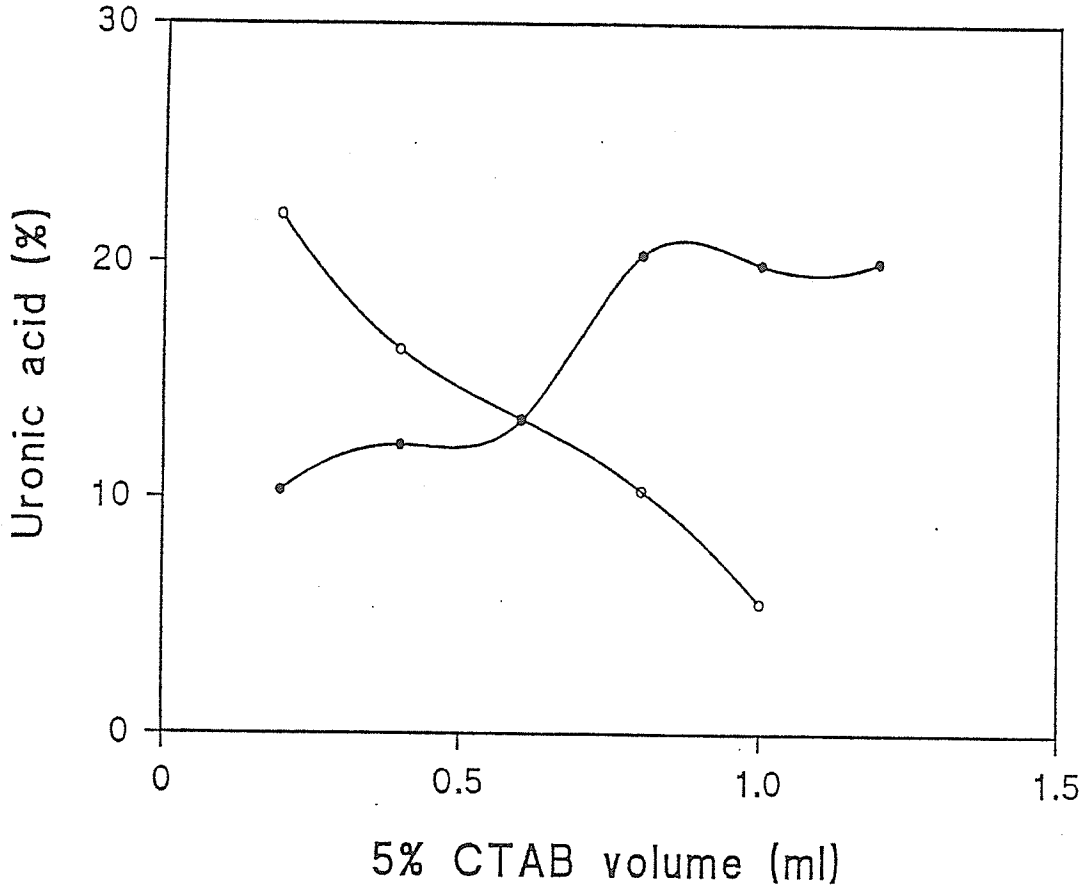


Fig. 4.1. Uronic acid content of the CTAB-precipitated (WSCP, ●–●) and the CTAB-soluble (WSCS, ○–○) fractions as a function of CTAB (5%) added.

There were no pectic type polysaccharides associated with the WSCS fraction. A possible explanation could be that the pectic polysaccharides were selectively precipitated by CTAB under optimum conditions. The presence of glucuronic acid in the WSCP fraction may be due to its association to the pectic polysaccharides or coprecipitation of glucuronic acid containing polymers with the pectic polysaccharides. The separation of WS into CTAB-precipitated and CTAB-soluble sub-fractions allowed an investigation of the influence of the chemical composition and linkage patterns on the physical properties of these materials.

Table 4.1 shows that both WSCP and WSCS were mainly composed of glucose (22.9-24.2% in WSCP, 24.7-26.2% in WSCS), galactose (21.4-22.1% in WSCP and 20.3-21.0% in WSCS), mannose (6.3% in WSCP and 11% in WSCS), rhamnose (12.6% in WSCP and 3.5% in WSCS), arabinose (6.1% in WSCP and 8.9% in WSCS) and xylose (3.8% in WSCP and 3.3% in WSCS). Prolonged hydrolysis significantly increased the rhamnose content in the WSCP fraction indicating that rhamnose was closely associated with the uronic acids. This was further supported by the methylation analysis shown in Table 4.2. All other sugar contents decreased slightly on prolonged hydrolysis which can be attributed to the decomposition of the released sugars not closely associated to the acidic monosaccharide residues. The pre-treatment of the samples with 72% H_2SO_4 was found essential for the complete release of glucose which would otherwise only yield 2 to 3%.

Table 4.1. Composition of the water-soluble CTAB-precipitated (WSCP) and CTAB-soluble (WSCS) fractions of yellow mustard mucilage

	WSCP		WSCS	
Yield (%)	52.0		34.0	
Uronic Acid (%)[a]	22.72±2.04		12.50±0.56	
Protein (%)[a]	1.84±0.06		3.28±0.10	
Monosaccharide[a]	2 h[b]	6 h	2 h	6 h
Rhamnose (%)	4.75±0.40	12.67±0.34	2.58±0.16	3.55±0.04
Arabinose (%)	6.12±0.42	5.23±0.01	8.88±0.16	7.34±0.30
Xylose (%)	3.82±0.11	2.62±0.13	4.34±0.01	3.17±0.28
Mannose (%)	6.15±0.40	6.35±0.08	10.98±0.06	10.50±0.05
Galactose (%)	22.14±0.34	21.44±0.18	21.00±0.11	20.29±0.19
Glucose (%)	24.23±0.07	22.86±0.28	26.24±0.14	24.74±0.27

a: n=2, mean±SD.
b: hydrolysis time.

The ^{13}C NMR spectra (Fig.4.2) confirmed that WSCP contained two uronic acids (δ=174.95 ppm, galacturonic acid and δ =175.75 ppm, glucuronic acid) and rhamnose (δ=17.26 ppm) while WSCS only contained glucuronic acid (δ=175.74 ppm) with the absence of galacturonic acid and rhamnose signals. The anomeric regions (δ=90-109 ppm) of the ^{13}C NMR spectra of WSCP and WSCS were significantly different from each other although WSCP and WSCS both had 102.7 and 103.5 ppm peaks as the most abundant components. Chemical shifts between δ=108 to 109.5 ppm can be attributed to the α 1-C of arabinoses which are weak in WSCP but significantly stronger in WSCS. Moreover, the resonances at δ=103.5 ppm and 102.7 ppm could be attributed to 1- C of β galactose and 1-C of 1,4-linked β-D-glucose, respectively (Bock *et al.*, 1984, Doco *et al.*, 1990 and Goldberg *et al.*, 1991). Two peaks were absent for WSCP (δ = 104.66 and 100.18 ppm) compared to WSCS, although an additional peak was evident at δ = 97.845 ppm for WSCP.

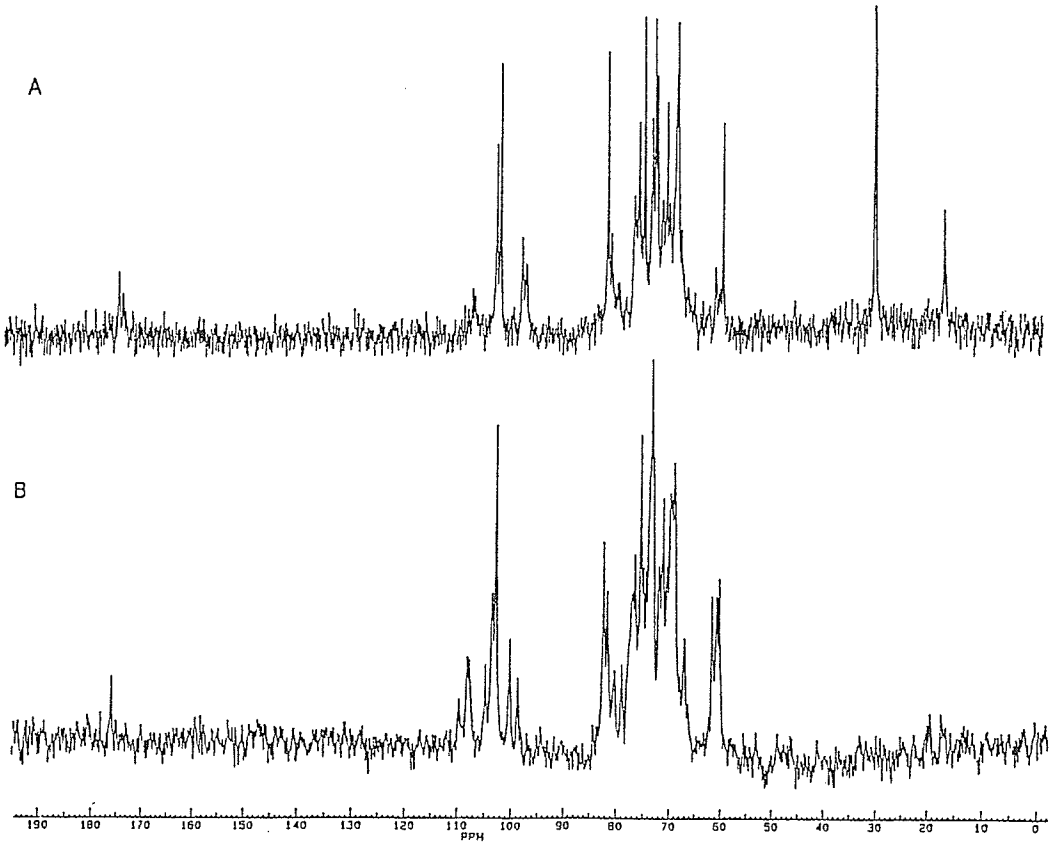


Fig. 4.2. Comparison of ^{13}C-NMR spectra of the CTAB-precipitated (WSCP, A) and the CTAB-soluble (WSCS, B) fractions of water-soluble yellow mustard mucilage. Acetone was used as an internal standard (δ= 30.511 ppm).

The relative intensities of glucuronic acid in both WSCP and WSCS were in agreement with the methylation results where glucuronic acid was higher in both WSCP (8.9%) and WSCS (9.3%) compared to galacturonic acid which was 6.5% in WSCP only (Table 4.2). There appeared to be some differences in resonances within the region δ = 50 to 90 ppm of WSCP and WSCS, although further fractionation and purification of the polymeric constituents of these fractions are necessary for a complete assignment of the ^{13}C NMR spectra.

The gel chromatographic profiles (Fig. 4.3) showed high molecular weight regions (at the void volume) for both WSCP and WSCS fractions which were composed mainly of neutral species. In addition to the high molecular weight neutral fraction, WSCP contained an acidic fraction with a peak elution volume smaller than that of Dextran T-500 as well as a well distributed acidic fraction with a much larger peak elution volume (between T-70 and T-500). WSCS exhibited a low molecular weight fraction (less than T-70) as the major component which contained high amounts of glucuronic acid (Fig. 4.3).

4.3.2 Methylation Analysis

Table 4.2 shows the relative retention time and molar proportion of partially permethylated acetyl alditols (PPAA) of WSCP and WSCS. Mass spectra obtained were compared to the literature (Carpita and Shea, 1990) and the corresponding diagnostic fragment ions for each derivative are also presented. Two uronic acids were determined following reduction of the carboxyl group with 1.0M lithium triethylborodeuteride in tetrahydrofuran (O'Neill *et al.*, 1990). A non-reducing end glucuronic acid was found having a relative retention time of Rf=2.35 on the SP-2330 capillary column (30m x 0.75mm). The diagnostic fragment ions for carboxyl reduced non-reducing end glucuronic acid were 118, 131, 162, 191 and 235

Table 4.2. Relative retention times and molar ratios of partially permethylated acetyl alditols of the water-soluble CTAB-precipitated (WSCP) and CTAB-soluble (WSCS) fractions of yellow mustard mucilage

	Rf[a]	Molar ratio (%)[b]				Diagnostic fragment ions
		WSCP	WSCPR[c]	WSCS	WSCSR[c]	
2,3,5-Me_3-Ara	0·66	3·1	1·1	3·7	2·6	117 118 161 162
2,3-Me_2-Ara	1·19	7·0	3·2	7·1	6·7	118 129 189
Total methyl ethers of arabinose		10·1	4·3	10·8	9·3	
2,3-Me_2-Xyl	1·32	5·7	3·1	5·3	3·1	118 129 189
Xyl $(acet)_5$	2·55	trace	8·0	trace	trace	115 145 188 218 290
Total methyl ethers of xylose		6·5	11·1	6·4	3·6	
2,3,4,6-Me_4-Glc	1·00	trace	trace	2·0	2·2	118 129 145 162
2,3,6-Me_3-Glc	1·82	17·1	10·5	32·4	22·7	118 162 233
2,3-Me_2-Glc	2·35	5·7	3·9	8·3	7·8	118 201 261
Total methyl ethers of glucose		23·9	14·4	42·7	32·7	
2,3,4-Me_3-Glc ($6D_2$)	1·74	n.d.	8·9	n.d.	9·3	118 131 162 191 235
2,3,4,6-Me_4-Gal	1·17	5·9	1·9	3·0	3·0	118 129 145 162
3,4,6-Me_3-Gal	1·71	2·6	trace	6·5	8·4	129 130 161 190
2,3,4-Me_3-Gal	2·03	26·5	22·1	14·6	16·8	118 129 189 233
2,4-Me_2-Gal	2·67	4·0	2·8	3·6	3·1	118 129 189 234
Total methyl ethers of galactose		39·0	26·8	29·3	31·3	
2,3-Me_2-Gal ($6D_2$)	2·62	n.d.	6·5	n.d.	trace	118 129 203 263
2,3,6-Me_3-Man	1·63	5·5	4·0	8·9	8·4	118 162 233
3,4-Me_2-Rham	0·94	4·9	3·5	trace	n.d.	131 190
3-Me-Rham	1·48	10·2	20·3	1·9	3·5	130 143 190 203
Total methyl ethers of rhamnose		15·1	23·8	1·9	3·5	

[a]Typical relative retention time of partially permethylated acetyl alditols on a SP-2330 glass capillary column (30 m × 0·75 mm), programmed from 160 to 210°C at 2°C per min; carrier gas: helium, 15 psi.
[b]Relative molar ratio, calculated from the ratio of peak areas.
[c]WSCPR and WSCSR are carboxyl-reduced WSCP and WSCS, respectively.
n.d. = Not determined.

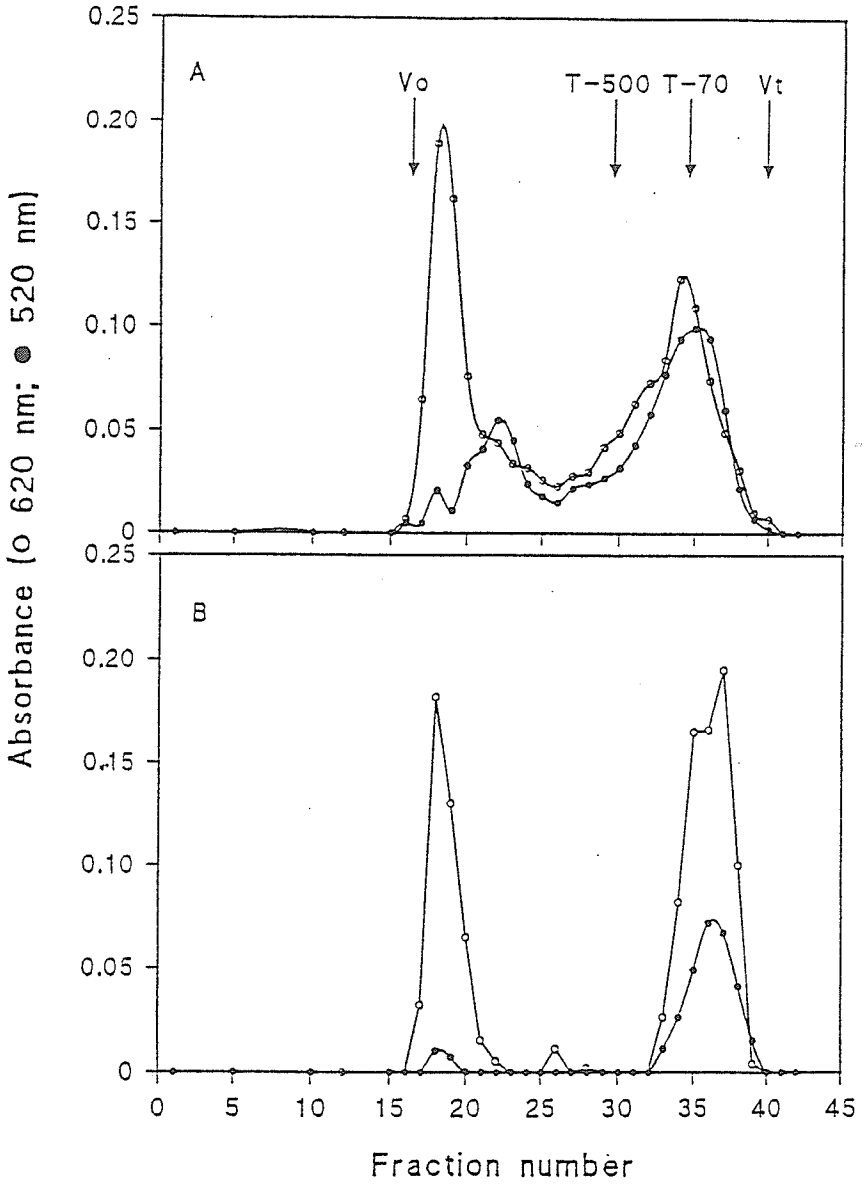


Fig. 4.3. Gel filtration chromatography of the CTAB-precipitated (WSCP, A) and the CTAB-soluble (WSCS, B) fractions of water-soluble yellow mustard mucilage on a Sephacryl HR S-500 column (1.6x70 cm) eluted with 0.1 N NaCl solution, flow rate 1 ml/min.temperature 23°C; arrows indicate peak elution volumes of dextran standards (Blue dextran T-200, Vo; T-500; T-70; Glucose, Vt) used as molecular weight markers ϕ—○, total carbohydrate; ●—● uronic acids).

(Table 4.2). The presence of 1,4-linked galacturonic acid was established by identification of the deuterated residue with a Rf=2.62 and diagnostic fragments 118, 129, 203 and 263.

Two major polysaccharides were apparent in the WSCP fraction, a pectic-like polysaccharide and a neutral polysaccharide. In the pectic polysaccharide of WSCP, 1,2,4-linked rhamnose and fully substituted xylose were closely associated with 1,4-linked galacturonic acid and possibly glucuronic acid. This conclusion was based on the significant increases of 3-Me-rham (10.2 to 20.3%) and xyl $(acetate)_5$ (trace to 8.0%) upon reduction of the carboxyl group of the uronic acids. Among the residues identified, a substantial amount of 1,4-linked β–D-glucose was found (17.1%, Table 4.2) which originated primarily from the high molecular size eluting species in Fig. 4.3A as shown from the monosaccharide analysis (Table 4.3).

Table 4.3. Monosaccharide (neutral) molar ratios of yellow mustard mucilage water-soluble CTAB-precipitated (WSCP) and CTAB-soluble (WSCS) fractions collected from gel filtration chromatography

Sugars[a]	WSCP-H[b]	WSCP-L[b]	WSCS-H[b]	WSCS-L[b]
Rhamnose	1.05	0.81	0.10	0.28
Arabinose	0.50	0.18	1.33	0.30
Xylose	0.00	0.07	0.22	0.10
Mannose	0.40	0.15	0.54	0.44
Galactose	1.00	1.00	1.00	1.00
Glucose	3.42	0.27	1.51	0.42

[a]: Sugar molar ratios were calculated from peak height.
[b]: Collected fractions from gel filtration chromatography (Fig. 4.3): H, higher molecular size peak; L, lower molecular size peak.

In contrast to WSCP, WSCS appeared to consist of polysaccharides containing non-reducing end glucuronic acid with trace amount of pectic polysaccharides. In addition, 1,4-linked β-D-glucose (22.7%) was found as the major component of WSCS, particularly

in the high molecular size material eluting in the void volume of Sephacryl S-500 column (Fig. 4.3B) as indicated by monosaccharide analysis (Table 4.3). Other sugars present in WSCS fraction were 1,6-linked galactose (16.8%), 1,4-linked mannose (8.4%) and non-reducing end glucuronic acid (9.3%). It appeared that some of the 1,2 and 1,6-linked galactose were connected with the non-reducing end glucuronic acid, as shown by their slight increase upon reduction of the carboxyl group of glucuronic acid after methylation (Table 4.2).

Although WSCP and WSCS are still heterogeneous mixtures of polysaccharides and their structural information is limited, there seem to be significant differences in chemical composition, molecular size distribution as well as linkage pattern among these fractions.

4.3.3 Rheological Properties

4.3.3.1 Steady shear rheological test

The shear rate dependent flow behaviour of WS, WSCP and WSCS is shown in Fig. 4.4. No Newtonian plateau is evident at concentrations between 0.5% to 2.0% for all samples over the shear rate range investigated (0.1 to 1162 s^{-1}). Newtonian (upper) plateaus were observed initially at 0.3% concentration sothat both the zero-shear-rate viscosity and the shear rate value ($\dot{\gamma}$) at which the onset shear-thinning behaviour occurs could be evaluated as shown in Table 4.4. The zero-shear-rate viscosity was found highest for WS, followed by WSCP and WSCS, while the $\dot{\gamma}$ value was highest for WSCS, lowest for WSCP and in between for WS. The lowest $\dot{\gamma}$ value for WSCP suggested that WSCP solutions were the most elastic, while WSCS the least. Both WSCP and WSCS appeared to contribute to the viscoelastic character of WS solutions.

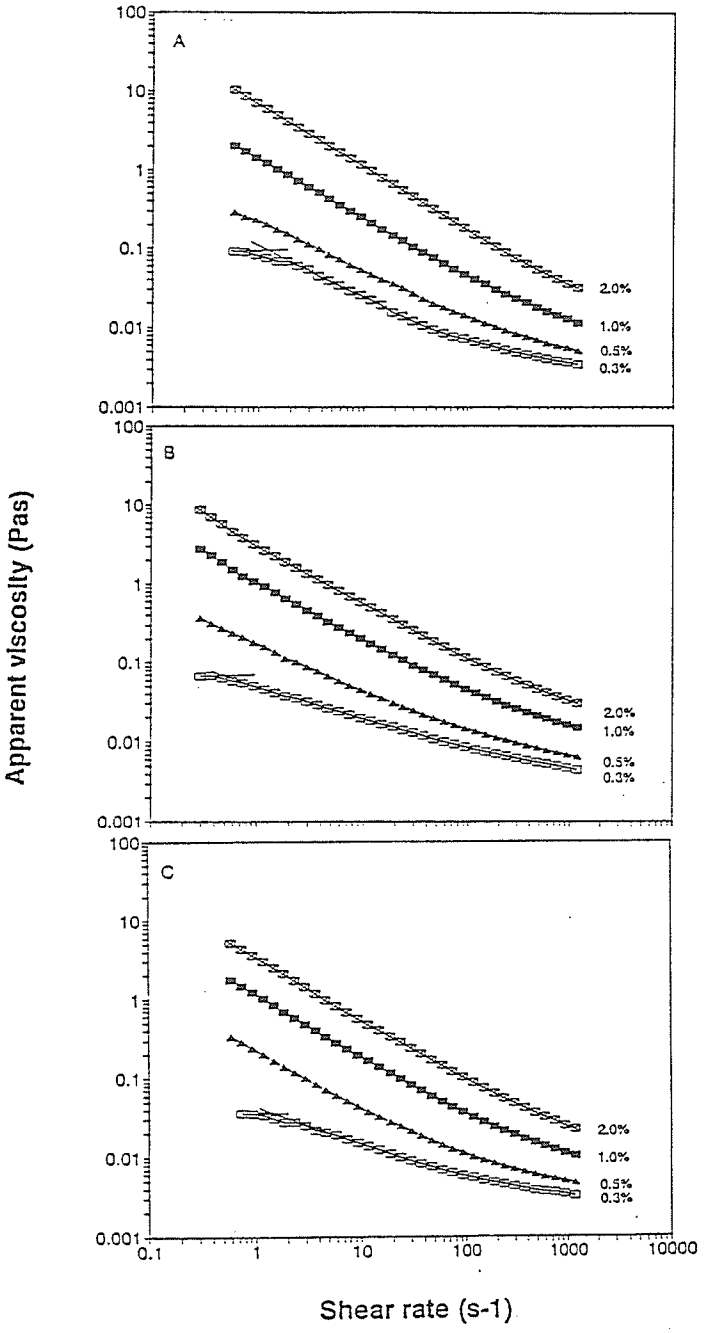


Fig. 4.4. Steady shear flow curves of the yellow mustard mucilage water-soluble fraction (WS, A) and its sub-fractions: the CTAB-precipitated fraction (WSCP, B) and the CTAB-soluble fraction (WSCS, C) at concentrations between 0.3 and 2.0%, 22.0±0.1°C.

Table 4.4. Comparison of zero-shear-rate viscosity (η_o) and shear-rate ($\dot{\gamma}$) value at which onset shear thinning occurred for the water-solubie fraction (WS) and its sub-fractions: CTAB-precipitated fraction (WSCP) and CTAB-soluble fraction (WSCS) at 0.3% (22°C)

	η_o (mPa s)	$\dot{\gamma}$ value (s^{-1})
WS	94.4	1.16
WSCP	68.2	0.46
WSCS	36.4	1.47

By applying the power law model (Witcomb *et al.*, 1980), the consistency index (K) and flow index (n), defined from the equation $\eta = K\dot{\gamma}^{1-1}$, were obtained (Table 4.5). As concentration increases, the K increases while n decreases. The increases in K with increasing concentration suggest that a more viscous system is obtained at higher concentrations. On the other hand, the decrease in n with increasing concentration implies a more pronounced shear thinning of the system. The WS exhibited the highest K value and the lowest n value, as compared to those of WSCP and WSCS, suggesting the presence of polysaccharide-polysaccharide interactions between some of the components present in WSCP and WSCS. This phenomenon is particularly significant at 2.0% polymer solutions.

Table 4.5. n and *K* values[a] of the water-soluble fraction (WS) and its sub-fractions: CTAB-precipitated fraction (WSCP) and CTAB-soluble fraction (WSCS) at different concentrations (22°C)

Concentration (%)	WSCP		WSCS		WS	
	n	*K*(Pa s)	n	*K*(Pa s)	n	*K*(Pa s)
0.3	0.668	0.041	0.641	0.033	0.537	0.070
0.5	0.557	0.119	0.473	0.138	0.468	0.173
1.0	0.407	0.740	0.331	0.845	0.321	1.120
2.0	0.340	2.506	0.285	2.764	0.224	6.440

[a]: Parameters n and *K* were calculated using the power-law model: $\eta = k\dot{\gamma}^{n-1}$. (Witcomb *et al.*, 1980).

4.3.3.2 Dynamic oscillatory shear experiments

The viscoelastic spectra of WS, WSCP and WSCS at 2.0% (w/w) are presented in Fig. 4.5. The viscoelastic behaviour of yellow mustard mucilage and its fractions are typical of a "weak gel" with G' > G" over the frequency range investigated (Cui *et al.*, 1993a). A typical liquid system has $G' \alpha \omega^2$ and $G'' \alpha \omega^1$, while for a "weak gel" system, both G' and G" are only slightly dependent on frequency (Navarini *et al.*, 1992). Both WSCP and WSCS exhibited similar dynamic rheological patterns to that of WS while the maximum frequency dependence was approximately $G' \alpha \omega^{0.15}$ and $G'' \alpha \omega^{0.25}$ for WSCP, $G' \omega^{0.23}$ and $G'' \alpha \omega^{0.22}$ for WSCS as compared to $G' \alpha \omega^{0.16}$ and $G'' \alpha \omega^{0.21}$ for WS.

Comparison of storage and loss moduli, phase angle (δ) and dynamic viscosity (η') vs frequency (f) of WSCP and WSCS to those of WS are illustrated in Fig.4.6. WSCP was found to be more similar to WS than WSCS in terms of storage and loss moduli, phase angle (δ) and dynamic viscosity (η'), although phase angle deviation was observed for WSCP compared to that of WS at the higher frequency range (Fig. 4.6C). The storage modulus and dynamic viscosity (η') of WSCP are superimposible with that of WS while the G' and η' of WSCS were lower (Fig.4.6A and 4.6D). In Fig.4.6B, the loss modulus G" of WSCP generally resembled that of WS although G" of WSCP was slightly lower at low frequencies and slightly higher at high frequencies than that of WS with a cross point at 3 Hz. Similar to G', the G" of WSCS was also lower than that of WS as shown in Fig.4. 6B. The phase angle (δ) describes the extent of departure of a viscoelastic system from an ideal elastic system. Fig. 4.6C illustrates the changes of δ as a function of frequency in which WSCP exhibited the lowest phase angle values at low frequencies and the highest phase angle values at higher frequencies. In contrast to WSCP, the phase angle

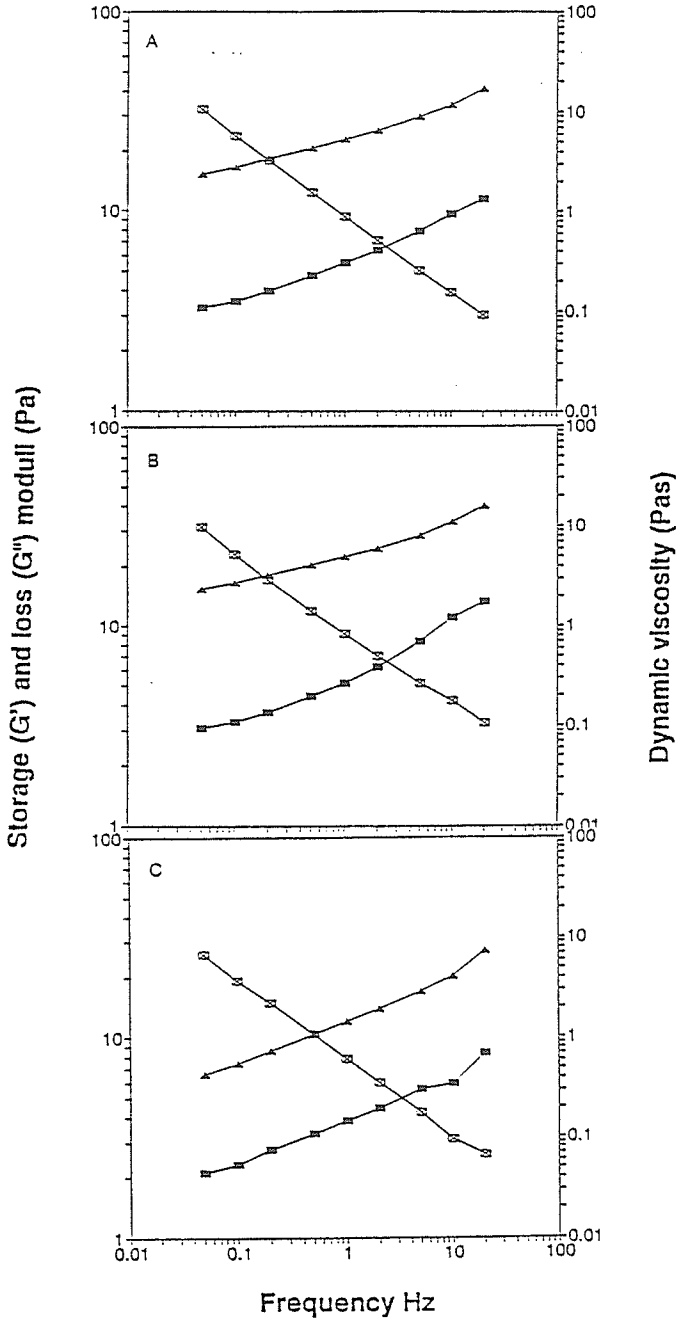


Fig. 4.5. Frequency dependence of storage (G', ▲) and loss (G", ■) moduli, and dynamic viscosity (η', ⊟) of yellow mustard mucilage water-soluble fraction (WS, A) and its sub-fractions: the CTAB-precipitated fraction (WSCP, B) and the CTAB-soluble fraction (WSCS, C) at concentration 2.0%, 22.0±0.1°C.

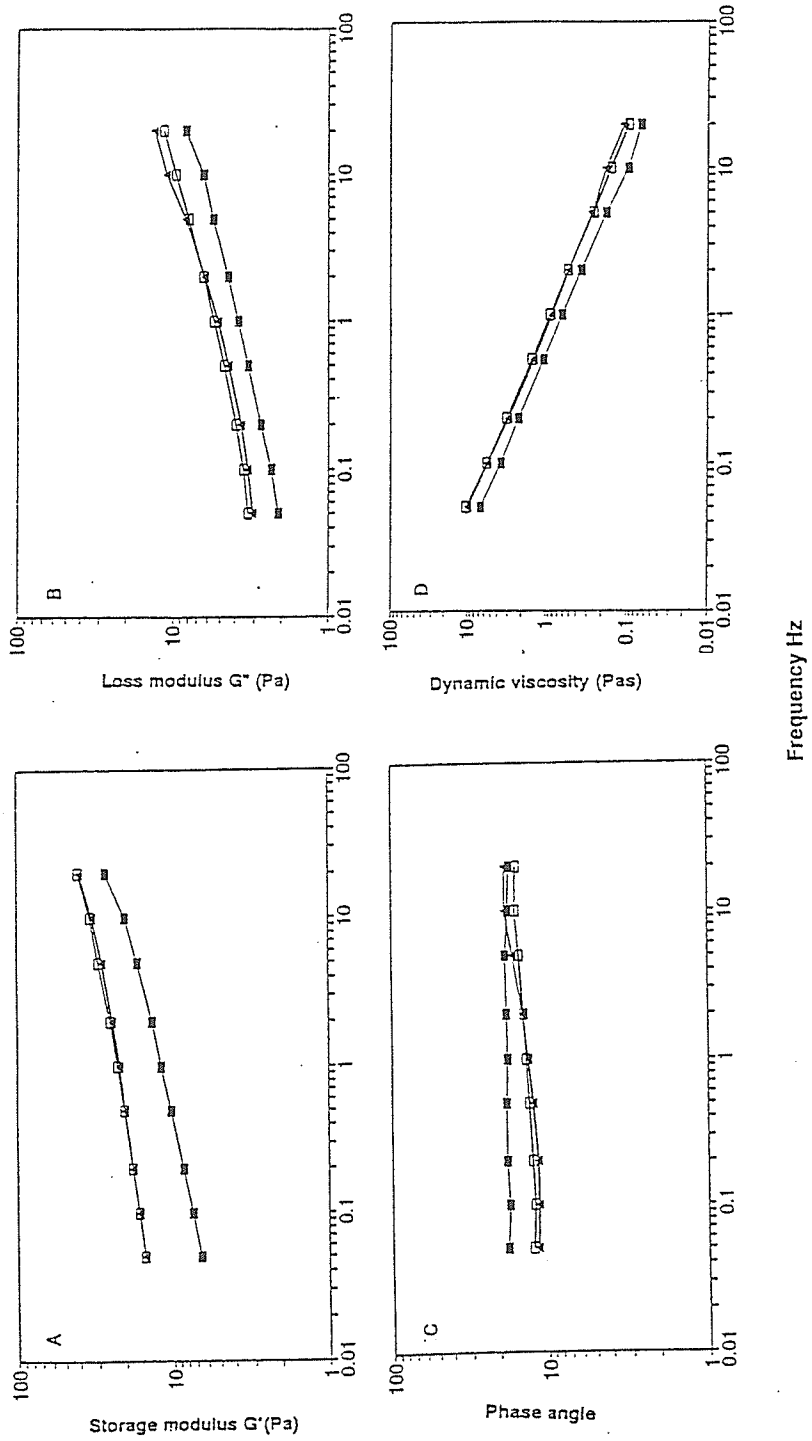


Fig. 4.6. Comparison of storage modulus (A), loss modulus (B), phase angle (C) and dynamic viscosity (D) as a funcion of frequency for the water-soluble fraction (WS, ⇆) and its sub-fractions: CTAB-precipitated fraction (WSCP, ▲) and CTAB-soluble fraction (WSCS, -■-) at concentration 2.0%, 22.0±0.1°C.

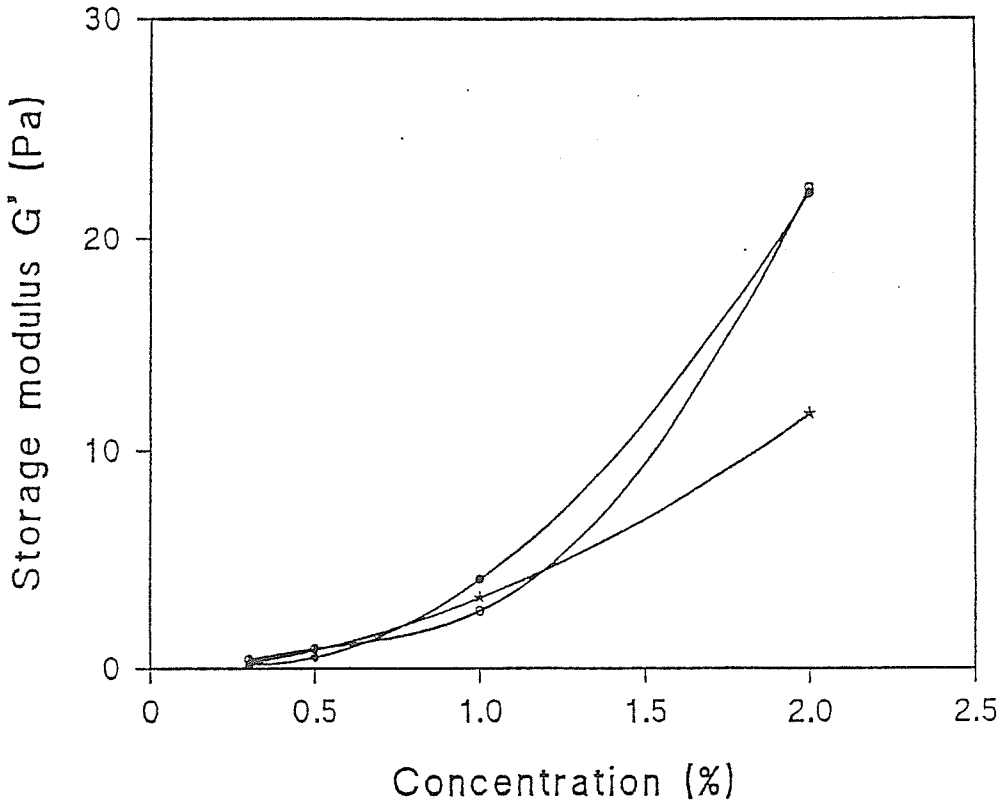


Fig. 4.7. Increase of storage modulus as a funcion of concentration of the water-soluble (WS, ○-○) fraction of yellow mustard mucilage and its sub-fractions: CTAB-precipitated fraction (WSCP, ●-●) and CTAB-soluble fraction (WSCS, ★-★).

values for WSCS remained constant over the frequency range investigated. Fig. 4.7 shows the changes of G' as a function of polymer concentration. Compared to WSCP, whose G' coincides with that of WS, the G' of WSCS appeared similar to that of WS only at low concentrations, but was substantially lower than WS at higher concentrations (e.g. 2.0%). The non-linear increase of G' of WSCP and WS may indicate the development of a more ordered network structure in WS and WSCP solutions at higher concentrations.

The relationship of apparent viscosity (η) and complex viscosity (η^*, defined as $\eta^*=[G'^2+G''^2]^{0.5}/\omega$), can be used diagnostically to distinguish normal polysaccharide solutions from "weak gels". For a "weak gel" system, the complex viscosity $\eta^*(\omega)$ is substantially higher than the apparent viscosity $\eta(\dot{\gamma})$ at equivalent values of frequency and shear rate. In contrast, the two viscosities coincide in a normal polysaccharide solution; this is known as the Cox-Merz rule (Navarini *et al.*, 1992; Morris, 1990 and Cox and Merz, 1958). The apparent viscosity $\eta(\dot{\gamma})$ and complex viscosity $\eta^*(\omega)$ of mustard mucilage samples are presented in Fig. 4.8. The complex viscosity $\eta^*(\omega)$ of WS is substantially higher than the apparent viscosity $\eta(\dot{\gamma})$ at equivalent values of frequency and shear rate thus exhibiting the typical behaviour of a "weak gel" (Fig.4.8A). The extent of $\eta^*(\omega)$ over $\eta(\dot{\gamma})$ for WSCP is greater than that for WS, indicating a more ordered "weak gel" structure or more elastic character for the WSCP (Fig. 4.8B). This is in agreement with the results of Fig. 4.6C as WSCP exhibited the smallest phase angle. The Cox-Merz rule was also tested for the WSCS over the whole range of frequencies and shear rates investigated; again, $\eta^*(\omega)$ and $\eta(\dot{\gamma})$ were not superimposible as shown in Fig. 4.8C. The degree of departure of $\eta^*(\omega)$ from $\eta(\dot{\gamma})$, however, was much smaller for WSCS compared to WSCP. Such a departure from the Cox-Merz superimposability has been attributed to the making and breaking of non-covalent (hydrogen) bonds (Morris, 1981). The rheological properties discussed above

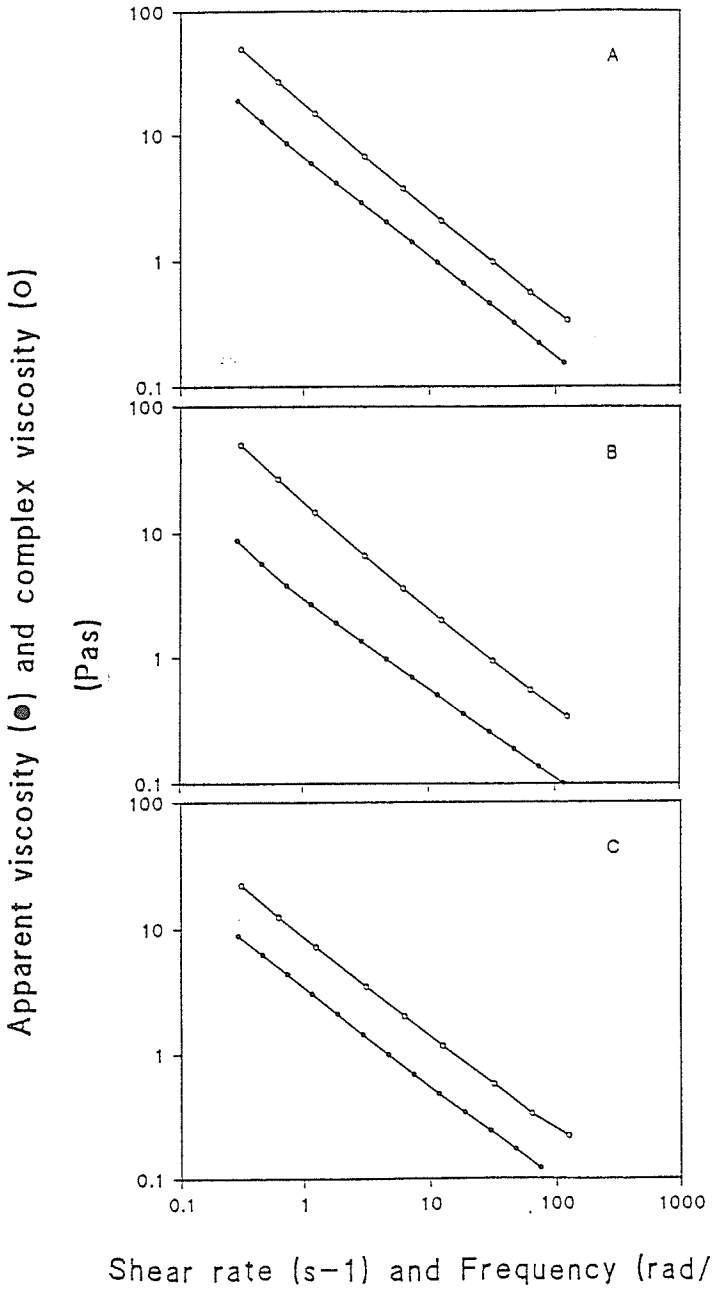


Fig. 4.8. Cox-Merz plot for 2% solutions or dispersions of the water-soluble (WS, A) fraction of yellow mustard mucilage and its sub-fractions: CTAB-precipitated fraction (WSCP, B) and CTAB-soluble fraction (WSCS, C) at 22.0°C.

suggested that both WSCP and WSCS contribute to the rheological properties of WS. Being the major fraction, WSCP contributes to both the shear thinning and the "weak gel" properties of WS, while WSCS contributes more to the viscous properties of WS solutions.

The effect of temperature, pH and co-solutes on "apparent viscosity" is shown in Fig. 4.9. The effect of temperature on viscosity of WSCP and WSCS obeyed the expected trend of decreasing viscosity with increasing temperature (Fig. 4.9A). The influence of pH on viscosity of WSCP and WSCS is in agreement with previous reports (Cui *et al.*, 1993a; Weber *et al.*, 1974); the lowest viscosity was observed between pH 3 to 7 and the highest viscosity at the low pH region (Fig. 4.9B). The substantial increase in viscosity in the low pH region could be attributed to the reduction of repulsion forces between polymer chains which allows interchain associations between polymer molecules thereby increasing viscosity. Addition of sucrose resulted in enhanced viscosity for both WSCP and WSCS as shown in Fig. 4.9C. It appeared the initial addition of sucrose brought about a faster increase in viscosity for WSCP with a possible turning point at ~0.4M sugar concentration; above this concentration, the rate of increase in viscosity became smaller. In contrast to WSCP, sucrose caused an almost linear increase in viscosity for WSCS. The effect of sucrose on the apparent viscosity of polysaccharide solutions can be attributed to a concentrating effect as well as improved polymer-solvent interactions (Dea *et al.*, 1977). The responses on apparent viscosity of WSCP and WSCS to the addition of salt are different as shown in Fig. 4.9D. The initial addition of NaCl resulted in a reduction in viscosity for the WSCP solutions up to 0.25 M salt concentration. Further addition of NaCl resulted in a rapid recovery of viscosity with the increase of viscosity being more pronounced at a higher concentration range (~1.0M). The initial reduction in viscosity on addition of NaCl is attributed to the progressive suppression of intramolecular charge-charge repulsion and

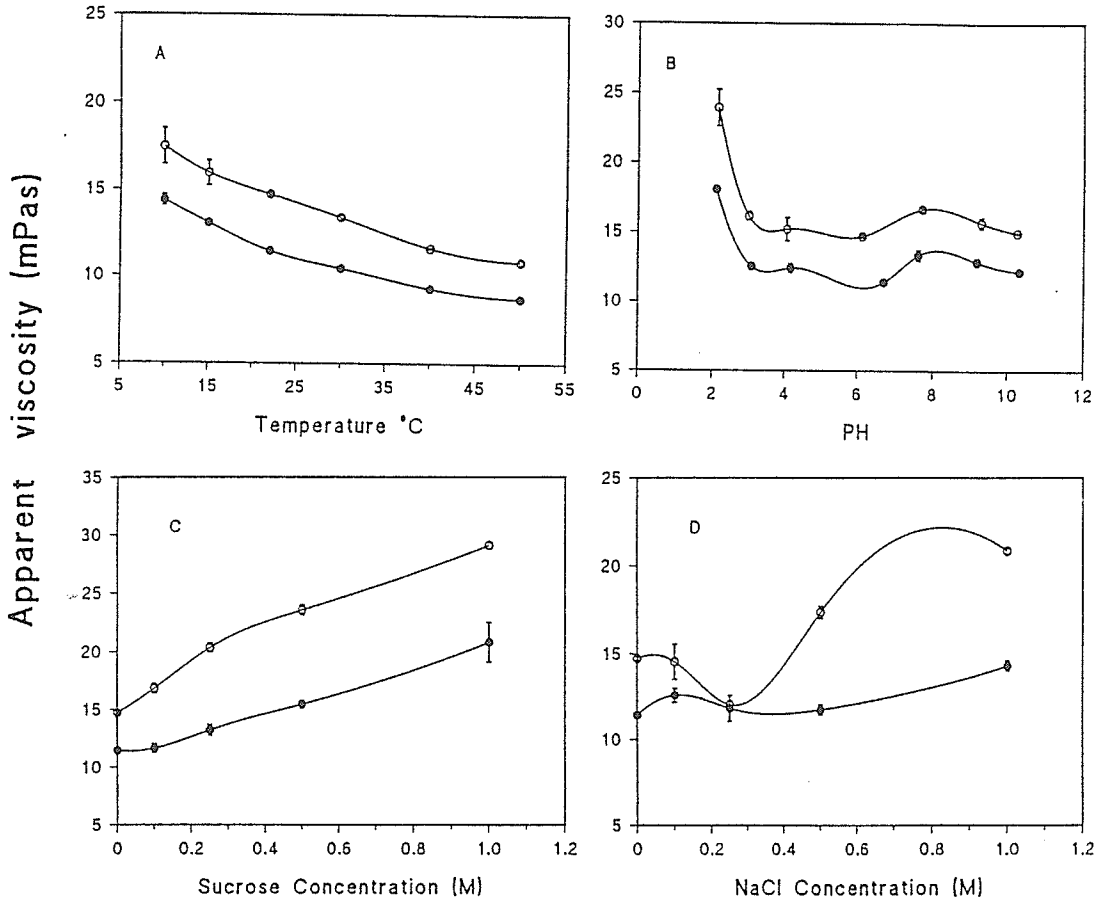


Fig. 4.9. Effect of temperature (A), pH (B), sucrose (C) and salt (D) concentrations on the apparent viscosity of water-soluble yellow mustard mucilage sub-fractions: CTAB-precipitated fraction (WSCP, ○–○) and CTAB-soluble fraction (WSCS, ●–●) at 0.5% (w/w) polymer concentration, 22.0°C, shear rate 92.32 s^{-1}.

consequent contraction of the polysaccharide molecules (Morris, 1990). As the salt conncentration increased beyond a certain point, intermolecular charge-charge repulsions are suppressed and intermolecular associations could occur which result in the recovery of viscosity. Further increase in viscosity at higher salt concentrations could be also due to a concentrating effect in addition to charge suppression. In contrast to WSCP, WSCS exhibited fairly stable viscosity upon addition of salt. The initial addition of salt even increased the viscosity slightly. This is possibly due to the non-reducing end glucuronic acid which is attached as side residue on the polysaccharide chain. The initial addition of NaCl would suppress the charge-induced intermolecular repulsions, and thereby enhance the intermolecular interactions between the polymer chains. Nevertheless, the overall response of WSCS solutions to added NaCl is minor compared to the WSCP and this may reflect the relatively fewer acidic groups present in WSCS.

CHAPTER 5

FRACTIONATION, STRUCTURAL ANALYSIS AND RHEOLOGICAL PROPERTIES OF WATER-SOLUBLE YELLOW MUSTARD (*Sinapis alba* L.) POLYSACCHARIDES

5.1 INTRODUCTION

Interesting rheological and interfacial properties of yellow mustard (*Sinapis alba* L.) mucilage and its fractions were previously reported (Chapter 3). Under optimized conditions, the water-soluble polysaccharides, responsible for most of the rheological properties of yellow mustard mucilage, were separated into a CTAB-precipitated fraction (WSCP) and a CTAB-soluble fraction (WSCS) (Chapter 4). The WSCP fraction was found to be the major component (52.0%) exhibiting more pronouced "weak gel" characteristics compared to WSCS, the minor fraction (34.0%) (Cui *et al.*, 1993b). The primary structures of WSCP and WSCS were examined by ^{13}C NMR spectroscopy and methylation analysis. The WSCP was composed of two major polysaccharides: an acidic polysaccharide containing 1,4-linked galacturonic acid, 1,2 and 1,2,4-linked rhamnose and 1,2 and/or 1,6 linked β-galactose; and a neutral polysaccharide with a predominant 1,4-linked β-D-glucose

backbone. WSCS also consisted of a similar 1,4-linked β-D-glucose based neutral polysaccharide but lacked pectic material (Cui *et al.*, 1993b). Both WSCP and WSCS were heterogeneous mixtures of polysaccharides so that their structures and the influence of structure on physical properties remain unclear. This paper reports on further fractionation and purification of the WSCP and WSCS polysaccharides by DEAE-high capacity cellulose ion exchange chromatography, their rheological properties and structural characterization of the purified sub-fractions by methylation analysis and ^{13}C NMR spectroscopy.

5.2 MATERIALS AND METHODS

5.2.1 Fractionation

Yellow mustard mucilage water-soluble CTAB-precipitated (WSCP) and CTAB-soluble (WSCS) fractions were separated as described in Chapter 4. All chemicals were of reagent grade unless otherwise specified.

5.2.2 Ion Exchange Chromatography

Ion exchange chromatography was carried out on a DEAE-high capacity ion exchange cellulose (Pierce Chemical Co., Rockford, Illinois, U.S.A.) column (2.6x50 cm). The resin was pre-equilibrated with 25 mM NaAc buffer (pH 5.0) prior to use (Kato *et al.*, 1991). A sample (240 mg) was dissolved in 500 ml distilled water, then adjusted to the same buffer concentration (25 mM NaAc buffer, pH 5.0) and loaded onto the column. The column was first eluted with buffer (25 mM NaAc, pH 5.0), followed by a stepwise increase of ionic strength: 0.2 M, 0.5 M and 1.0 M NaCl for WSCP and 0.1 M, 0.2 M and 1.0 M NaCl for WSCS, in the same buffer, respectively. Finally, the column was eluted

with 6.0 M urea. Carbohydrates and uronic acids were monitored using the methods described by Dubois *et al.* (1956) and Blumenkrantz and Asboe-Hansen (1973), respectively.

5.2.3 Rheological Measurements

Rheological properties were determined on a Bohlin VOR Rheometer (Bohlin Reologi, Sweden). A concentric cylinder geometry, with a cylinder height of 30 mm and radii of the inner and outer cylinders of 14 and 15.4 mm respectively, was used for the rheological measurements. In steady shear tests, samples at 0.5% (w/w) concentration were subjected to shear sweeps between 0.01 and 1164 sec^{-1}.

5.2.4 Gel Filtration Chromatography

Gel filtration chromatography was conducted on a Sephacryl S-500 (HR, 2.6x98 cm) column which eluted with 0.1 M NaCl solution (60ml/h, 25°C). Samples dissolved in the same buffer (1mg/ml) were applied onto the column, and 5 ml fractions collected. D-Glucose was used to determine the total volume, while linear dextrans T-500 (MW 460,000) and T-70 (MW 69,000) (Pharmacia Ltd, Montreal, PQ) were used as relative molecular weight markers. Carbohydrate and uronic acids were also monitored (Dubois *et al.*, 1956; Blumenkrantz and Asboe-Hansen, 1973).

5.2.5 Monosaccharide Analysis and ^{13}C NMR Spectroscopy

Neutral monosaccharides in the hydrolyzates were determined following the procedure described by Englyst *et al.* (1982) on a SP-2330 glass capillary column, 30 m X 0.75 mm ID. All samples were treated with 72% H_2SO_4 at 35°C for 30 min prior to the hydrolysis with 2M H_2SO_4 at 100°C. The ^{13}C NMR spectra of the polysaccharide fractions were

recorded on a Bruker AMX 500 spectrometer at 65°C, approximately 5% (w/w) solutions in D_2O using 5 mm NMR test tubes.

5.2.6 Methylation Analysis

Methylation analysis was carried out as described by Ciucanu and Kerek (1984). The reduction of carboxyl group after methylation was performed according to O'Neill *et al.* (1990). Qualitative and quantitative measurements of partially permethylated acetyl alditols were performed as described previously in Chapter 4.

5.3 RESULTS AND DISCUSSION

5.3.1 Fractionation and Ion Exchange Chromatography

Fig. 5.1 shows the general fractionation scheme used to separate yellow mustard mucilage into its sub-fractions. Extracted yellow mustard mucilage was dialysed and fractionated by centrifugation into a water-soluble fraction and a water-insoluble fraction as described earlier (Chapter 3). The water-soluble fraction, the major component of yellow mustard mucilage exhibiting strong shear thinning behaviour in solution, was further separated into a CTAB-precipitated fraction (WSCP) and a CTAB-soluble fraction (WSCS) by complexation of CTAB (hexadecyltrimethylammonium bromide) with the acidic polysaccharides (Cui *et al.*, 1993b). The WSCP and WSCS fractions were further fractionated by ion-exchange chromatography on a DEAE-high capacity cellulose column into five sub-fractions respectively (Fig. 5.1). The ion exchange chromatography profiles are presented in Fig. 5.2 and Fig. 5.3, respectively. The DEAE-cellulose column was initially equilibrated with sodium acetate buffer (25mM, pH 5.0) prior to use. Samples were first dissolved in

water, then adjusted to the appropriate ionic strength with sodium acetate buffer before being introduced onto the column. The column was eluted with the same buffer until eluted carbohydrates were no longer detected by the phenol-sulfuric acid method (Dubois *et al.*, 1956). Subsequently a stepwise increase in ionic strength (0.2, 0.5 and 1.0M NaCl, Fig. 5.2) of the elution buffer (pH 5.0) was applied for WSCP based on the results of preliminary experiments. Five sub-fractions were thus obtained for WSCP including WSCP-I (buffer elution), WSCP-II (0.2 M NaCl buffer elution), WSCP-III (0.5 M NaCl buffer elution), WSCP-IV (1.0 M NaCl buffer elution) and WSCP-V (6.0 M Urea elution) (Fig. 5.1). In a similar manner, WSCS was also separated into five fractions with a stepwise increase of the ionic strength (0.1, 0.2 and 1.0M NaCl) using the same buffer system (Fig. 5.3). As a result, five fractions were obtained: WSCS-I (buffer elution), WSCS-II (0.1M NaCl buffer elution), WSCS-III (0.2M NaCl buffer elution), WSCS-IV (1.0M NaCl buffer elution) and WSCS-V (6.0 M urea elution) as shown in Fig. 5.1. Each fraction collected was concentrated by rotary evaporation at 40°C, dialysed and freeze-dried. The approximate recovery of each fraction is shown in Table 5.1.

Table 5.1. Recovery (%) of fractions from the DEAE-high capacity cellulose ion exchange colum

WSCP Fraction	Recovery (%)	WSCS Fraction	Recovery (%)
WSCP-I	15.2	WSCS-I	9.2
WSCP-II	22.4	WSCS-II	28.3
WSCP-III	14.5	WSCS-III	12.2
WSCP-IV	6.1	WSCS-IV	22.9
WSCP-V	44.2	WSCS-V	31.6

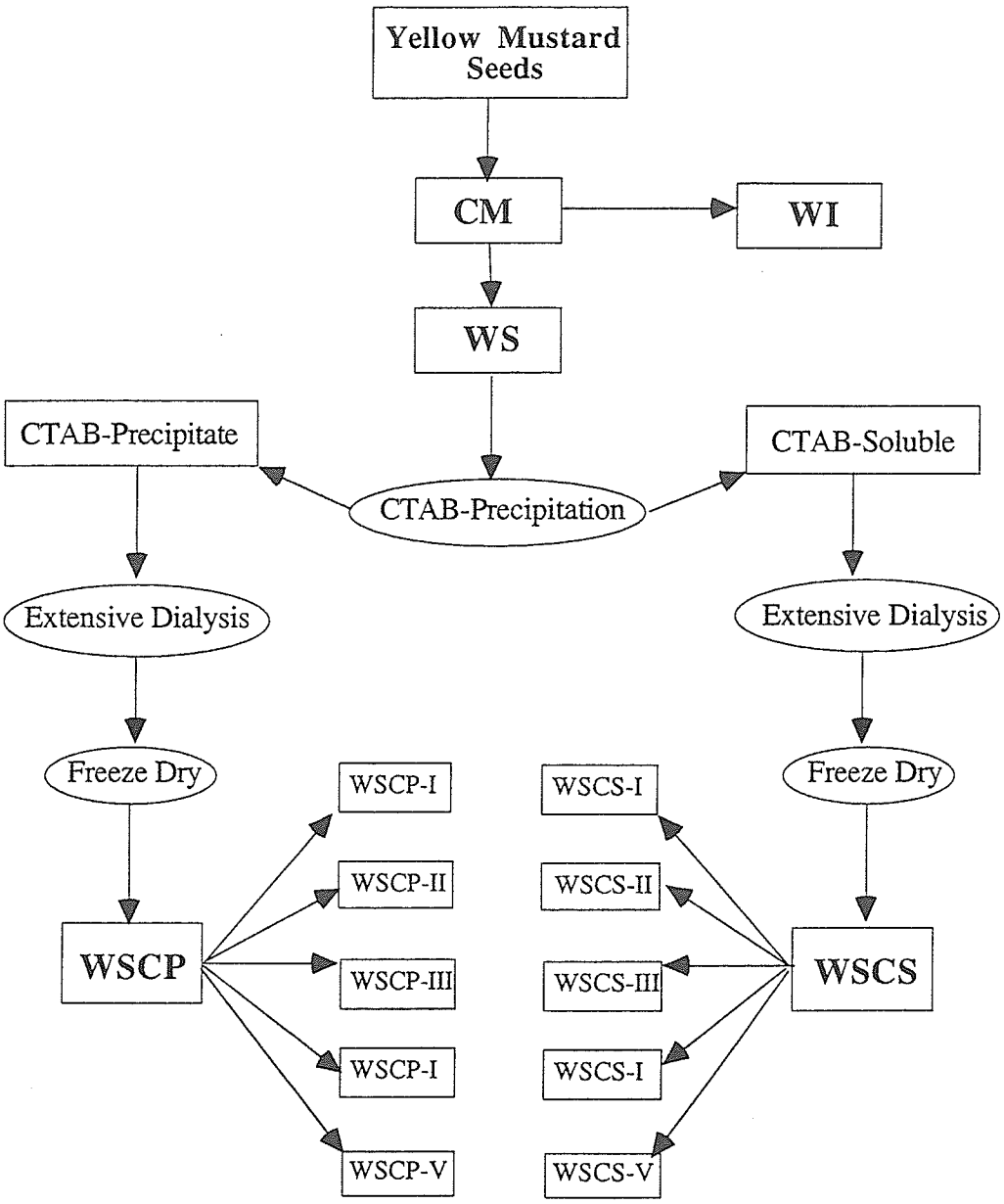


Fig. 5.1 Flow chart of isolation of yellow mustard mucilage and its fractions.

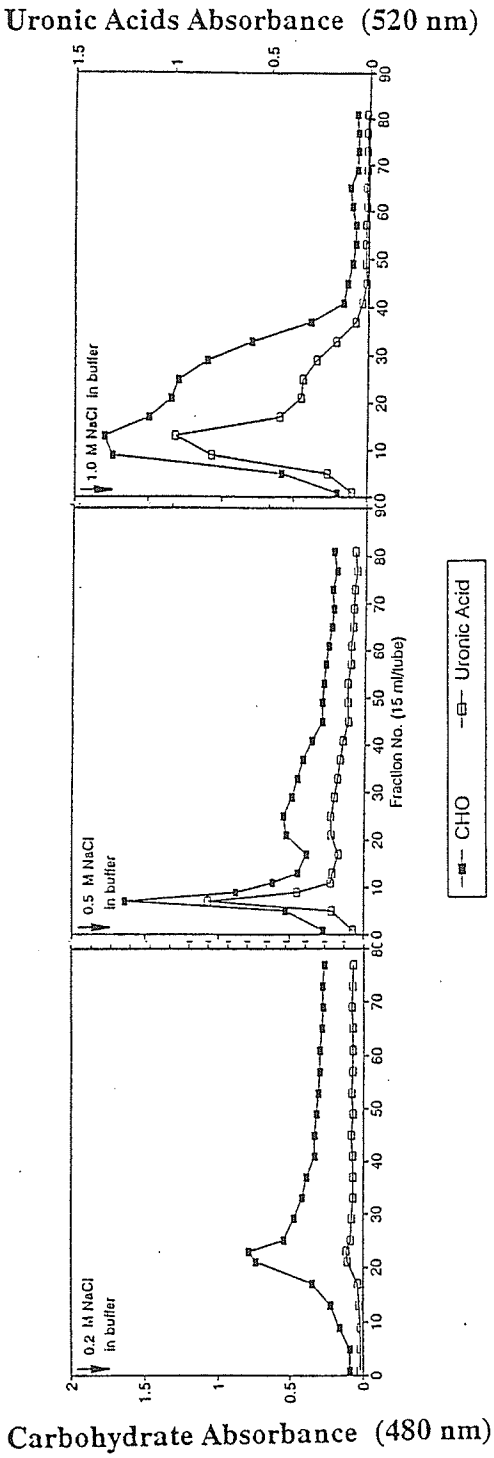


Fig. 5.2. Ion exchange chromatographic profiles of WSCP fractions on high-capacity DEAE-Cellulose column (2.6x50cm) after the neutral fraction was eluted with buffer.

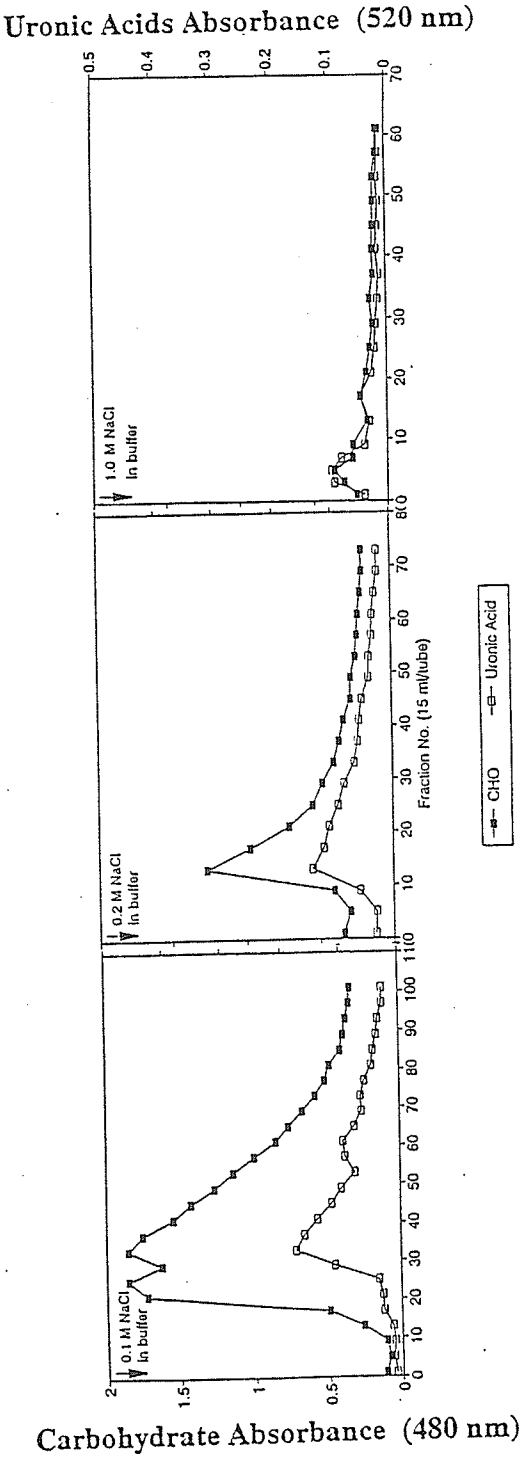


Fig. 5.3. Ion exchange chromatographic profiles of WSCS fractions on high-capacity DEAE-Cellulose column (2.6x50cm) after the neutral fraction was eluted with buffer.

5.3.2 Rheological Properties

The results of steady shear rheological tests for the WSCP and WSCS sub-fractions are presented in Fig. 5.4 and 5.5, respectively. WSCP-I and WSCP-III exhibited typical shear thinning behaviour where the apparent viscosity decreased as the shear rate increased (Morris *et al.*, 1981). The shear thinning ability was greater for WSCP-I compared to WSCP-III by exhibiting higher viscosity at low shear rates and lower viscosity values at higher shear rates. The WSCP-III exhibited a slightly more shear thinning behaviour at lower shear rates while the reverse was observed at higher shear rates, as shown in Fig.5.4. This may indicate that the flow curve of WSCP-III solutions under the test conditions was close to the second Newtonian-like plateau at the high shear rate region (Morris *et al.*, 1981). The strong shear thinning behaviour of WSCP-I may be partly related to its solubility as it was not possible to redissolve it completely in aqueous solutions to obtain a ^{13}C NMR spectrum or a gel filtration chromatographic profile for this fraction. The rest of the WSCP series (WSCP-II, WSCP-IV and WSCP-V) exhibited typical Newtonian behaviour in aqueous solutions under the test conditions where the apparent viscosity was independent of the shear rate. The rank of the apparent viscosity among these fractions was in the order of WSCP-IV > WSCP-V > WSCP-II, as shown in Fig. 5.4.

In Fig. 5.5, only WSCS-I was found to exhibit shear thinning behaviour in the solutions tested. The shear thinning behaviour of WSCS-I was weaker compared to that of WSCP-I and WSCP-III by exhibiting lower apparent viscosity over the entire shear rate range investigated. All the other fractions from the WSCS series showed Newtonian behaviour and their apparent viscosity followed the order of WSCS-V > WSCS-III > WSCS-II > WSCS-IV.

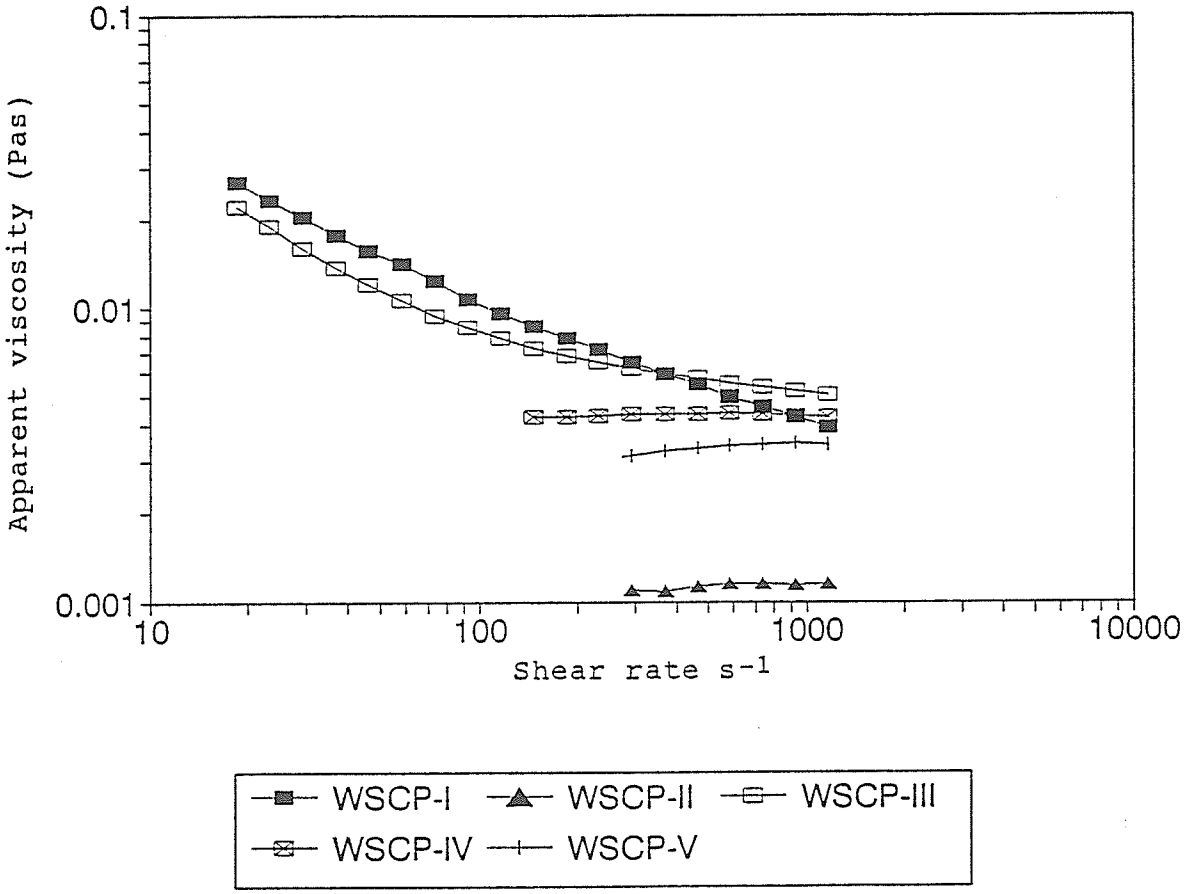


Fig. 5.4. Steady shear rheological profiles of WSCP series at 0.5% (w/w) polymer concentration, 22°C.

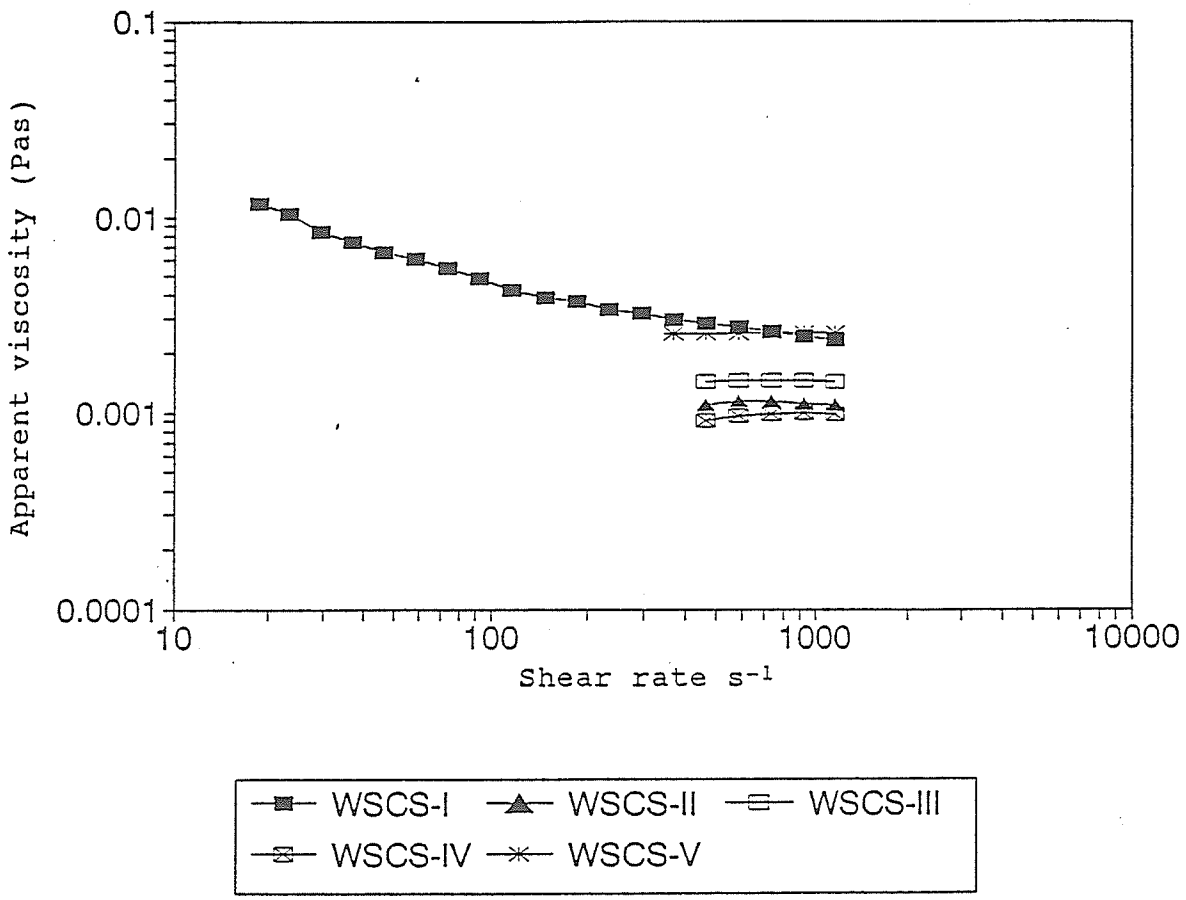


Fig. 5.5. Steady shear rheological profiles of WSCS series at 0.5% (w/w) polymer concentration, 22°C.

The observations for the rheological properties of the isolated fractions were in agreement with data in Chapter 4 indicating that WSCP contribute more to the pronounced shear thinning properties of water-soluble yellow mustard polysaccharides than WSCS (Cui *et al.*, 1993b).

5.3.3 Gel Filtration Chromatography

Fig. 5.6 and 5.7 show the molecular weight distributions of the isolated WSCP and WSCS sub-fractions. The WSCP-I profile was not determined due to poor solubility of this fraction. Among the rest of the WSCP series, WSCP-II contained lower amounts of uronic acids compared to WSCP-III and WSCP-IV. The curve for uronic acids coincided with that for carbohydrate in WSCP-III, indicating its relative homogeneity . The chemical homogeneity of WSCP-III was confirmed by monosaccharide analysis, methylation and ^{13}C NMR spectrum (Table 5.2, 5.3 and Fig. 5.8). WSCP- IV contained the second largest proportion of uronic acid, although the eluting peak position for carbohydrates came later than the uronic acid which may reflect the heterogeneity of WSCP-IV. In Fig.5.6, WSCP-V was eluted from the ion exchange column by 6.0M urea solution almost free of uronic acids. The peak elution volume was highest for WSCS-II, followed by WSCP-V, WSCP-IV and WSCP-III. This order of the elution volume corresponds with the increase in apparent viscosity (Fig. 5.4) suggesting that polysaccharide distribution of higher molecular size contributed to a higher apparent viscosity. This is in agreement with the report of Izydorczyk and Biliaderis (1992) that higher molecular weight pantosans contributed to higher viscosity (shear thinning) in solutions.

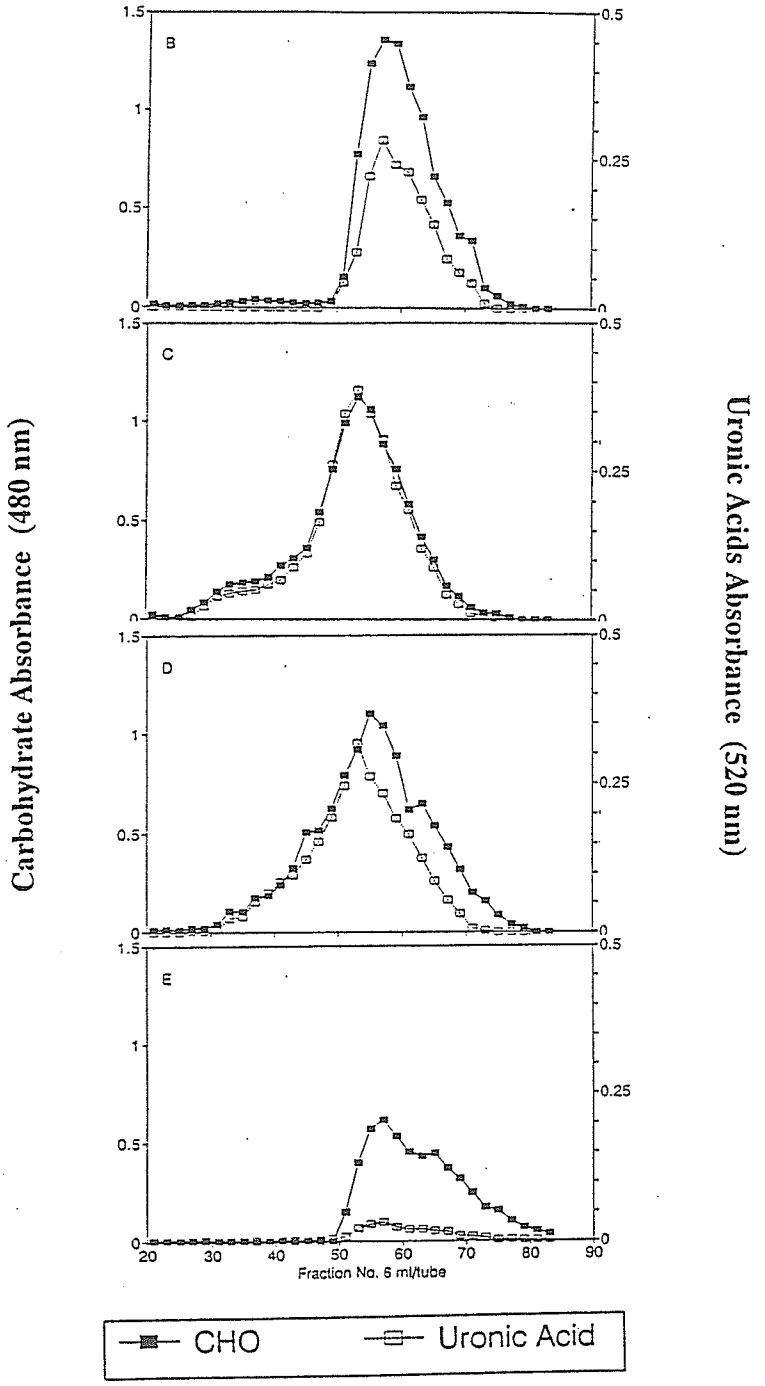


Fig. 5.6. Gel filtration chromatographic profiles of WSCS fractions on Sephacryl S-500 (2.6x98cm, 6ml/tube, 60ml/h) (A: WSCP-I, not determined; B: WSCP-II; C: WSCP-III; D: WSCP-IV and E: WSCP-V).

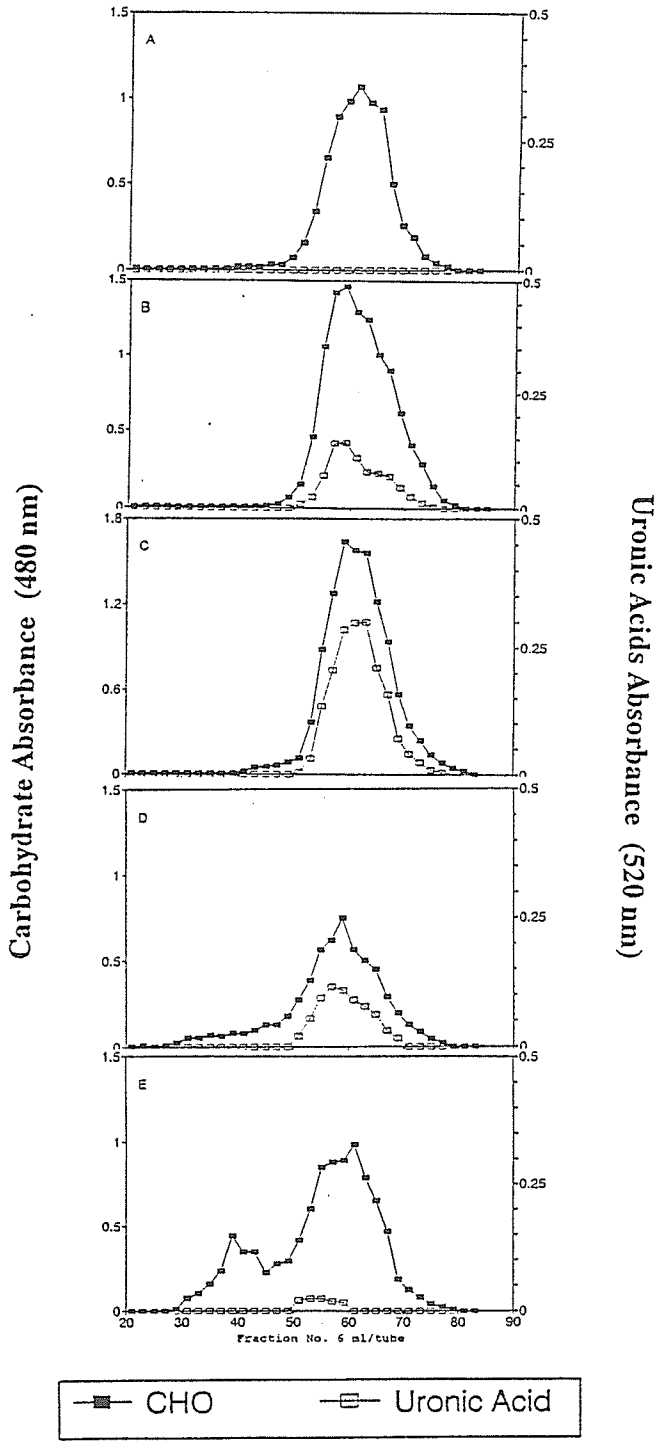


Fig. 5.7. Gel filtration chromatographic profiles of WSCS fractions on Sephacryl S-500 (2.6x98cm, 6ml/tube, 60ml/h) (A: WSCS-I; B: WSCS-II; C: WSCS-III; D: WSCS-IV and E: WSCS-V).

In Fig. 5.7, WSCS-I appeared to be a major neutral peak with no detectable uronic acids. The uronic acids were higher in WSCS- III and WSCS-IV compared to WSCS-II although WSCS-IV exhibited a wider elution peak. There were two major peaks in the WSCS-V profile, peak a: fraction No. 35-45 and peak b: fraction No. 50- 66. The presence of component with high molecular size (lower elution number) in the WSCS-V could explain why the apparent solution viscosity of this fraction coincided with that of WSCS-I over the high shear rate region (Fig.5.5).

5.3.4 Monosaccharide Analysis

Table 5.2 shows the molar ratios of neutral monosaccharides for the WSCP and WSCS sub-fractions. In the WSCP series, WSCP-I contained mainly glucose, followed by rhamnose, galactose, mannose, a small amount of arabinose and a trace amount of xylose. With increased ionic strength (using different NaCl concentrations, 0.1 to 1 M in buffer), the acidic fractions eluted from the ion exchange column were composed typically of galactose and rhamnose. WSCP-III was obtained as a relatively homogeneous fraction and contained mainly galactose and rhamnose with only trace amount of glucose, mannose and arabinose. This is in agreement with the gel filtration profile of WSCP-III where it appeared relatively homogeneous, as shown in Fig. 5.6. WSCP-II and WSCP-IV, however, were less homogeneous containing a fair amount of glucose and mannose. The monosaccharide composition of WSCP-V was predominated by glucose, followed by rhamnose, galactose, mannose with a trace amount of arabinose. In the WSCS series, WSCS-I contained much more glucose than the WSCP-I fraction (glucose:galactose ratio 16.4:1 and 2.4:1 respectively). With increased ionic strength the order of elution by ion exchange chromatography was WSCS-II, WSCS-III and WSCS-IV respectively (Fig. 5.3). These three fractions were

composed mainly of galactose, rhamnose, mannose, glucose together with small amounts of xylose and arabinose. It appeared that none of the three fractions were homogeneous although WSCS-III contained smaller amounts of glucose and mannose. The WSCS-V fraction was similar to WSCS-I as it was composed mainly of glucose with smaller amounts of mannose, xylose, arabinose, rhamnose and galactose.

Table 5.2. Neutral monosaccharide molar ratios of fractions isolated by ion exchange chromatography from yellow mustard mucilage water-soluble CTAB precipitated (WSCP) and water-soluble CTAB soluble (WSCS) fractions

	WSCP					WSCS				
	I[a]	II	III	IV	V	I	II	III	IV	V
Rha	2.0	0.3	0.4	1.1	2.3	3.5	0.4	0.5	0.6	1.1
Ara	0.3	tr	tr	tr	0.1	tr	0.1	0.3	3.0	1.1
Xyl	tr	0	0	0	0	tr	0.2	0.2	tr	1.1
Man	0.5	0.1	tr	0.2	0.7	tr	0.6	0.3	0.4	1.5
Gal	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glc	2.7	0.2	tr	0.2	6.7	16.4	0.5	0.3	0.6	12.8

[a]: I to V were isolated by ion exchange chromatography:
WSCP: I, eluted with buffer (50 mM NaAc, pH 5.0);
II, eluted with 0.2M NaCl in buffer;
III, eluted with 0.5M NaCl in buffer;
IV, eluted with 1.0M NaCl in buffer;
V, eluted with 6.0M Urea.
WSCS: I, eluted with buffer (50 mM NaAc, pH 5.0);
II, eluted with 0.1M NaCl in buffer;
III, eluted with 0.2M NaCl in buffer;
IV, eluted with 1.0M NaCl in buffer;
V, eluted with 6.0M Urea.
b: tr: Trace amount, less than 0.05.
c: Molar ratios expressed relative to galactose.

5.3.5 Methylation Analysis

The methylation analysis of WSCP I to V is summarized in Table 5.3. WSCP-I contained 46% of glucose primarily as 1,4 linkage (44.5%) together with a small amount of non-reducing end glucose (1.6%) and trace amounts of 1,4,6 linkages. This is in agreement with the monosaccharide analysis that glucose was the predominant sugar. The second largest component of WSCP-I, xylose, was mainly composed of 1,4 linked xylopyranose (18.7%) and a small amount of all substituted xylose (3.9%). In addition, 5.9% of 1,6 linked galactose and 3% of 1,2 linked galactose together with trace amount of 1,3,6 linked galactose were also found in the WSCP-I fraction.

The methylation results in Table 5.3 shows that WSCP-III is mainly a pectic-like polysaccharide which is in agreement with previous report that WSCP contained a pectic polysaccharide (Cui *et al.* 1993b). Its major components are 1,6-linked galactose (22.9%), 1,2 linked (11%) and 1,2,4-linked (17%) rhamnoses. The 1,4-linked galacturonic acid content was 13.6% with similar amount of non-reducing end glucuronic acid (13.3%).

Compared to WSCP-III, WSCP-II and WSCP-IV were less homogeneous containing fairly high amounts of methyl ethers of glucose (20.8% for WSCP-II and 10.2% for WSCP-IV). In addition, the 1,4 linked galacturonic acid contents of WSCP-II and WSCP-IV (5.4 and 5.6%, respectively) was much lower than that for WSCP-III (13.6%). The non-reducing end glucuronic acid content decreased (WSCP-II:15.9%; WSCP-III: 13.3%; WSCP-IV: trace) as the elution ionic strength increased from 0.2 M to 1.0 M NaCl in the buffer used for the ion exchange chromatography. This may be due to the non-reducing end glucuronic acid having a lower capacity to bind with the DEAE ions, making it easier for it to be washed out of the column. The methylation analysis also revealed that WSCP-V was composed solely of 1,4-linked β-D-glucan.

Table 5.3. Molar ratios of partially permethylated acetyl alditols of the water-soluble CTAB-precipitated (WSCP) fractions of yellow mustard mucilage

	Molar Ratio (%)[a]				
	WSCP-I	WSCP-II[b]	WSCP-III	WSCP-IV	WSCP-V
2,3,5-Me_3-Ara	2.8	0.8	0.4	3.6	0
2,3-Me_2-Ara	4.3	2.7	2.5	4.0	0
Total methyl ethers of Ara	7.1	3.5	2.9	7.6	0
2,3-Me_2-Xyl	18.7	4.0	1.3	1.8	0
Xyl $(acet)_5$	3.9	0.6	tr	15.3	0
Total methyl ethers of Xyl	22.6	4.6	1.3	17.1	0
2,3,4,6-Me_4-Glc	1.6	1.4	0	4.2	1.8
2,3,6-Me_3-Glc	44.5	13.9	1.0	4.3	81.3
2,3-Me_2-Glc	tr	5.5	0	1.7	0
Total methyl ethers of Glc	46.1	20.8	1.0	10.2	83.1
2,3,4-Me_3-Glc (6D_2)	n.d.	15.9	13.3	n.d.	n.d.
2,3,4,6-Me_4-Gal	n.d.	5.5	2.3	4.0	0
3,4,6-Me_3-Gal	3.0	0	2.4	4.6	0
2,3,4-Me_3-Gal	5.9	23.8	22.9	39.4	0
2,4-Me_2-Gal	tr	0.8	0.6	tr	0
Total methyl ethers of Gal	8.9	30.1	28.2	48.0	0
2,3-Me_2-Gal (6D_2)	n.d.	5.4	13.6	5.6	n.d.
2,3,6-Me_3-Man	4.5	5.3	0.2	0	0
3,4-Me_2-Rham	1.2	2.1	11.0	0	0
3-Me -Rham	tr	5.6	17.7	4.9	0
Total methyl ethers of Rham	1.2	7.7	28.7	4.9	0

[a]: Relative molar ratio calculated from the ratio of peak heights.
[b]: WSCP-II, III and IV were carboxyl reduced (O'Neil, 1990).
n.d.: not determined.
tr: trace amount.

Table 5.4 represents the molar ratios of partially methylated alditol acetates for WSCS-I to WSCS-V. Both WSCS-I and WSCS-V are neutral polysaccharides composed primarily of 1,4-linked β-D-glucan (66% and 72%, respectively) with small amount of branches (4% and 5%, respectively). Other components were present in a similar ratio among the two fractions. This is in agreement with the monosaccharide analysis (Table 5.2) in which both WSCS-I and WSCS-V contained mainly glucose with traces of other components.

Increasing ionic strength during ion exchange chromatography, the resulting WSCS-II and WSCS-III sub-fractions exhibited a similar ratio of non-reducing end glucuronic acid (9.0 and 9.7%, respectively) while the content of 1,4-linked galacturonic acid increased from trace amounts in WSCS-II to 3.3% in WSCS-III. The increase in galacturonic acid with increasing elution ionic strength indicated that galacturonic acid in the polysaccharide favored its binding to the DEAE-cellulose. This is in agreement with the DEAE-cellulose ion exchange chromatographic results for WSCP series which showed that less glucuronic acid and more galacturonic acid favored retention of the polymer on the column under the conditions investigated. In addition, the presence of 1,4-linked mannose was greater in WSCS-II (8.7%) than WSCS-III (3%) while the amount of 1,4-linked xylopyranose was lower in WSCS-II (2%) than in WSCS-III (5.2%). Neither of these fractions appeared to be homogeneous since they both contained 14 to 28% of glucose in agreement with the results of monosaccharide analysis (Table 5.2).

Table 5.4. Molar ratios of partially methylated alditol acetates of the water-soluble CTAB-precipitated (WSCS) fractions of yellow mustard mucilage

	Molar Ratio (%)[a]				
	WSCS-I	WSCS-II[b]	WSCS-III	WSCS-IV	WSCS-V
2,3,5-Me_3-Ara	1.8	1.0	0.6	tr	tr
2,3-Me_2-Ara	1.6	5.8	6.2	tr	1.2
Total methyl ethers of Ara	3.4	6.8	6.8	--	1.2
2,3-Me_2-Xyl	tr	2.0	5.2	tr	1.0
Xyl $(acet)_5$	2.3	0.3	0.3	tr	2.0
Total methyl ethers of Xyl	2.3	2.3	5.5	--	3.0
2,3,4,6-Me_4-Glc	3.2	2.4	4.2	0	1.8
2,3,6-Me_3-Glc	66.0	17.5	4.9	tr	71.6
2,3-Me_2-Glc	4.1	8.4	4.9	0	5.2
Total methyl ethers of Glc	73.3	27.9	14.0	--	78.6
2,3,4-Me_3-Glc (6D_2)	n.d.	9.0	9.7	6.0	n.d.
2,3,4,6-Me_4-Gal	1.6	2.0	4.0	tr	0.4
3,4,6-Me_3-Gal	0.8	8.8	2.1	tr	0.5
2,3,4-Me_3-Gal	1.4	12.4	12.1	21.8	1.5
2,4-Me_2-Gal	6.9	0.4	2.8	tr	7.6
Total methyl ethers of Gal	10.7	23.6	21.0	21.8	10.0
2,3-Me_2-Gal (6D_2)	n.d.	tr	3.3	2.4	n.d.
2,3,6-Me_3-Man	1.2	8.7	1.3	0	1.0
3,4-Me_2-Rham	0.6	1.2	3.4	tr	0
3-Me -Rham	tr	0.4	4.7	tr	1.1
Total methyl ethers of Rham	0.6	1.6	8.1	--	1.1

[a]: Relative molar ratio calculated from the ratio of peak heights.
[b]: WSCS-II, III and IV were carboxyl reduced (O'Neil, 1990).
n.d.: not determined.
tr: trace amount.

5.3.6 NMR Spectroscopy

^{13}C NMR spectra of WSCP-II to V are presented in Fig. 5.8. The spectrum of WSCP-I was not determined because of poor solubility. In Fig. 5.8B, two uronic acid peaks were identified with almost equal intensity and could be attributed to non-reducing end glucuronic acid (δ 176.1ppm) and1,4-linked galacturonic acid (δ 175.2 ppm) in WSCP-III. This is consistent with the methylation data, in which, glucuronic acid and 1,4-linked galacturonic acid content accounted for 13.3% and 13.6% respectively (Table 5.3). A resonance at δ 17.8 ppm is due to the C-6 methyl group of rhamnose. In the anomeric carbon resonance region, (δ 90 to 110 ppm), two strong peaks were identified at δ 104.1 ppm and 103.4 ppm and two medium intensity peaks with chemical shifts of 99.17 ppm and 98.55 ppm. Weak absorbance at chemical shifts 108-110 ppm could be due to trace amounts of arabinose present which is common to pectic polysaccharides (Aspinall, 1989).

The results in Fig. 5.8 show that both WSCP-II and WSCP-IV exhibited similar spectrum patterns to that of WSCP-III, but, their anomeric resonance region was more complex. This is in agreement with the monosaccharide analysis and methylation analysis that WSCP-III is more homogeneous than WSCP-II and WSCP- IV by containing only trace amounts of glucose.

The ^{13}C NMR spectrum for WSCP-V in Fig. 5.8. appeared to be rather simple. There was only one single major absorbance in the anomeric region which can be attributed to the C-1 of 1,4-linked β-D-glucose residue while the chemical shift at 60.97 ppm was caused by the C-6 of the same sugar. The C-4 resonance located at 79.37 ppm and the chemical shifts of 75.69 ppm, 74.97 ppm and 73.84 ppm could be attributed to C-5, C-3 and C-2 of the same residue, respectively (Bock *et al.*, 1984; Grimmecke *et al.*, 1991).

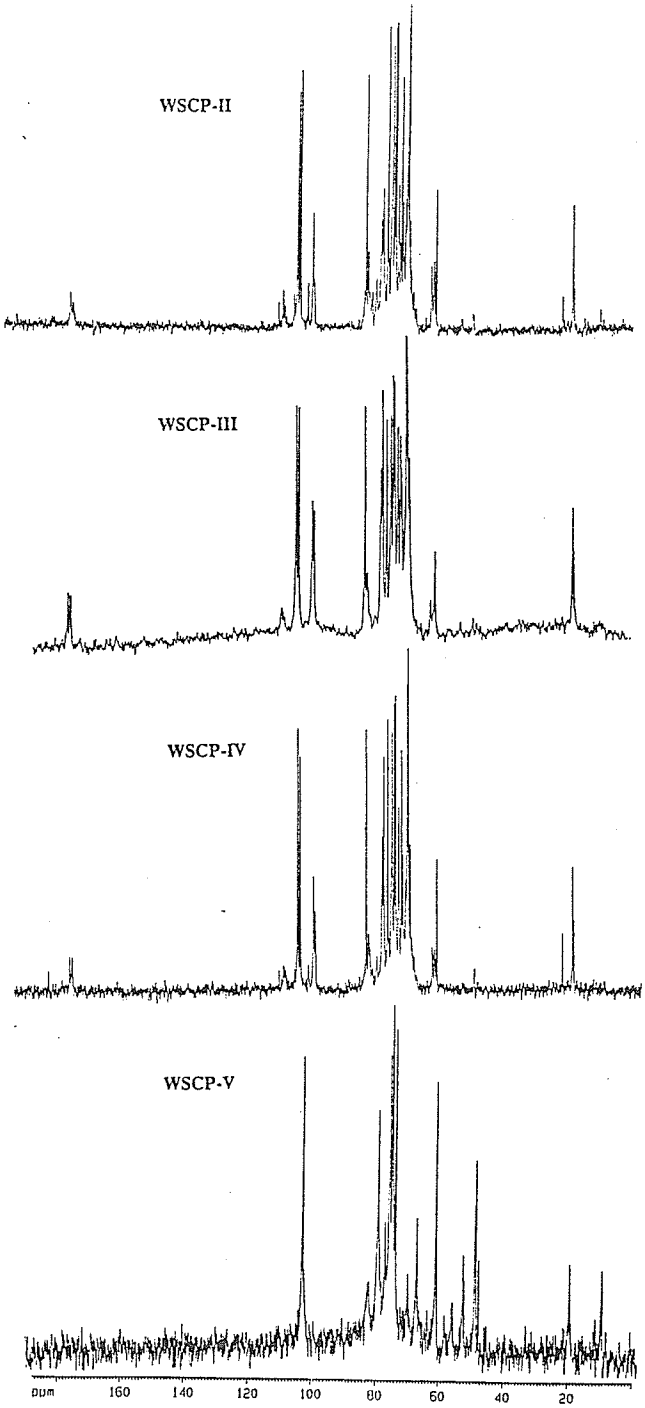


Fig. 5.8. Comparison of ^{13}C NMR spectra of WSCP-II to WSCP-V.

Other resonances over the 47- 55 ppm region could be due to the presence of methyl or ethyl ethers (Tezuka *et al.*, 1991). The presence of these groups could explain the solubility of the cellulose-like material in aqueous solution.

The ^{13}C NMR spectra of WSCS-I to WSCS-V are shown in Fig. 5.9. The spectrum of WSCS-I and WSCS-V are similar to that of WSCP-V, typical of cellulose-like structure. It appeared that the relative resonance intensity of the methyl ether groups (45-55 ppm) is higher for WSCS-V as compared to WSCS-I. This could be related to their flow behaviour in solution as WSCS-I contained less methyl groups and exhibited shear thinning properties while WSCS-V contained relative more methyl groups and exhibited Newtonian flow behaviour. A higher content of methyl groups would favor the solubilization of the cellulose-like material while a lower content of methyl ether groups may favor network development (solid-like character) in the solutions of this material. Only a non-reducing end glucuronic acid resonance was identified for WSCS-II (Fig. 5.9B), which is in agreement with the methylation analysis where only trace amounts of 1,4-linked galacturonic acid were found (Table 5.4). The content of 1,4-linked galacturonic acid increased from WSCS-II to WSCS-IV, consistent with the methylation analysis which supports the conclusion that DEAE-cellulose column selectively retained polysaccharides containing 1,4-linked galacturonic acid. The anomeric regions of WSCS-II to WSCS-IV are quite complicated although some reductions in resonance intensities were observed in these regions. This diversity in the anomeric region reflected the lack of homogeneity for these fractions as confirmed by monosaccharide and methylation analyses (Table 5.2 and 5.4).

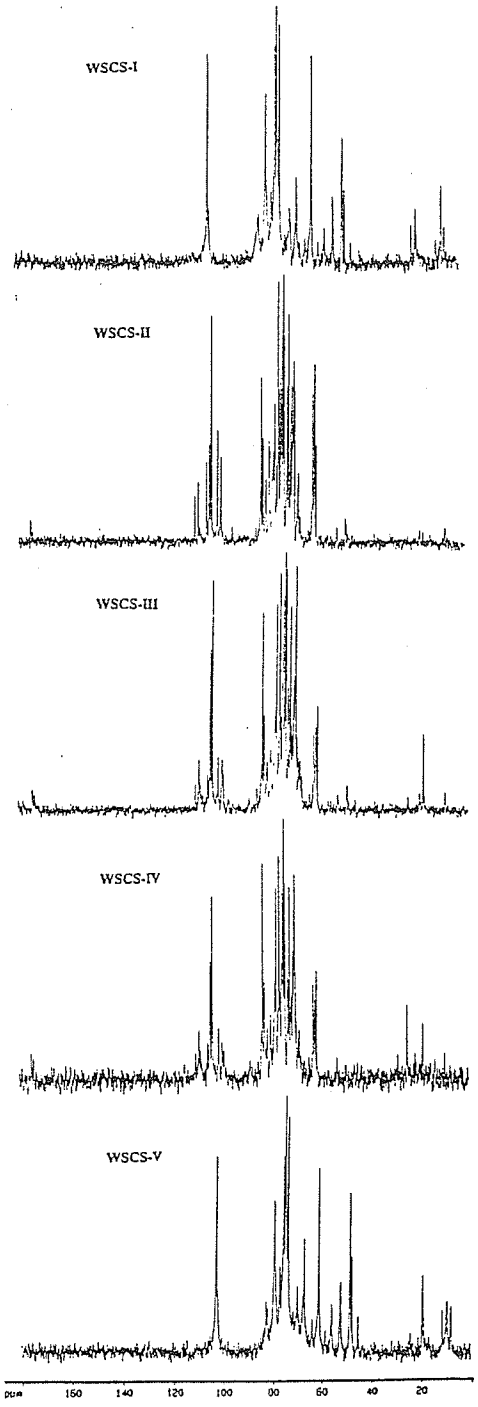


Fig. 5.9. Comparison of ^{13}C NMR spectra of WSCS-I to WSCS-V.

5.3.7 Conclusions

The water-soluble yellow mustard polysaccharides were further fractionated into ten sub-fractions by DEAE-high capacity ion exchange chromatography. Of the isolated fractions, WSCP-I, WSCP- III and WSCS-I were identified as those responsible for the pronounced shear thinning properties of yellow mustard mucilage solutions (Cui *et al.*, 1993a and 1993b). All the other fractions exhibited Newtonian-like behaviour in solution under the conditions investigated. Methylation analysis revealed that WSCP-I was mainly composed of 1,4-linked β-D-glucose together with a small amount of 1,4-linked xylopyranose and all substituted xylose. WSCP-III was a relative homogeneous pectic-like polysaccharide containing 1,6-linked galactose (22.9%), 1,2-linked (11%) and 1,2,4-linked (17%) rhamnose together with 13.3% of non-reducing end glucuronic acid and 13.6% 1,4-linked galacturonic acid as shown by methylation analysis and ^{13}C NMR spectrum. The structure of WSCS-I is rather simple containing primarily 1,4-linked β-D-glucose. This study revealed, for the first time, the relationship between the internal structure of some purified yellow mustard mucilage fractions and their rheological properties, particularly those exhibiting strong shear thinning behaviour.

CHAPTER 6

STRUCTURAL ANALYSIS OF A PECTIC POLYSACCHARIDE FROM YELLOW MUSTARD (*Sinapis alba L.*) MUCILAGE: PARTIAL HYDROLYSIS, ISOLATION OF OLIGOSACCHARIDES AND NMR AND FAB-MS CHARACTERIZATIONS

6.1 INTRODUCTION

A pectic polysaccharide isolated from yellow mustard mucilage was a contributing component to the pronounced shear thinning behaviour of water-soluble yellow mustard polysaccharides (Chapter 4 and 5). Methylation analysis and NMR spectroscopy of this material suggested a typical pectic linkage pattern which contained 1,4-linked D-galacturonic acid, 1,2 and 1,2,4-linked rhamnose. In addition, 1,6-linked galactose and a non-reducing end glucuronic acid were found associated with this polymer (Cui *et al.*, 1993b). This chapter reports the further structural analysis of this material by partial hydrolysis of the polysaccharide, isolation of the hydrolysed oligosaccharides and structural characterization of the oligosaccharides by FAB-MS and NMR spectroscopy.

6.2 MATERIAL AND METHODS

6.2.1 Materials

A pectic polysaccharide (WSCP-III) was prepared from yellow mustard mucilage by anion exchange chromatography of the water-soluble CTAB-precipitate fraction (WSCP) as described in Chapter 4 and 5. All chemicals were of reagent grade unless otherwise specified.

6.2.2 Partial Hydrolysis

The polysaccharide WSCP-III was dissolved in 30 ml of hot water (100°C), then, trifluoroacetic acid was added to a concentration of 0.4M. The solution was heated at 100°C for 2.5 h. and the cooled hydrolyzate concentrated to dryness by rotary evaporation (O'Neill *et al.*, 1990).

6.2.3 Anion Exchange Chromatography

The dried hydrolyzate dissolved in 20 mM NaAc buffer (pH 5.0) was then applied onto a DEAE-Sephadex A-25 (Kato and Nevins, 1992) column (1.6x20 cm) equilibrated with the same buffer. The column was sequentially eluted (1ml/min) with buffer containing 0.1M, 0.3M and 0.5M NaCl respectively, as shown in Fig. 6.4. The neutral fractions (eluted with buffer alone) was monitored for sugars by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Following stepwise change in ionic strength (NaCl) the eluted acidic fractions were detected using the method described by Blumenkrantz and Asboe-Hansen (1973). Appropriate fractions were combined and concentrated on a rotary evaporator at 40°C (F-I: fractions 22-26; F-II: fractions 29- 33; F-III: fractions 35-39; F-IV: fractions 44-49).

6.2.4 Gel Filtration Chromatography

Selected fractions identified in Fig. 6.4 (F-I, II and III) were applied on a Bio-Gel P-2 column (2.5x100 cm). The column was eluted with 25mM NaCl (1ml/min) and monitored by the methods described previously (Dubois *et al.*, 1956; Blumenkrantz and Asboe-Hansen, 1973). The oligosaccharide fractions collected were reduced by borohydride (Kiefer *et al.*, 1990) and desalted on a Bio-Gel P-2 column (1.6x90 cm) by eluting with distilled water.

6.2.5 Methylation Analysis

Methylation analysis was carried out by the method of Ciucanu and Kerek (1984). The reduction of carboxyl groups was carried out after methylation according to O'Neill *et al.* (1990). Qualitative and quantitative measurements of partially permethylated acetyl alditols were performed as described previously (Cui *et al.*, 1993b).

6.2.6 NMR Spectroscopy

NMR spectra were recorded at 65°C on Bruker AM300 and AMX500 spectrometers using 4% carbohydrate solutions in D_2O (5 and 10 mm tube). Internal p-dioxane was used as an internal chemical shift reference for ^{13}C spectra. Reported values have been converted to the TMS scale.

Homonuclear correlation (COSY) spectra (Aue *et al.*, 1976), and NOE correlation (NOESY) spectra (Bodenhausen *et al.*, 1985) were recorded with F2 time domains of 1024 points and F1 time domains of 256 points. Zero filling in F1 yielded a 512 (real) matrices after transformation. A 90° mixing pulse was employed for the COSY spectra. A 100 ms mixing time was employed for the NOESY spectra. The COSY spectra were recorded in the magnitude mode while NOESY spectra were recorded in the phase sensitive mode

employing time proportional phase increments for F1 quadrature detection.

Hetereonuclear correlation spectra were recorded with the proton detected single quantum coherence (HSQC) experiment (Bodenhausen and Ruben, 1980), with an F2 time domain of 4096 points and F1 time domain of 256 points. Zero filling in F1 and F2 resulted in a 4096 (real) by 512 (real) matrix after transformation.

Difference NOE experiments were performed with a spectral width of ca. 4000 Hz and a real frequency domain data size of 32K points, resulting in a digital resolution of 0.12 Hz per point. Frequency list cycling was employed to distribute long-term changes in homogeneity equally among all spectra. Multiplets were irradiated by stepping the decoupler frequency between each line of the multiplet at 200 ms intervals (Kinns and Sanders, 1984), and each multiplet was irradiated for a total of 5 s. The irradiating field strength (calculated from the 90° pulse length and expressed as $\beta B2/2\pi$) was ca. 7 Hz. At least 512 transients (32 transients per irradiation point with 16 loops through the frequency list) were acquired for each irradiation point in order to ensure adequate signal-to-noise ratio and cancellation of unenhanced peaks. A control spectrum subtracted from each spectrum, and NOE values were determined by careful integration of the resulting difference spectrum. Using these techniques, NOE enhancements of less that 1% could be easily observed.

6.2.7 Fast Atom Bombardment Mass Spectra (FAB-MS)

The FAB MS was recorded on a VG 7070 E-HF mass spectrometer with a DATA SYSTEM 11-250J. Samples were suspended in glycerol matrix and xenon atoms at 8Kev were employed as source ions (Dell, 1987).

6.3 RESULTS AND DISCUSSION

6.3.1 Methylation and NMR Spectra of the Pectic Polysaccharide

A pectic polysaccharide WSCP-III from yellow mustard mucilage was fractionated by ion exchange chromatography and purified by gel filtration chromatography as described earlier (Chapter 5). The purified pectic polysaccharide was analyzed by methylation analysis and NMR spectroscopy, as presented in Table 6.1 and Fig. 6.1 to 6.3. In Table 6.1, the ratio of 1,4-linked galacturonic acid, non-reducing end glucuronic acid, 1,6-linked galactose, 1,2,4-linked rhamnose and 1,2-linked rhamnose were ≈0.9:0.8:1.5:1:0.5. From the ^{1}H and ^{13}C NMR spectra of WSCP-III, it could be confirmed that WSCP-III mainly contained four monosaccharides (δ 4.5, 4.7, 5.3 and 5.6 ppm in ^{1}H spectrum; δ 104.2, 103.4, 99.2 and 98.5 ppm in ^{13}C spectrum) as shown in Fig. 6.1. Two resonances at δ 176.2 and 175.5ppm were assigned to the C6 of non-reducing end glucuronic acid and 1,4-linked β-D-galacturonic acid, respectively. While two resonances at δ 20.75 and 17.85 ppm were about 2:1 in peak intensity which could be attributed to 1,2,4-linked and 1,2-linked rhamnose, respectively.

Table 6.1. Molar ratios of partially permethylated acetyl alditols (PPAA) of the pectic polysaccharide from y ellow mustard mucilage

PPAA	2,3-Me_2-Gal 6D_2	2,3,4-Me_3-Glc 6D_2	2,3,4-Me_3-Gal	3-Me-Rham	3,4-Me_2-Rham
Ratio	0.9	0.8	1.5	1.0	0.5

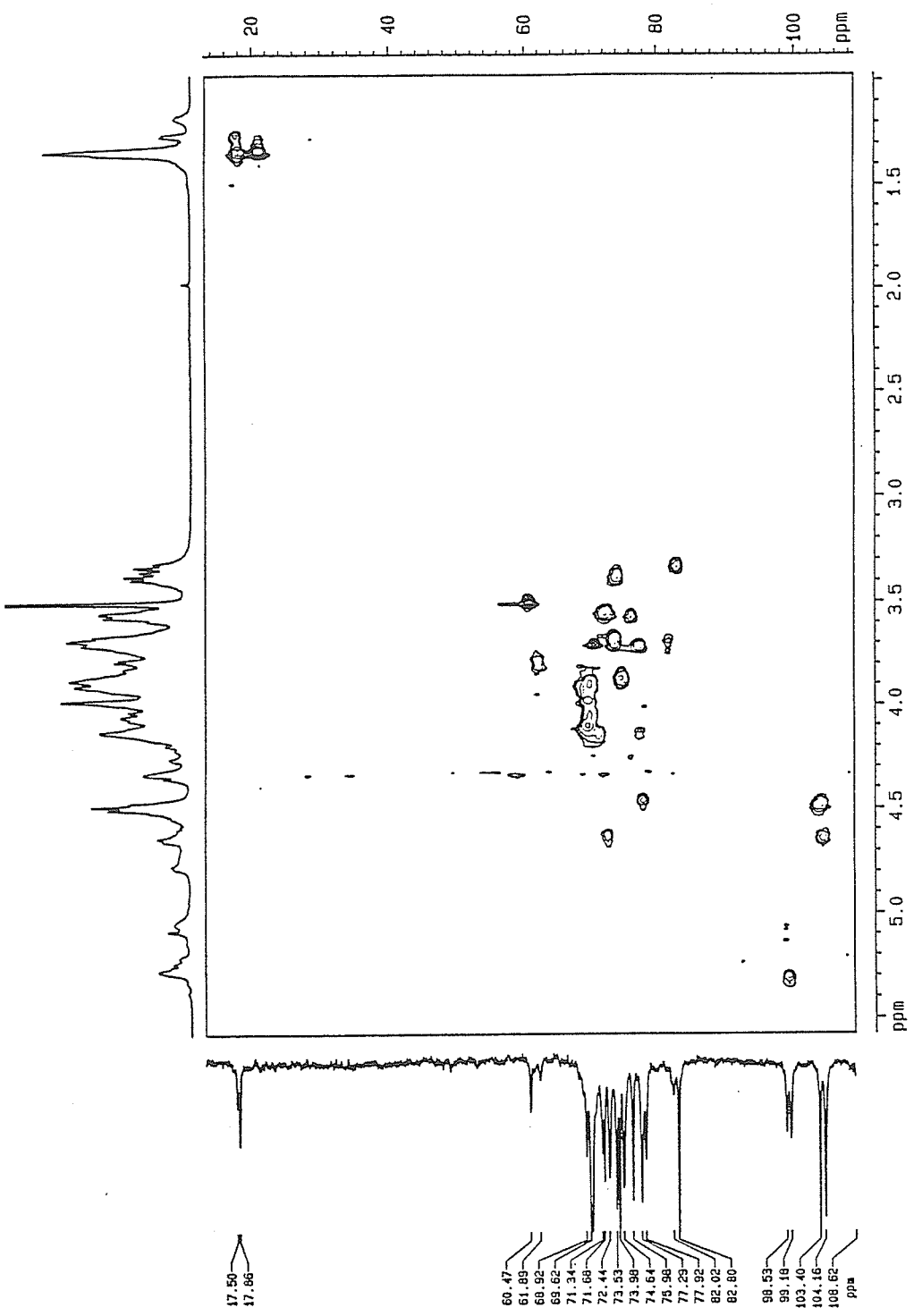


Fig. 6.1. ^{1}H and ^{13}C heteronuclear correlation spectrum of a pectic polysasccharide from yellow mustard.

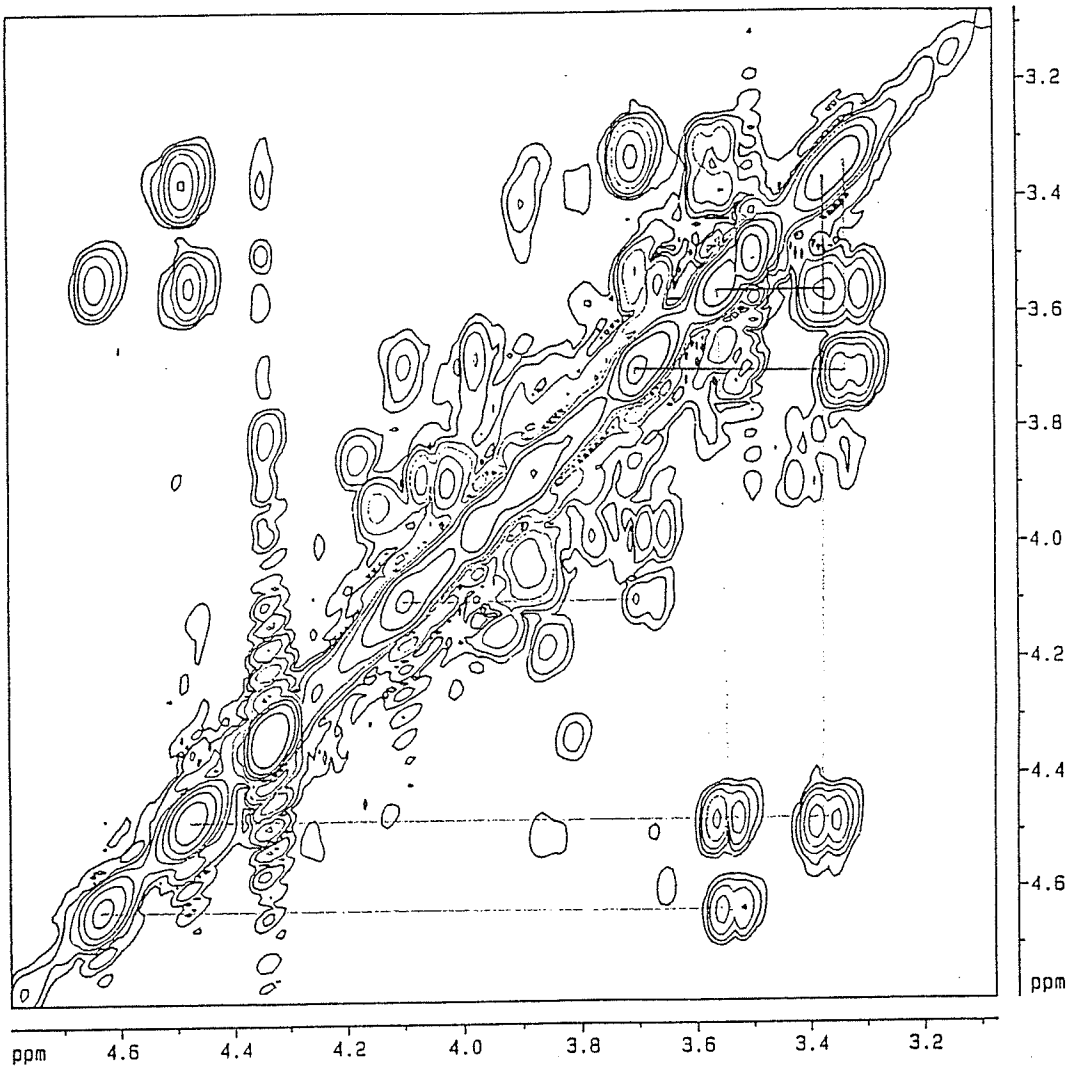


Fig. 6.2. ^{1}H and ^{1}H homonuclear correlation spectrum (COSY) of WSCP-III from yellow mustard.

This is in agreement with the results of methylation analysis that the ratio of 1,2,4-linked rhamnose to that of 1,2-linked rhamnose was 2:1 (Table 6.1). The assignment of proton signals in the ^{1}H NMR spectrum to their corresponding carbons in ^{13}C NMR spectrum was obtained by applying a heteronuclear ^{1}H-^{13}C shift-correlated spectroscopy, as shown in Fig. 6.1. However, further detailed assignment of the NMR spectra required an additional experiment, namely homonuclear ^{1}H-^{1}H shift-correlated spectroscopy (COSY) which has been widely used to determine the scalar coupling connectivities between sugar protons (Dabrowski, 1987, Koerner *et al.*, 1987 and Martin and Zektzer, 1988), as shown in Fig.6.2. The doted line indicated rhamnose connections from H1 to H6 while solid line demonstrated another connection which could be attributed to 1,4-linked galacturonic acid. The remaining connections could not be resolved due to complexity of the polysaccharide. The 2D NOESY experiment was carried out to provide information on the sequence and linkage site between monosaccharides as shown in Fig. 6.3. The complexity of the polysaccharide, however, made it extremely difficult to resolve the spectrum completely. Therefore, partial hydrolysis and the isolation of the resulting oligosaccharides were carried out to simplify the spectrum for further elucidation of the structure.

6.3.2 Partial Hydrolysis and Isolation of the Oligosaccharides

Approximately 30 mg of WSCP-III were dissolved in 20 ml of boiling water, and adjusted to 30ml with TFA at a final concentration of 0.4 M and hydrolysed at 100°C for 2.5 hr (O'Neil *et al.*, 1990). The hydrolyzate was concentrated to dryness by rotary evaporation under 40°C. The dried hydrolyzate was redissolved in NaAc buffer (25mM, pH 5.0) and loaded onto a pre-equilibrated DEAE-Sephadex A 25 (Kato and Nevins, 1992) column (1.6x25 cm). The column was first eluted with buffer, followed by stepwise

increase in ionic strength with NaCl, as shown in Fig. 6.4.

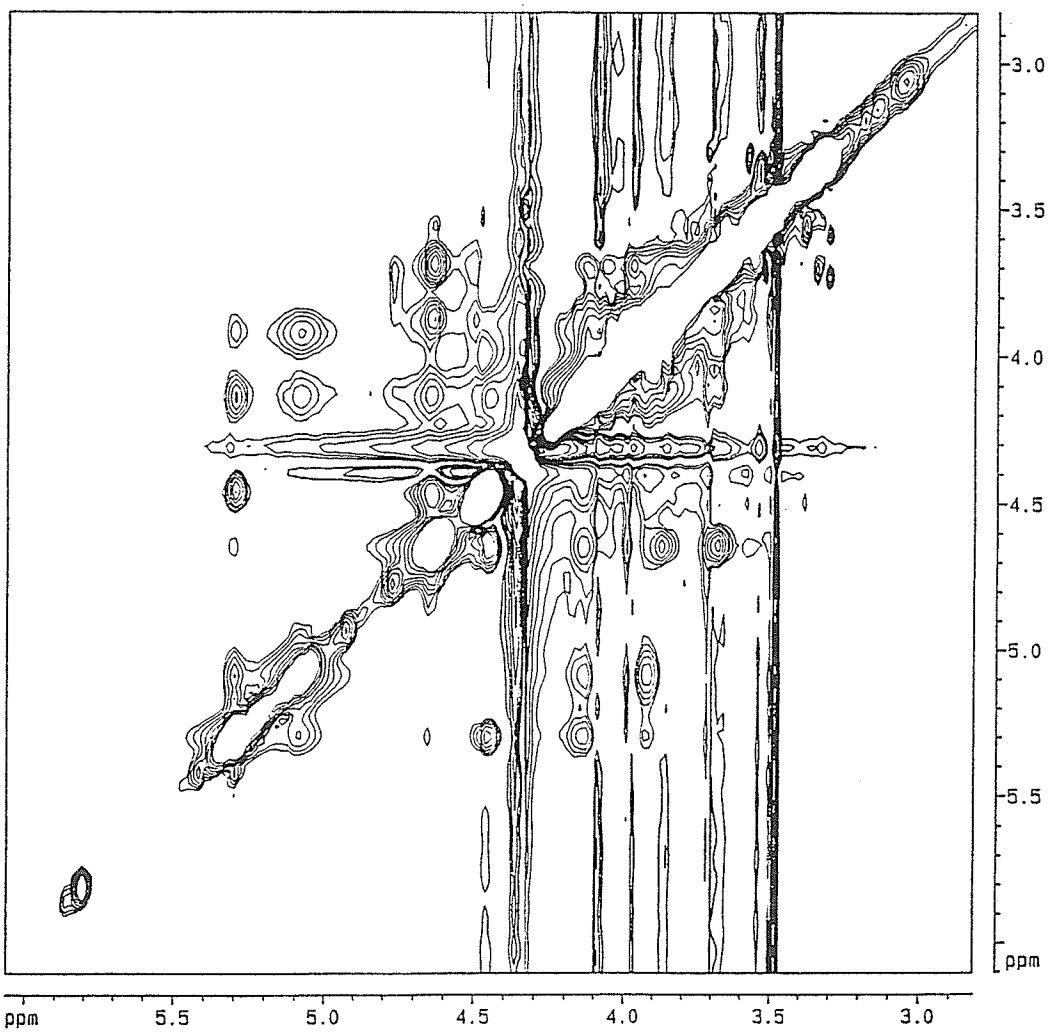


Fig. 6.3. NOESYspectrum of a pectic polysasccharide from yellow mustard mucilage.

Selected fractions from Fig. 6.4 were purified by gel filtration on a Bio-Gel P-2 column (2.5x95cm). The column was pre-equilibrated with NaAc buffer (25 mM, pH 5.0). Samples were dissolved in 1.0ml of the same buffer and loaded onto the column which was then eluted with the same buffer, as shown in Fig. 6.5. The uronic acids were determined by the method described previously (BluemanKrantz and Asboe-Hansen, 1973). Samples were collected as indicated in Fig. 6.5, concentrated and reduced to alditols with borohydride (Kiefer *et al.* 1990). The reduction of the reducing end was carried out to simplify the NMR spectrum (Kiefer *et al.*, 1990). The reduced oligosaccharides were desalted on a small Bio-Gel P-2 column (1.6x70cm) and eluted with distilled water. Samples were collected, concentrated and freeze-dried until further analyzed.

6.3.3 1D and 2D NMR Spectra of Oligosaccharides

The ^{1}H NMR spectra of F-I, F-II and F-III alditols are presented in Fig. 6.6. Peaks between δ 4.3 to 4.4 ppm were caused by HOD. The reduction of the reducing end simplified the spectra which otherwise would have produced two anomeric resonances for the α and β conformations of the oligomers. In the anomeric resonance region (δ 4.4 to 4.5 ppm), two doublets were observed for F-I while only one doublet was found for both F-II and F-III (Dabrowski, 1987). The presence of two doublets suggested a trisaccharide moiety for F-I while a single doublet indicated that both F-II and F-III were dimers since the reducing end C1 was reduced to alditol by borohydride (Kiefer *et al.*, 1990). Two similar well resolved triplets were observed for both F-II and F-III, which could be assigned to H2 (δ 3.38 ppm) and H4 (δ 3.30 ppm) of glucuronic acid and galacturonic acid

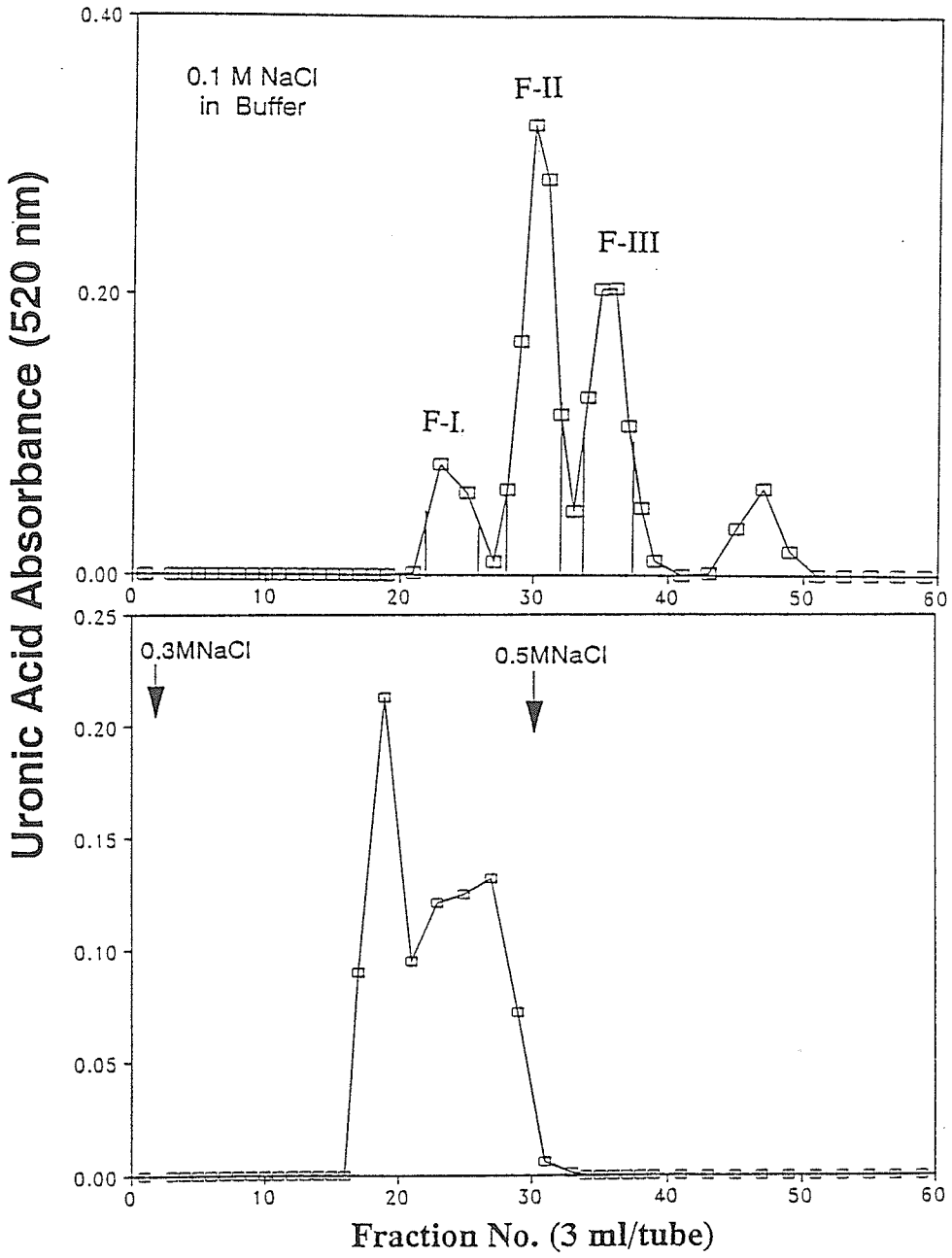


Fig.6.4.Ion exchange chromatographic profiles of oligosaccharides partially hydrolyzed from a pectic polysaccharide from yellow mustard mucilage on DEAE Sephadex A 25 eluted with salt in sodium acetate buffer (pH 5.0, 25 mM).

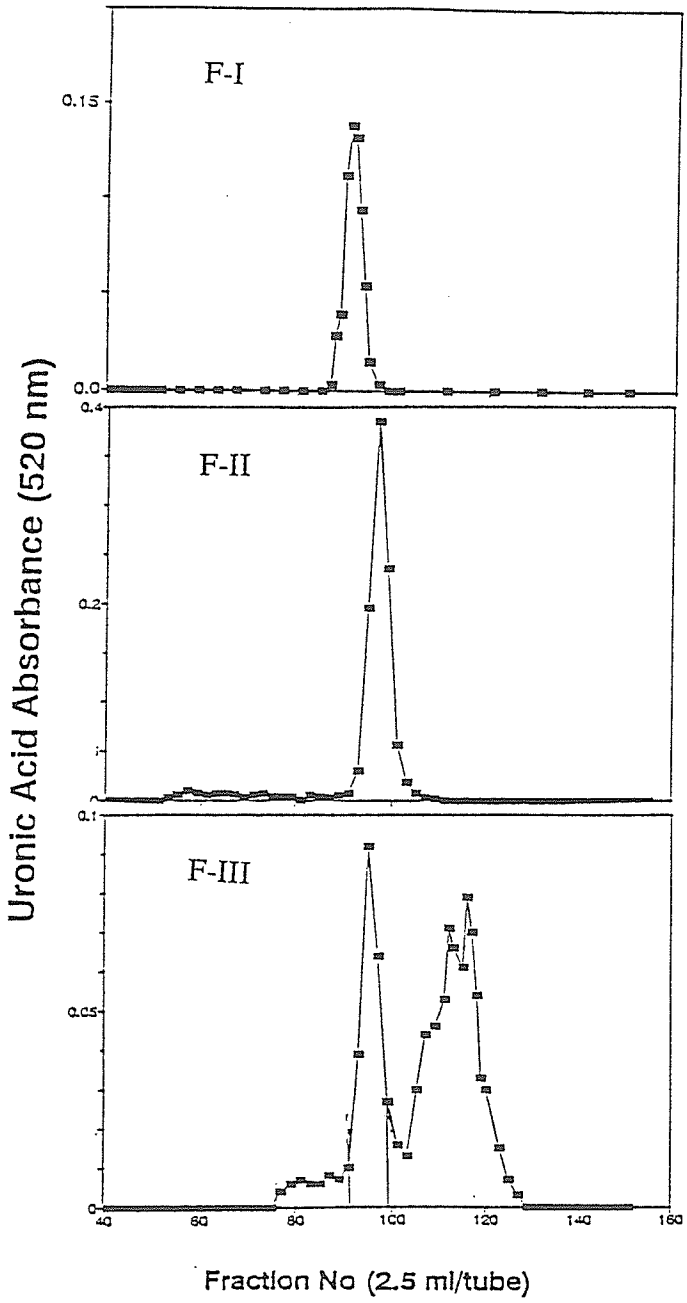


Fig.6.5. Gel filtration chromatographic profiles of F-I to III on Bio-Gel P-2 column (2.5x90cm, 50ml/h at 25°C).

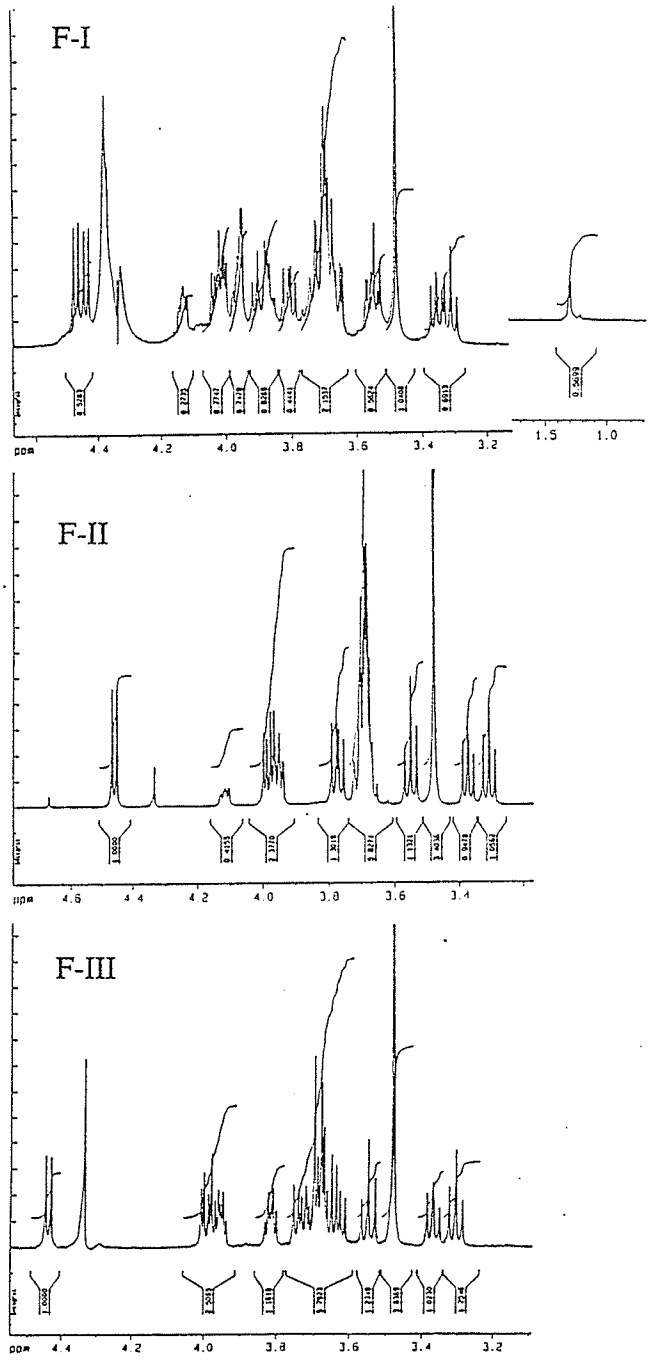


Fig. 6.6. ^{1}H NMR spectra of F-I to III

respectively, as confirmed by the COSY experiments (Fig. 6.8a-c). Two triplets were also observed for F-I, but they were much closer to each other as compared to F-II and F-III. These two triplets could be attributed to H2 (δ 3.36 ppm) and H4 (δ 3.31 ppm) of the 1,4-linked galacturonic acid, respectively. One additional triplet was observed for both F-II and F-III, which could be assigned to the H3 (δ 3.55 ppm) of the glucuronic and galacturonic acids while multiple resonances were found in the case of F-I at same chemical shift (δ 3.55 ppm). The triplets observed for H2, H3, and H4 of the uronic acids were due to co-couplings with their neighbours. A doublet resonance was found at high field (δ 1.33 ppm) for F-I, which could be attributed to the H6 of the rhamnose residue. This doublet was caused by its co-coupling with the H5. The lack of resonances at δ 1.33 ppm suggest the absence of a rhamnose residue in F-II and F-III. The remaining resonances could not be resolved due to overlap in the 1D ^{1}H NMR spectra. Therefore, 2D NMR spectroscopy was carried out for further assignment of the remaining resonances.

Heteronuclear correlation spectra of F-I, II and III are presented in Fig. 6.7a-c. Each proton was assigned to its corresponding carbon, as indicated by the vertical and horizontal lines (Fig. 6.7a). The complete assignment of all resonances were obtained with the assistance of homonuclear shift correlated spectroscopy (COSY) shown in Fig.6.8a-c and the data are summarized in Table 6.2. In the COSY spectrum of F-I, the scalar coupling connectivities of the galacturonic acid, rhamnose and galactose were demonstrated by dotted, broken and solid lines respectively, as shown in Fig. 6.8a. Also the COSY experiment of F-II (Fig. 6.8b) established the scalar coupling connectivities of the disaccharide which supported the complete assignment of the resonances (Table 6.2). In Fig. 6.8c, the connectivities of the galacturonic acid were resolved, however, the assignment of the connectivities of the

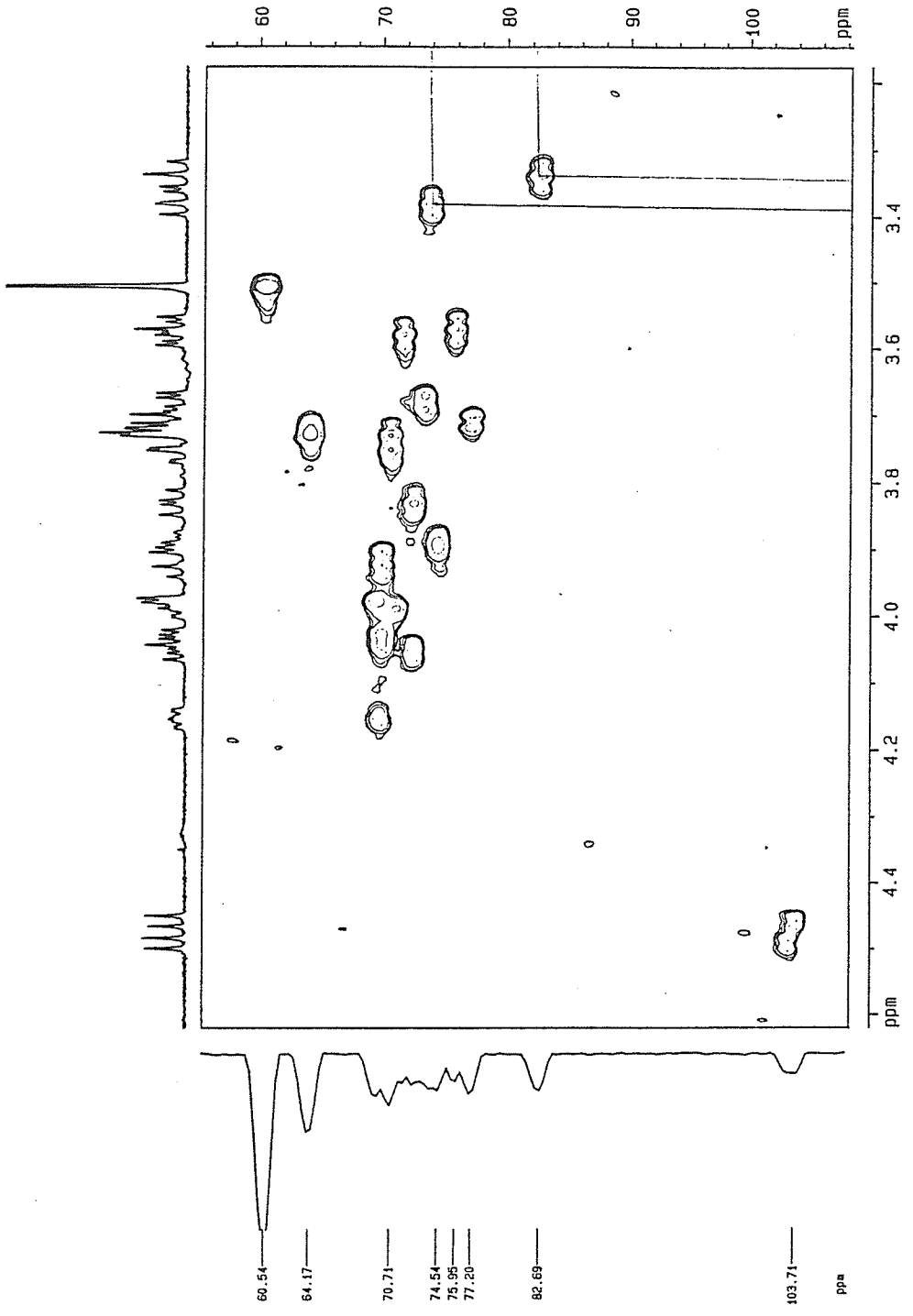


Fig.6.7a. H/C correlation spectrum of oligosaccharide F-I.

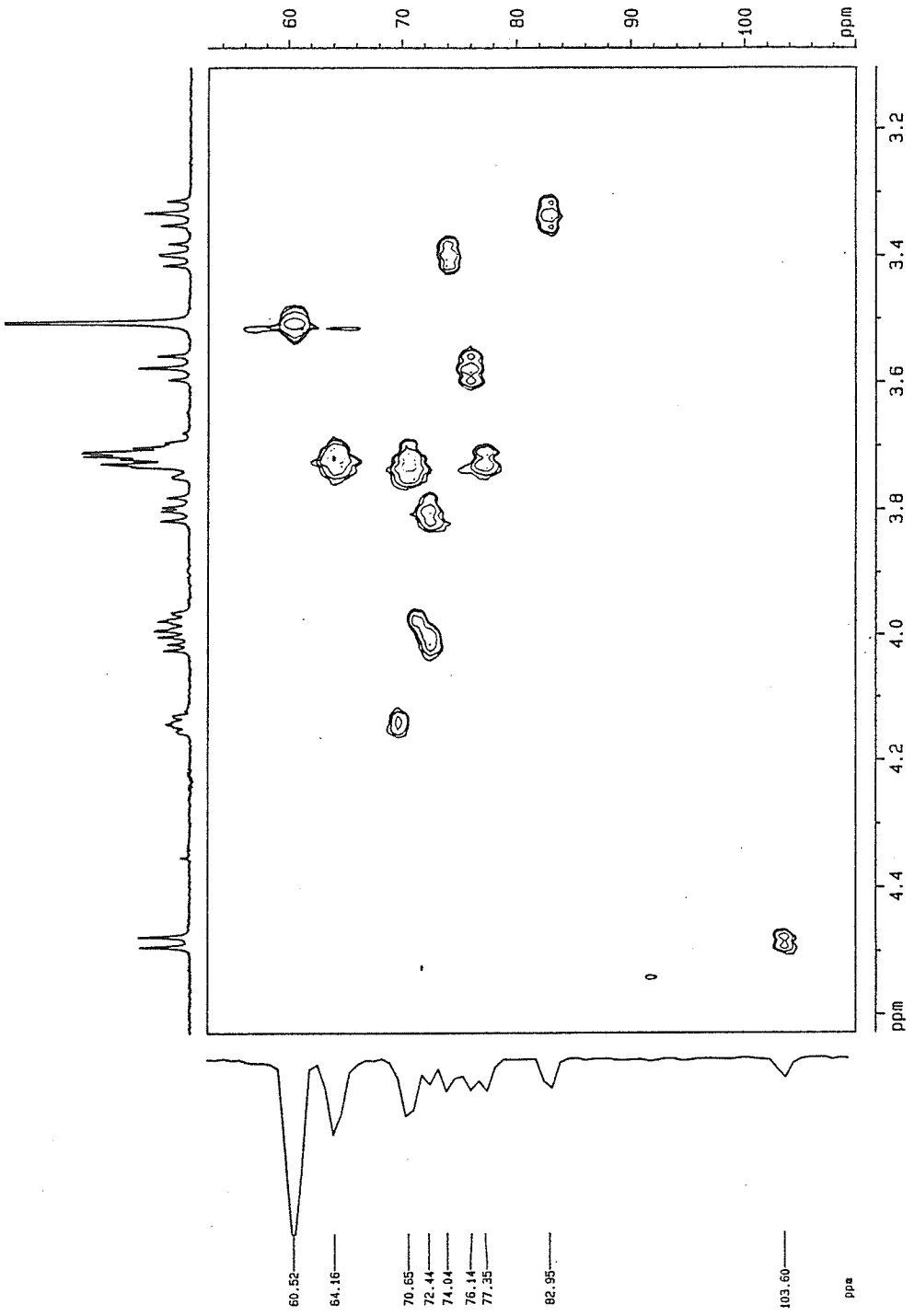


Fig.6.7b. H/C correlation spectrum of oligosaccharide F-II.

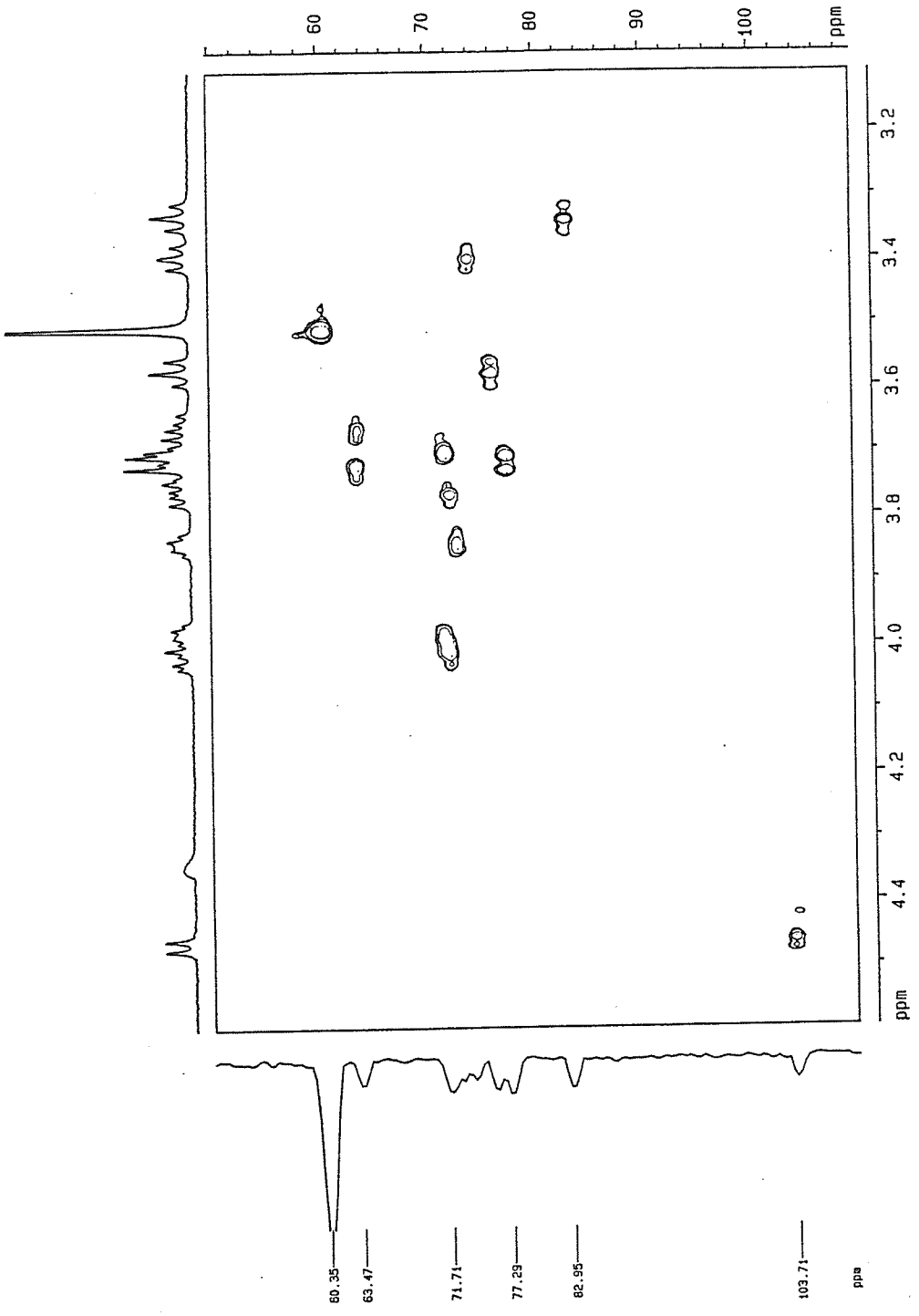


Fig.6.7c. H/C correlation spectrum of oligosaccharide F-III.

reducingend were only partially resolved and still require further experiment for complete assignment.

To determine the sequence and site of linkage, difference NOE experiments were performed, as presented in Fig. 6.9. In Fig. 6.9A(F-I low field), dipole (through-space) couplings were observed between the anomeric resonances (H1) and intra-residue resonances (H3, 3.55 ppm; H5, 3.68 ppm) and inter-residue resonance (H4 of rhamnose, 3.8 ppm). The intra-residue dipole couplings confirmed the assignment of resonances, while the inter-residue dipole coupling identified that galacturonic acid was linked to rhamnose at the 4 position. In a similar manner, the anomeric resonance (H1) of rhamnose was inter-residuely through-space coupled with the H6 (3.80 and 3.87 ppm) of the galactose in addition to its intra-residue dipole couplings (Fig. 6.9B). This experiment established that F-I was a trisaccharide: galacturonic acid linked at the 4 position of rhamnose, which was in turn, linked at the 6 position of galactol as shown in structure 1.

GalA1->4Rham1->6Galtol

(1)

In Fig. 6.10 B, the dipole couplings of the anomeric resonance (H1) of the glucuronic acid with both intra-residue and inter-residue resonances were observed. This supported the previous assignment of the resonances and also provided evidence that the glucuronic acid was linked at the 6 position of the reducing end galactol (structure 2).

GlcA1->6Galtol.

(2)

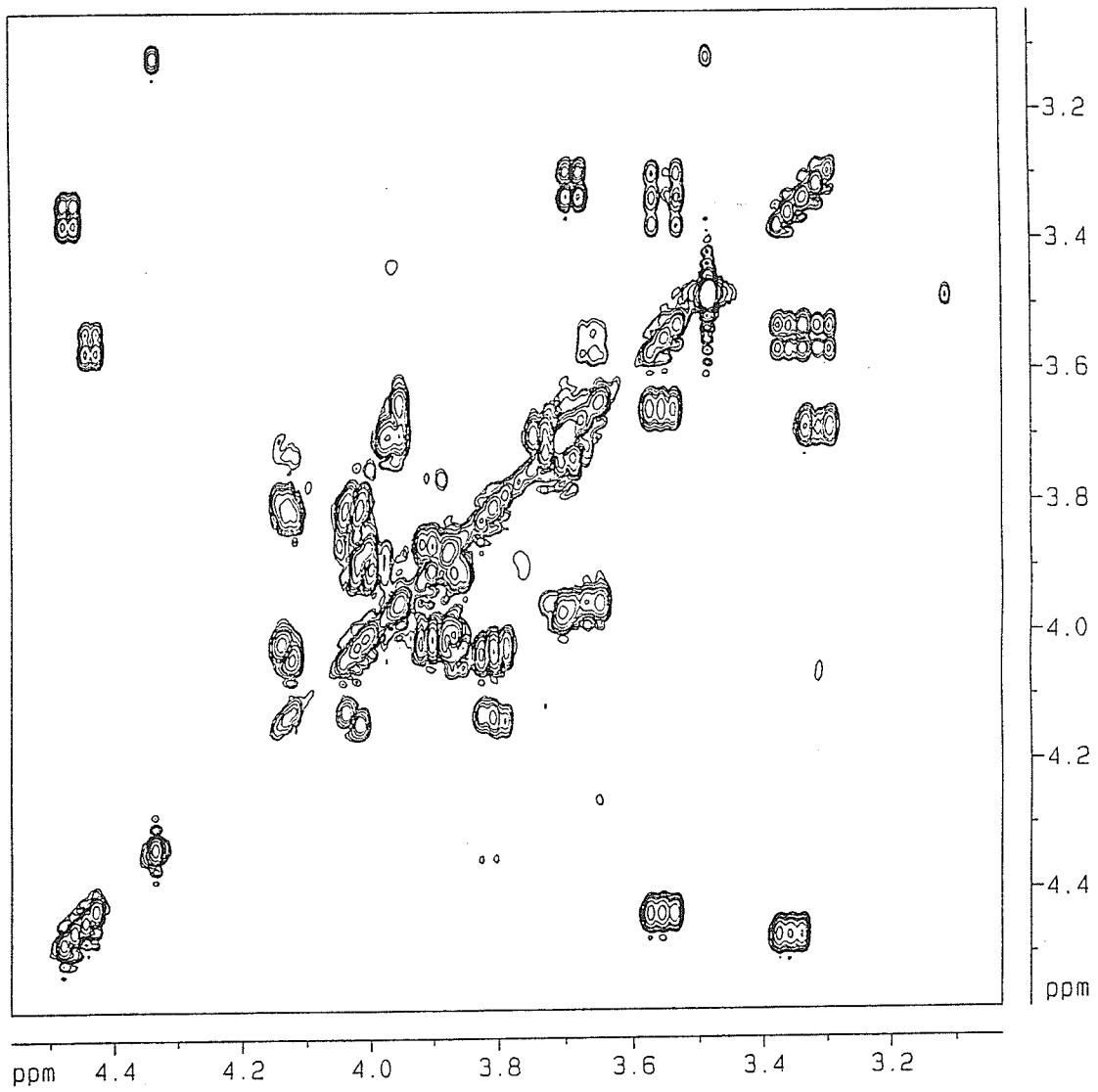


Fig.6.8a. COSY spectrum of oligosaccharide F-I.

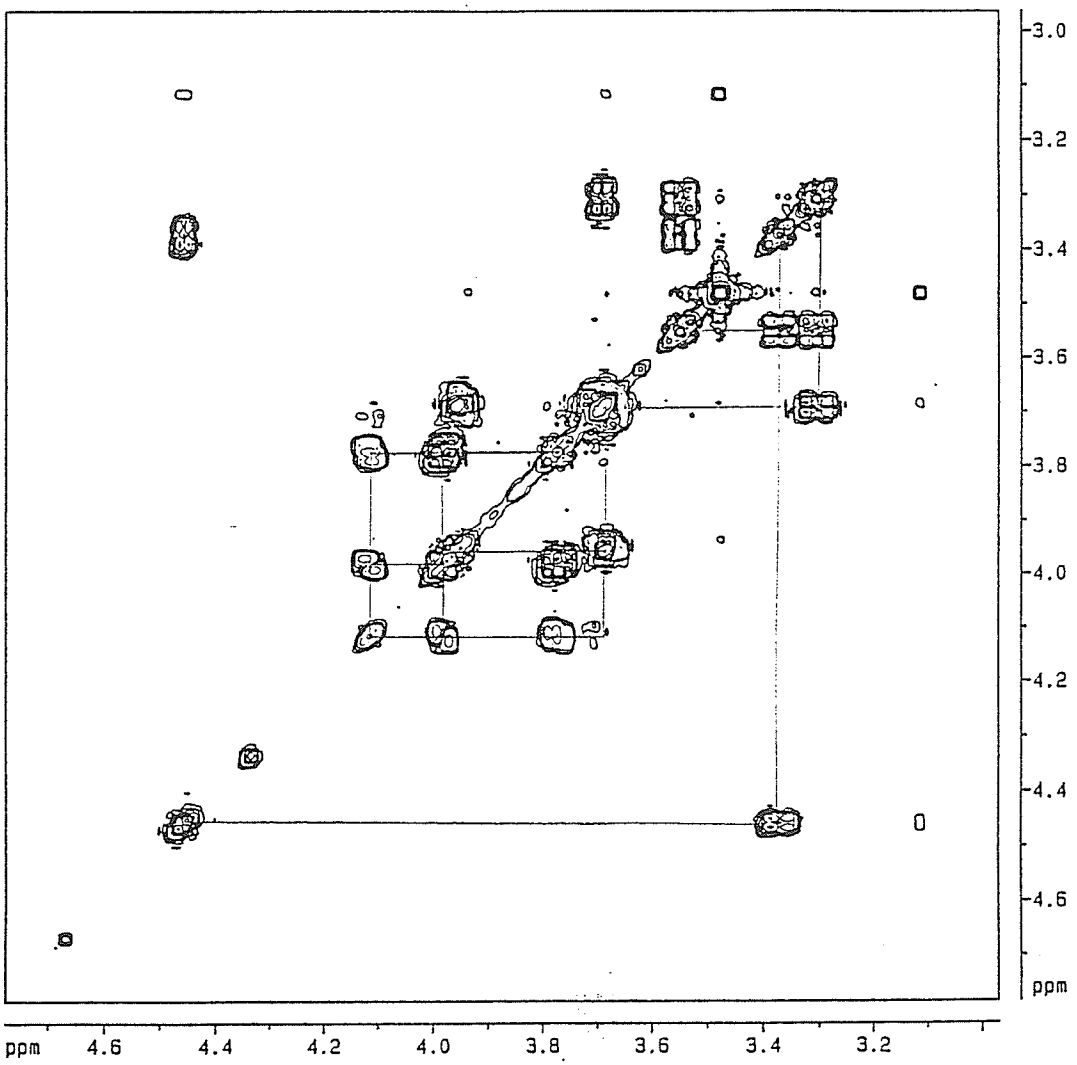


Fig.6.8b. COSY spectrum of oligosaccharide F-II.

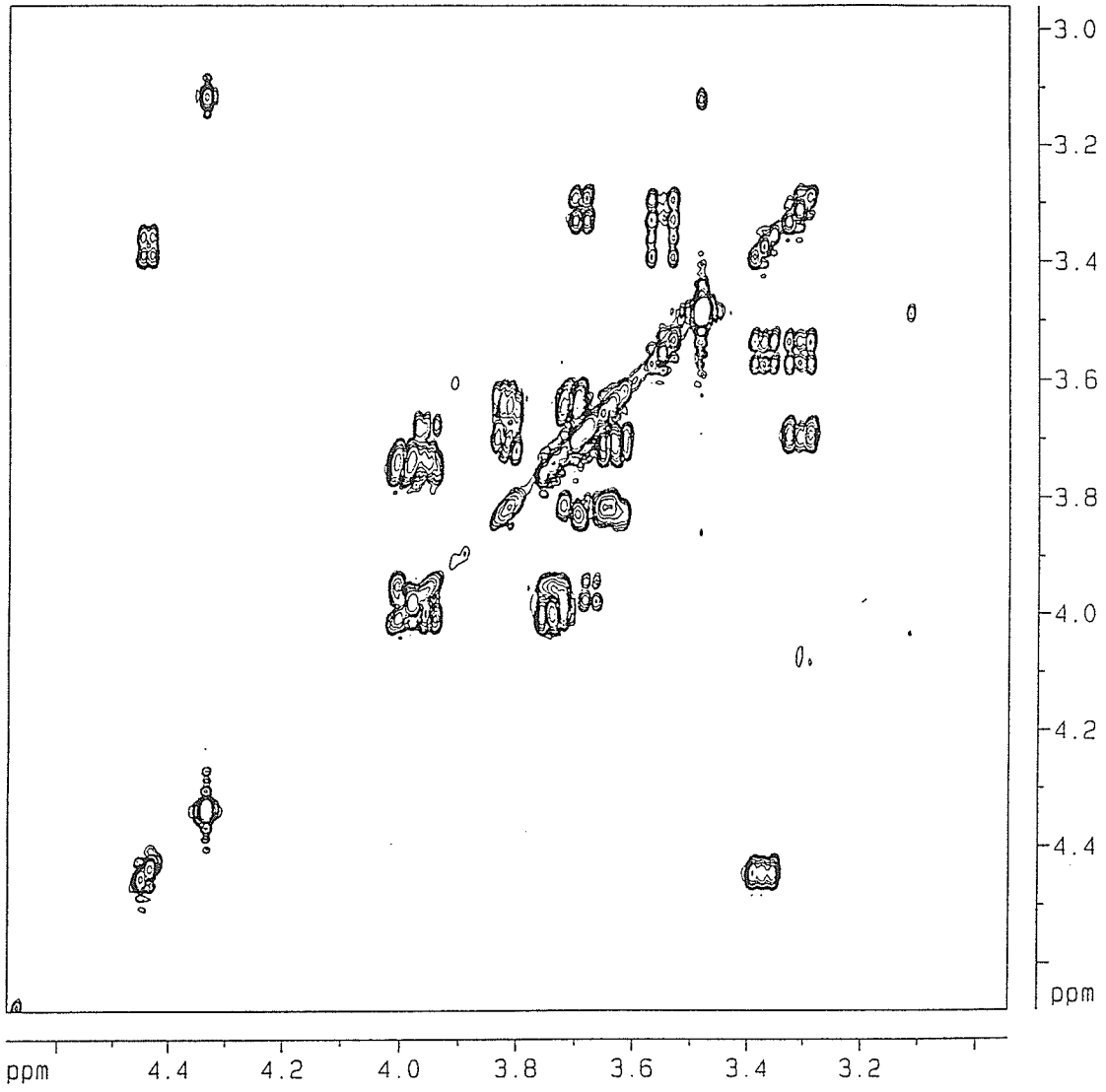


Fig.6.8c. COSY spectrum of oligosaccharide F-III.

Table 6.2. Assignment of ^{1}H and ^{13}C resonances of F-I, II and III.

	F-I		F-II		F-III	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
	(ppm)		(ppm)		(ppm)	
	GalA		GlcA		GalA	
1	103.0	4.50	103.0	4.48	103.0	4.46
2	74.0	3.39	74.2	3.39	94.0	3.38
3	76.0	3.57	76.0	3.56	76.0	3.57
4	82.8	3.35	83.0	3.34	83.0	3.33
5	77.3	3.72	77.5	3.71	72.5	3.68
6						
	Rham		Gal		GalA	
1	103.8	4.47	61.0	3.50	63.5[a]	3.65, 3.72
2	71.5	3.57	64.0	3.71	73.0	3.82
3	73.5	3.65	71.0	3.72	72.0	3.10
4	76.0	3.94	72.5	3.79	78.0	3.72
5	70.3	4.00	69.5	4.14	72.5	3.98
6	21.0	1.35	73.0	3.79, 3.97		
	Gal					
1	60.5	3.52				
2	64.0	3.70				
3	71.0	3.72				
4	72.5	3.79				
5	69.5	4.14				
6	73.0	3.80, 3.87				

[a]:tentative assignments.

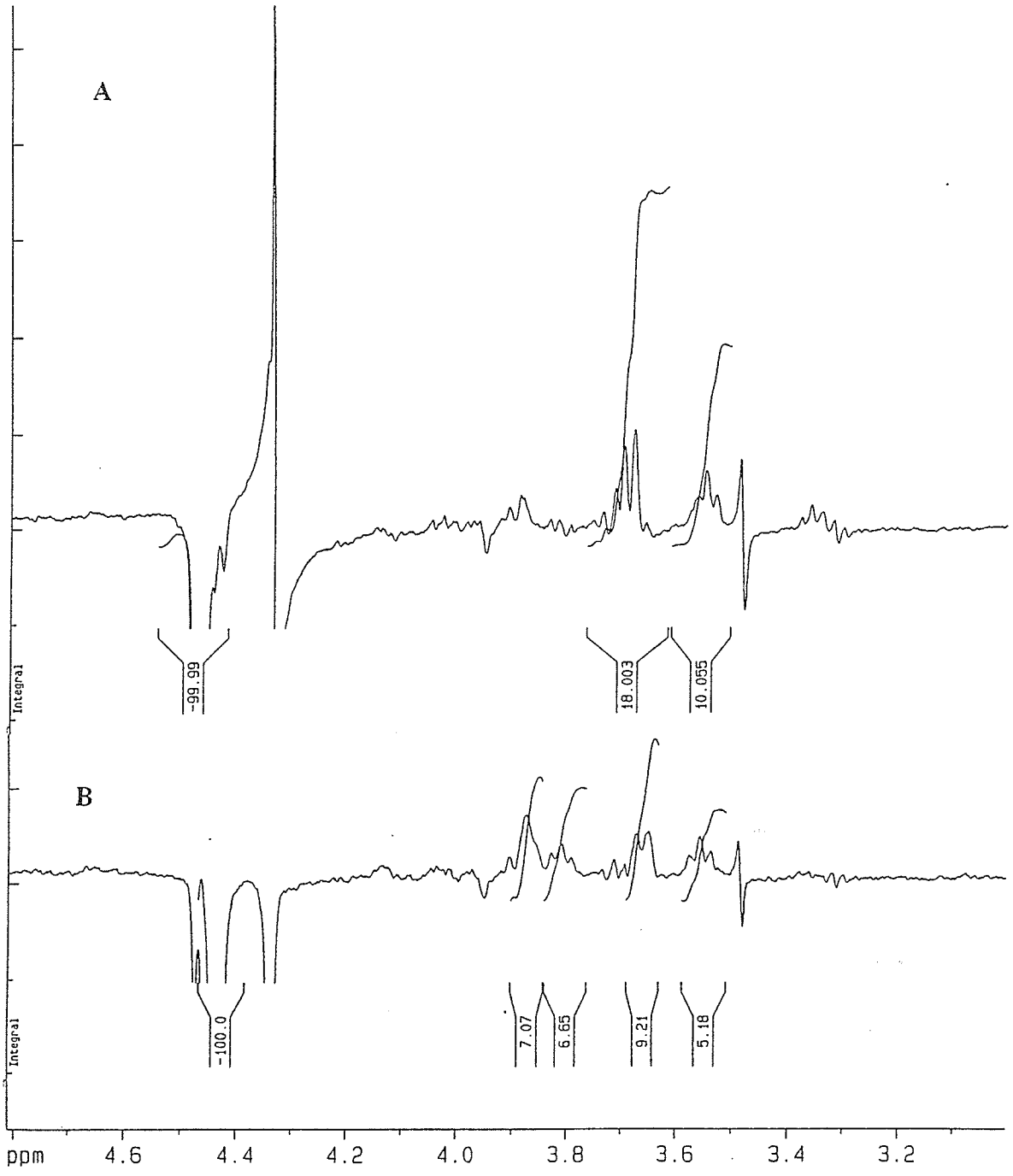


Fig. 6.9. Difference NOE spectra of F-I (A: low field; B: high field).

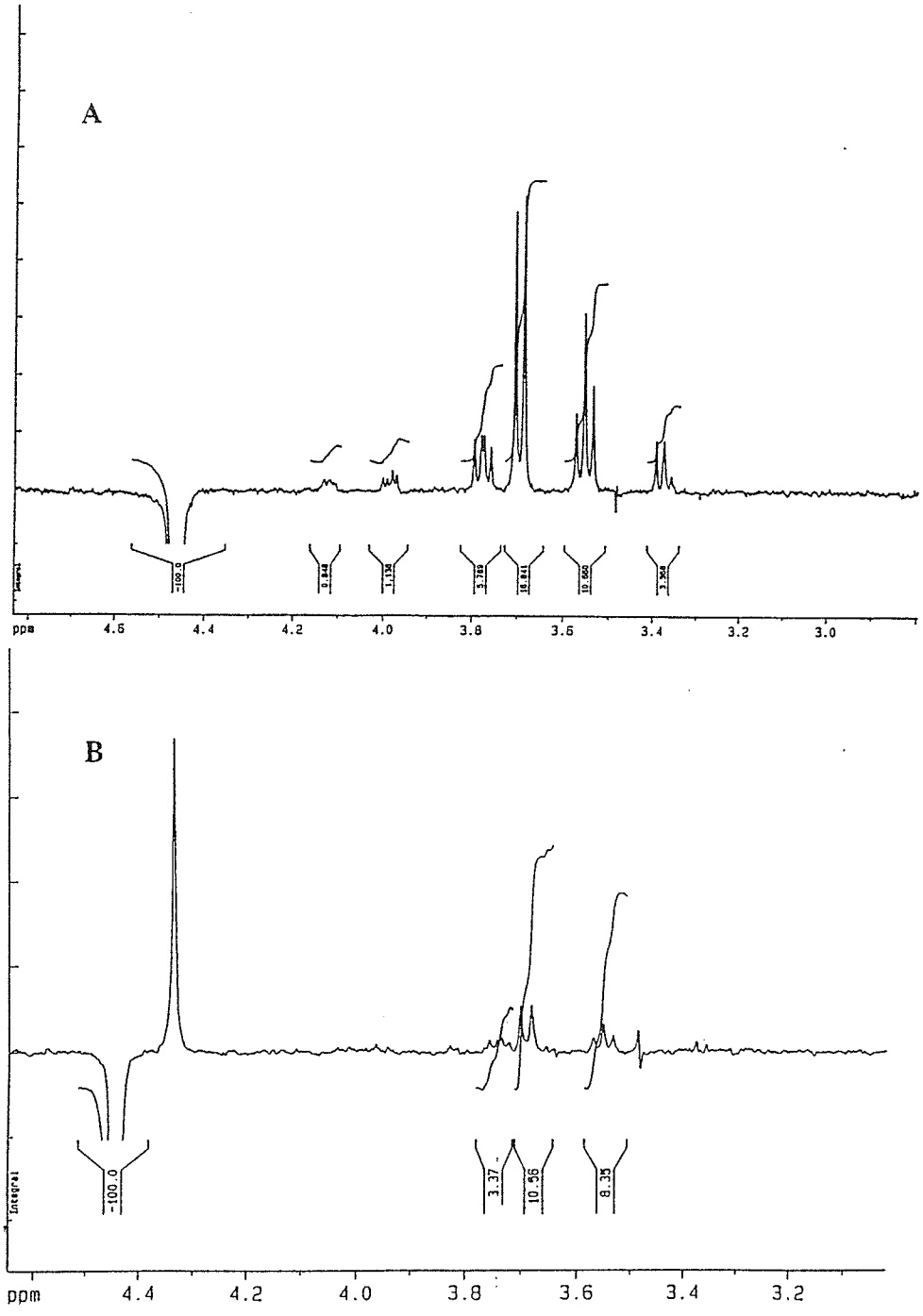


Fig. 6.10. Difference NOE spectra of F-II (A) and F-III (B).

The intra-residue dipole couplings of the anomeric resonance (H1) were observed for F-III which supported the assignment of the resonances (H3 at 3.57ppm and H5 at 3.68ppm). The inter-residue through space coupling was observed at δ 3.72 ppm. This allowed the assignment of the linkage site that non-reducing end galacturonic acid was linked at the 4 position of the reducing end galacturonic acid, as shown in structure 3.

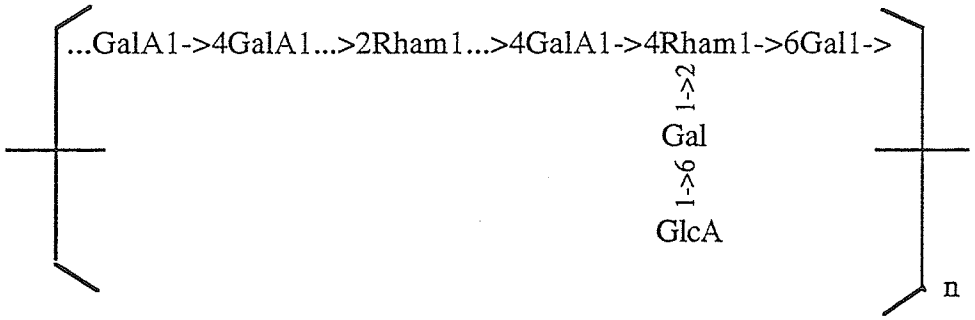
GalA1->4GalAtol

(3)

6.3.4 FAB-MS of Permethylated Oligosaccharide Alditols

FAB-MS of F-I after methylation confirmed that F-I was composed of three monosaccharide residues. Ion 658 could be attributed to the molecular ion while ion 426 was a fragment of the molecular ion (658) which lost a non-reducing end galacturonic acid through β-cleavage (Dell, 1987). Fragmentation via type D pathway resulted in an ion 454, which was 28 amu heavier than analogous pathway B ion. Ion 252 also arose by β-cleavage. This confirmed the sequence of F-I elucidated by NMR spectroscopy. The molecular ion of F-II was found as 486 $[M+H+H]^+$ which matched the formula of F-II (structure 2) after methylation. The presence of ion 426 was a consequence of fragmentation by losing the HCOOCH3 group while the presence of ion 252 was due to the loss of glucuronic acid through β-cleavage (Dell, 1987). This confirmed the sequence of F-II obtained from the NMR spectra. In the FAB-MS of F-III, ion 534 could be attributed to the molecular ion with two ammonium molecules (498+36). An ion at 484 could result from the loss of a CH_2 group from the molecular ion. While type A cleavage resulted in the ion of 233. This also is in agreement with the result of NMR spectra that F-III was composed of two galacturonic acids (structure 3). Considering the overall information, a possible average

structural unit for the pectic polysaccharide was proposed as follows (structure 4).



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CHAPTER 7

CHARACTERIZATION OF A 1,4-LINKED β-D-GLUCAN FROM YELLOW MUSTARD (*Sinapis alba L.*) MUCILAGE USING NMR SPECTROSCOPY

7.1 INTRODUCTION

A water-soluble 1,4-linked β-D-glucan (WSCS-I) isolated from yellow mustard (*Sinapis alba* L.) polysaccharides exhibited pronounced shear thinning behaviour in aqueous solution at 0.5% (Chapter 5). Although the linkage pattern was determined by methylation analysis, however, further structural information is required to understand its physical properties, particularly solubility and rheological behaviour in aqueous solution. This chapter focuses on the structural characterization of WSCS-I using one and two dimensional NMR spectroscopy.

7.2 MATERIALS AND METHODS

The water-soluble 1,4-linked β-D-glucan (WSCS-I) was isolated by ion exchange chromatography as described in Chapter 5. Methylation analysis was carried out according

to Ciucanu and Kerek (1984). All NMR spectra were recorded on a Bruker AMX 500 spectrometer according to the procedure described in Chapter 6.

7.3 RESULTS AND DISCUSSION

Methylation analysis revealed that WSCS-I contained mainly 1,4-linked β-D-glucose (Chapter 5). The six major resonances in the ^{13}C NMR spectrum of WSCS-I were assigned to C1 to C6 of the 1,4-linked β-D-glucose residue respectively by comparing with the literature data for cellulose-like materials as shown in Fig.7.1b (Defaye *et al.*, 1983; Bock et al., 1984). The major proton resonances in Fig. 7.1a were assigned with the assistance of the carbon-proton correlation spectrum (Fig. 7.2) and the results summarized in Table 7.1. The resonances of the anomeric proton at δ 4.54 ppm with a $H_{1,2}$ coupling constant of 7.5 Hz are in agreement with that of β conformation of D-glucose (Iwata *et al.*, 1992).

In addition to the resonances produced by the 1,4-linked β-D-glucan, there were some nonsugar resonances scattered at chemical shifts 9.04 ppm, 19.22 ppm and 47-55 ppm which could be attributed to CH_3 of methyl or ethyl groups while resonances between 65 to 70 could be due to the -O-CH_2- of the ethyl group (Kondo & Gray, 1991). The absence of resonances at 170 to 185 ppm confirmed these nonsugar resonances did not originate from acetyl groups (Iwata *et al.*, 1992). The assignment of nonsugar resonances in ^{1}H spectrum to its corresponding ^{13}C resonances is shown in Table 2.

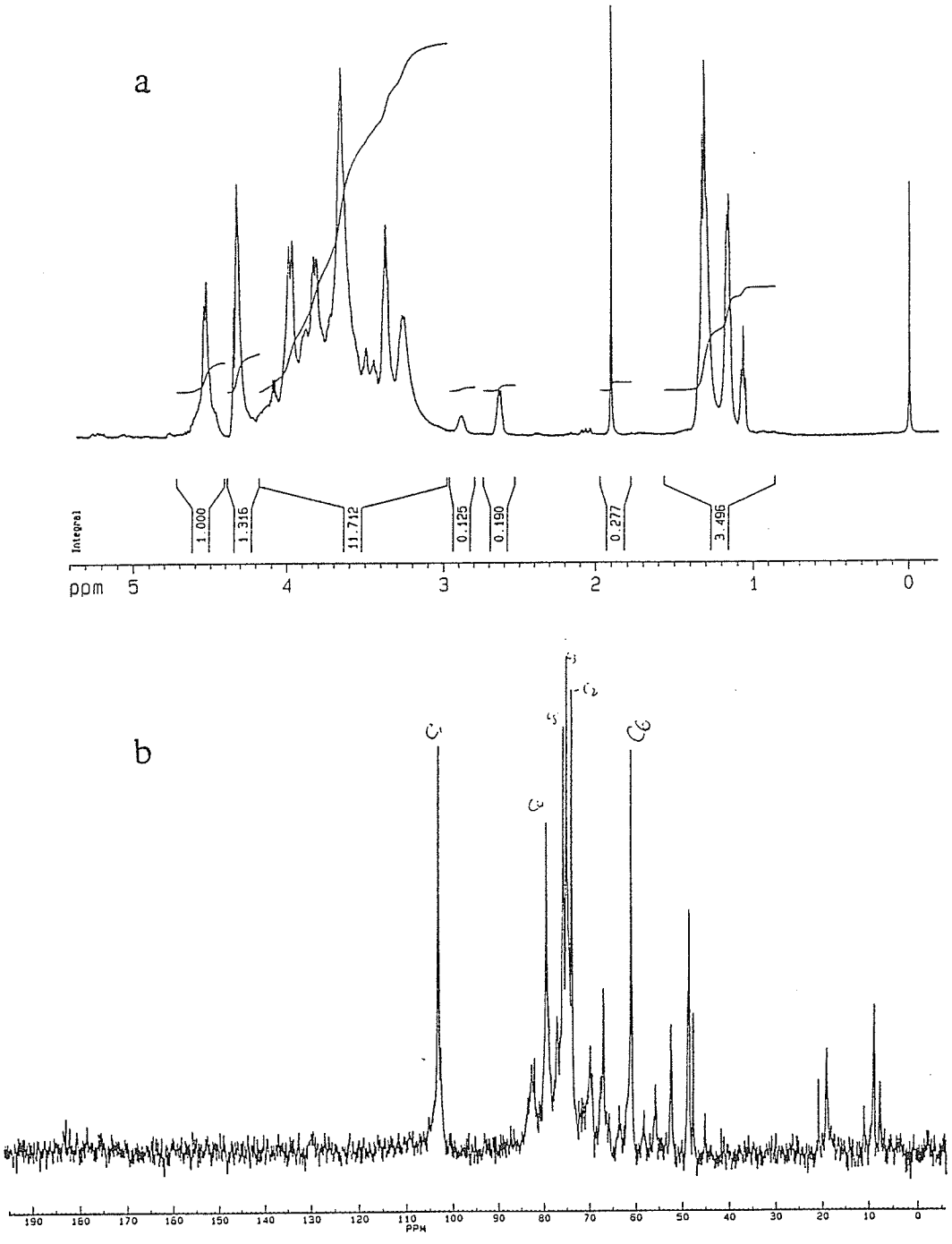


Fig. 7.1. ^{1}H (a) and ^{13}C (b) NMR spectra of 1,4-linked β-D-glucan from yellow mustard mucilage (WSCS-I).

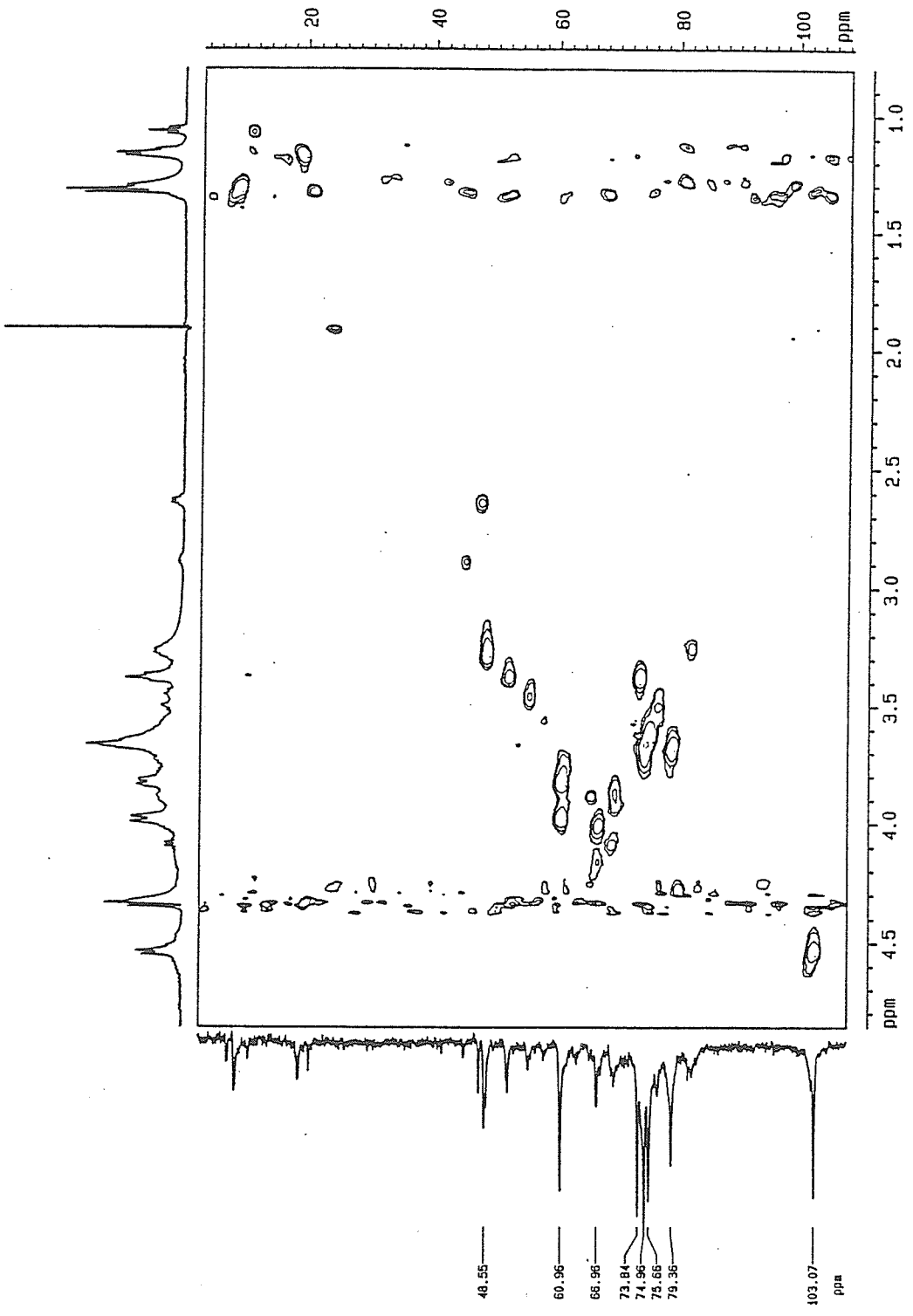


Fig. 7.2. H/C heteronuclear correlation NMR spectrum of 1,4-linked β-D-glucan from yellow mustard (WSCS-I).

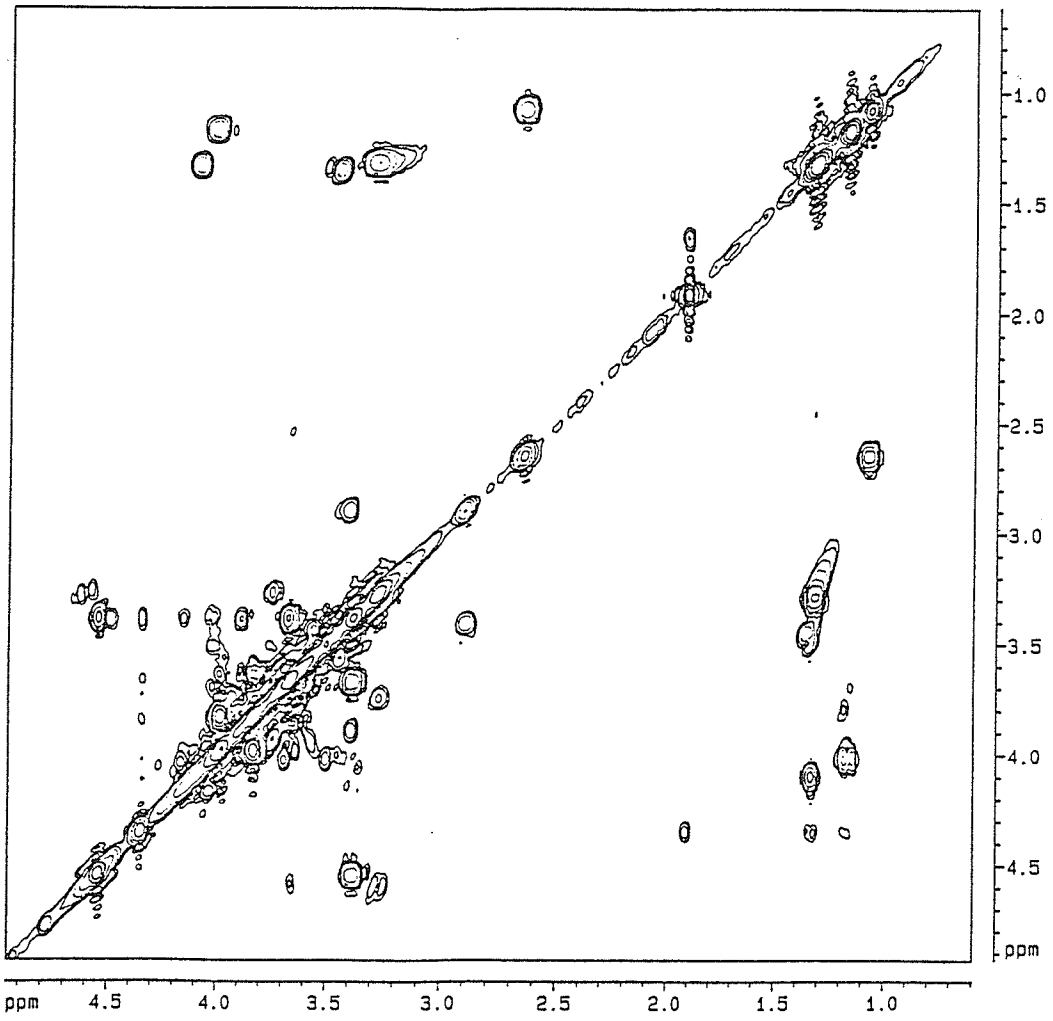


Fig. 7.3. Homonuclear shift correlated spectrum (COSY) of 1,4-linked β-D-glucan from yellow mustard (WSCS-I).

Table 7.1. Complete assignment of the major resonances of ^{1}H and ^{13}C spectra of WSCS-I.

	1(C,H)	2(C,H)	3(C,H)	4(C,H)	5(C,H)	6(C,H)
^{13}C ppm	103.08	73.84	74.95	79.36	75.67	60.96
^{1}H ppm	4.54	3.38	3.65	3.66	3.52	3.82,3.98
	$J_{1,2}$7.5 Hz					

Table 7.2. C/H correlations of unresolved nonsugar resonances.

H ppm	C ppm	possible assignment	referrence
1.07	11.0		
1.17	19.22	$-CH_3$	
1.32	9.04		Kondo and Gray
1.32	21.0	$-CH_3$	1991
2.65	47.65		
2.82	44.50		
3.265	48.50	methyl ethers	Tezuka et al.
3.32	52.40		1991
3.40	54.50		
3.88	63.50		
3.98	66.96	$O-CH_2$	Kondo and Gray
4.05	69.50		1991
3.88	69.79		
3.26	82.70		

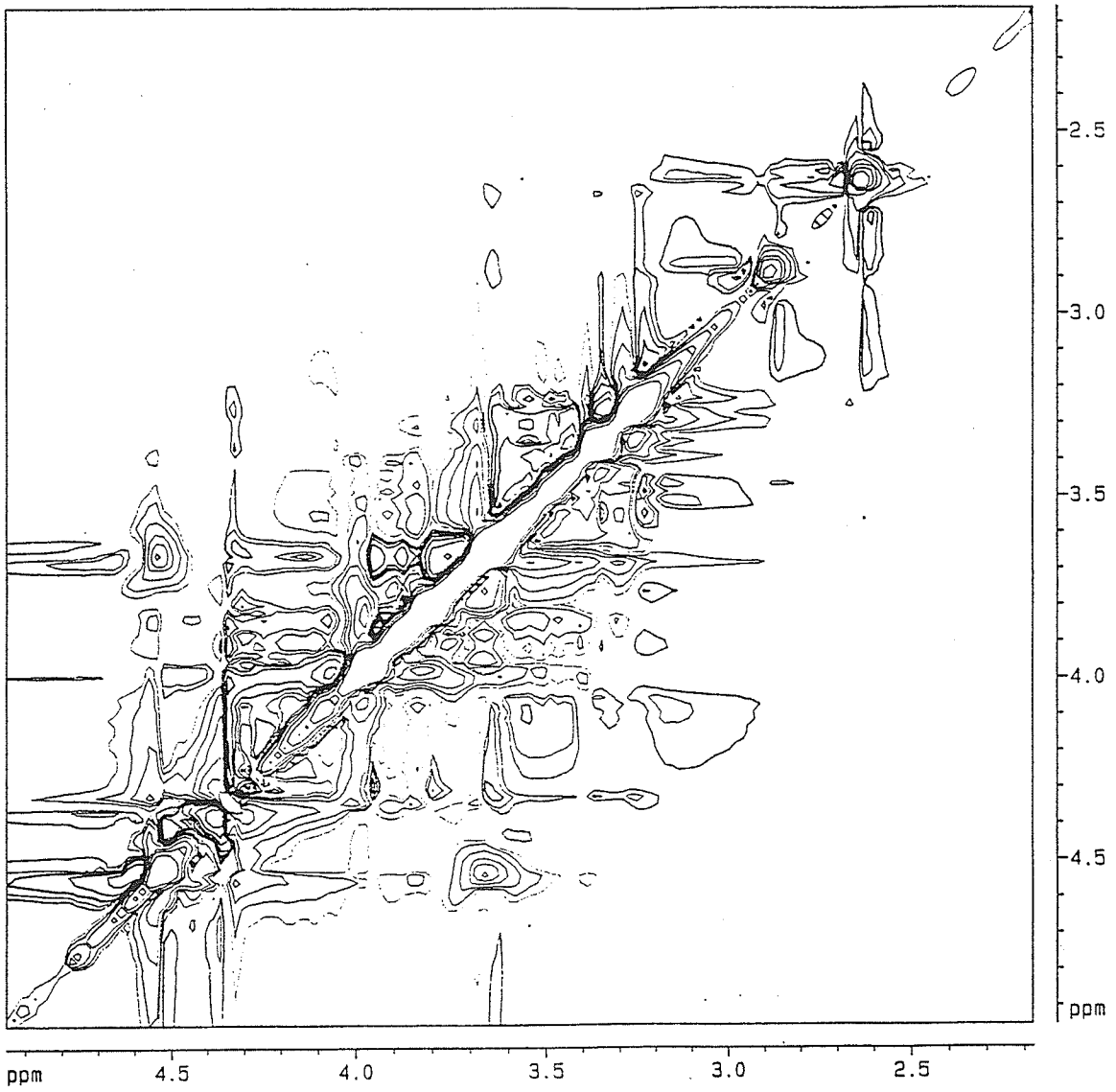


Fig. 7.4. NOESY of 1,4-linked β-D-glucan from yellow mustard.

The H-H homonuclear correlation (COSY) spectrum for the intra-residue connectivities of the 1,4-linked β-D-glucon is shown in Fig. 7.3. The connectivities of H1 and H2, H2 and H3 are obvious in Fig. 7.3 where H3 to H4 correlation cannot be observed due to close overlap of the two resonances (3.65 and 3.66 ppm respectively, Fig. 7.1a and Fig. 7.2). The nonsugar groups also showed some connectivities although further study is needed to elucidate their detailed structure in relation to the 1,4-linked β-D-glucopyranosyl backbone chain. The presence of nonsugar resonances might be the major factor contributing to the solubility of this material in aqueous solutions, because it is well known that pure 1,4-linked β-D-glucan (cellulose) is insoluble in water due to its stiff, compact structure. The presence of a nonsugar group on the cellulose-like backbone chain might act as "kink" which break down the conformational regularity of the backbone chain and prevent the normal intermolecular associations which produce insoluble cellulose fibers. 2D NOESY spectrum shown in Fig. 7.4 confirmed the 1,4-linked β-D-glucose sequence of WSCS-I on the basis of inter-residue dipole (through-space) correlation; the intra-residue dipole correlations was in agreement with the complete assignment for the ^{1}H and ^{13}C spectra shown in Table 7.1. A dipole correlation was observed between two resonances (2.85 and 3.39 ppm) which may indicate that the methyl group was attached on the 2 position of some of the 1,4-linked β-D-glucose residues. Some NOE correlations were also observed for the nonsugar resonances, however, it could not be resolved completely due to complexity of the spectrum.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Since the initial report of the presence of mucilage in yellow (or white) mustard seeds by Bailey and Norris (1932), there have been few studies carried out on its chemical composition or physical properties (Weber *et al.*, 1974; Woods and Downey, 1980; Vose, 1974; Theander *et al.*, 1977; Siddiqui *et al.*, 1986 and Anquilar and Ziegler, 1990). These studies, however, were based on mucilage extracted following the Weber's method (Weber *et al.*, 1974) which only yielded 2% mucilage. The low yield and limited information on the physical properties and chemical structure of yellow mustard mucilage has possibly limited its commercial exploitation and a theoretical understanding of the fuctional properties of this material. An improved extraction procedure developed in our laboratory more than doubled the yield of mucilage to 5% of yellow mustard seeds. The rheological data of the yellow mustard mucilage obtained by this method showed it exhibited shear thinning behaviour similar to that of xanthan gum. These results lead to the current study to: 1) maximize mucilage yield; 2) examine physical and chemical properties of the extracted mucilage; 3) separate yellow mustard mucilage into different fractions and evaluate the contribution of each fraction to the functional properties of yellow mustard mucilage and 4) characterize the linkage patterns and structural features of those polysaccharide fractions exhibiting shear thinning properties.

In the first phase of the study (Chapter 3), a 5% yield of crude mucilage (CM) was obtained from yellow mustard (*Sinapis alba*, L.) seeds compared to 2% reported previously

(Weber *et al.*, 1974 and Woods and Downey, 1980). CM was separated into a water-soluble fraction (WS, 55.6%) and a water-insoluble fraction (WI, 38.8%) by centrifugation. Proximate analysis of CM and its fractions revealed carbohydrates as the major component (80-94%) with ash (1.7-15%) and protein (2.2-4.4%) as minor constituents. Glucose (22-35%) was the major monosaccharide present followed by galactose (11-15%), mannose (6-6.4%), rhamnose (1.6-4.0%), arabinose (2.8-3.2%) and xylose (1.8-2.0%). These results were in general agreement with data reported by Siddiqui *et al.* (1986) but differed from those reported by other researchers (Vose, 1974 and Theander *et al.*, 1977). Differences in monosaccharide composition could be attributed to varietial differences and the method of extraction. CM and its fractions exhibited interfacial activity in terms of reducing water surface tension and showing both emulsion and foam capacity and stability. The emulsion capacity and stability of CM prior to dialysis were superior to the commmercial gums xanthan, guar and gum arabic. The substantial reduction of emulsion capacity and stability properties for CM after dialysis suggested that some small molecules made an important contribution to the emulsification properties of the crude yellow mustard mucilage. Shear thinning flow behaviours resembling xanthan gum were found for CM and its fractions in aqueous solutions or dispersions.

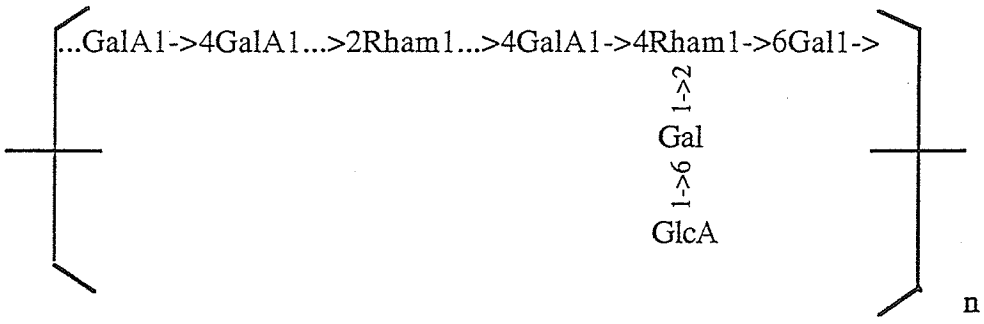
The second phase of this thesis focused on the water-soluble fraction (WS) as it was the major fraction of yellow mustard mucilage (55.6%) and exhibited pronounced shear thinning behaviour in aqueous solutions (Chapter 4). To evaluate the molecular basis for these rheological properties, WS was separated into a CTAB-precipitated fraction (WSCP, 52%) and a CTAB-soluble fraction (WSCS, 34%) by precipitation of the acidic polysaccharides with 5% CTAB under optimum conditions. The chemical structure and molecular size distributions of WSCP and WSCS were determined by methylation, ^{13}C

NMR and gel filtration chromatography while the rheological properties were examined by steady shear and dynamic oscillatory tests in aqueous solutions. The major fraction WSCP (52.0%) was mainly a mixture of pectic-like material, consisting primarily of galacturonic acid, glucuronic acid, galactose and rhamnose, and a 1,4-linked β-D-glucan. The minor fraction WSCS (34.0%) was also composed of at least two polysaccharide fractions differing in their molecular size and consisting mostly of neutral sugars and non-reducing end residues of glucuronic acid. The presence of glucuronic acid in yellow mustard mucilage was reported for the first time and confirmed by ^{13}C NMR spectrum. Both WSCP and WSCS contributed to the rheological properties of WS although they have different structures and molecular weight distributions. WSCP exhibited similar rheological behaviour to WS as assessed by both dynamic and steady shear flow measurements. In comparison, fraction WSCS showed only a steady shear rheological pattern similar to WS and was less affected by electrolytes. The minor response of WSCS to added salts reflected fewer acidic groups and absence of 1,4-linked galacturonic acid.

Although WSCP and WSCS were less complex compared to WS, they were still heterogeneous, limiting a full interpretation of the influence of structure on rheological properties. The third phase of this thesis aimed at further isolation and fractionation of WSCP and WSCS in order to examine more closely the relation of chemical structure to rheological properties (Chapter 5). Both WSCP and WSCS were separated into 5 sub-fractions by ion exchange chromatography on a DEAE-high capacity cellulose column. Of the ten sub-fractions obtained, two neutral fractions (WSCP-I and WSCS-I) and a pectic fraction (WSCP-III) continued to pocsses the typical shear thinning properties of yellow mustard mucilage. The remaining fractions exhibited Newtonian-like flow behaviour although their viscosities varied. Monosaccharide analysis, methylation analysis and/or ^{13}C NMR

spectra revealed that both WSCP-I and WSCS-I were composed mainly of 1,4-linked β-D- glucose although there were far more other sugars in WSCP-I compared to WSCS-I. WSCP-III, the pectic-like fraction exhibiting shear thinning behaviour in solution, appeared to be relatively homogeneous being composed of a non-reducing end glucuronic acid (13.3%), 1,4-linked galacturonic acid (13.6%), 1,6-linked galactose (22.9%), 1,2-linked (11%) and 1,2,4-linked (18%) rhamnose. WSCP-I was found as the major fraction responsible for the shear thinning properties of yellow mustard polysaccharides. The poor solubility of this fraction in aqueous solution, however, prevented further structural analysis of this material.

In the final phase of this thesis, further structural analysis of WSCP-III and WSCS-I, the two water-soluble fractions which exhibited shear thinning behaviour, was carried out. The structure of WSCP-III, a pectic polysaccharide,was determined by methylation analysis, FAB-MS and NMR spectroscopy (Chapter 6). The polymer was partially hydrolysed, and the resulting oligosaccharides isolated and purified by ion exchange and gel filtration. The purified oligosaccharides were characterized by FAB-MS and one and two-dimensional NMR spectroscopy. A possible average structural unit was proposed as follows:



(4)

WSCS-I, a water-soluble 1,4-linked β D-glucan exhibiting shear thinning behaviour, was also characterised by one and two dimensional NMR spectroscopy (Chapter 7). The complete assignment of the ^{1}H and ^{13}C resonances was achieved with the assistance of heteronuclear shift correlated experiments. The connectivities within residue were obtained by homonuclear corrrelation (COSY) while NOE correlation confirmed the 1,4-linked backbone structure. In addition, methyl and/or ethyl groups were found associated with the cellulose-like polysaccharide which presumably influence the solubility and flow behaviour of this material in aqueous solution.

In conclusion, a yield of yellow mustard mucilage was obtained at 5% of total seed weight. The rheological properties of yellow mustard mucilage resembled xanthan gum in aqueous solutions or dispersions in terms of viscoelastic behaviour of its aqueous dispersions. The removal of small molecules and minerals reduced emulsification capacity and stability substantially. The shear thinning behaviour of yellow mustard mucilage was attributed to three fractions: WSCP-I, WSCP-III and WSCS-I. The two neutral fractions were mainly composed of 1,4-linked β-D-glucan similar to the backbone chain of xanthan gum, xyloglucan and cellulose. The backbone of the pectic-like polysaccharide appeared to be composed of 1,4-linked galacturonic acid, 1,2-linked and 1,2,4-linked rhamnose and 1,6-linked galactose with branches of glucuronic acid attached to the backbone chain through 1,6-linked galactose at the 2 or 4 position of the 1,2,4-linked rhamnose residues. This pectic polysaccharide may be responsible for the sensitivity of yellow mustard mucilage to added salt.

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