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Hydrodynamic characterisation of the molar mass and gross conformation of corn cob heteroxylan AGX

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The water-soluble fraction of arabinoglucuronoxylan AGX, extracted from corn cob has been studied previously using a combination of static light scattering and viscometry (A. Ebringerova *et al.* (1992). *Carbohydr. Polym.* **19**, 99–105). The non-linearity of the resulting Mark–Houwink relation was interpreted as being due to possible self-association/aggregation phenomena. The aim of the present study is to investigate this possibility further, together with the size and gross conformation of xylan, using analytical ultracentrifugation and viscometry. Studies were conducted in a phosphate–chloride buffer (pH = 6.8, $I = 0.10$).

Sedimentation equilibrium in the analytical ultracentrifuge gave absolute weight- and z-average molar masses of $(1.50 \pm 0.15) \times 10^5$ g/mol and $(1.91 \pm 0.20) \times 10^5$ g/mol using, respectively, Rayleigh and Schlieren optical systems to record equilibrium solute distributions. A value for the second thermodynamic virial coefficient, B of $(2.28 \pm 0.05) \times 10^{-4}$ ml. mol/g², from the Rayleigh distribution and a similar value from the Schlieren optical records $\{\sim 2.6 \times 10^{-4}$ ml. mol/g²$\}$ were obtained. Capillary viscometry yielded an intrinsic viscosity $[\eta] = (41.6 \pm 2.2)$ ml/g and a Huggins constant K_η of $\sim (3.6 \pm 0.6)$ Sedimentation velocity using a novel application of the Beckman Optima XL-A ultracentrifuge gave an infinite dilution sedimentation coefficient $s^\circ_{20,w} = (0.99 \pm 0.20)S$. No direct evidence could be seen of any 'simple' self-association phenomena from neither the concentration dependence of the apparent molar masses, $M_{w,app}$, $M_{z,app}$, from the concentration dependence of $s_{20,w}$, nor from the shape of the sedimenting boundaries. Analysis of the conformation by the Π-function and the frictional ratio (in terms of the equivalent hydrodynamic ellipsoid) suggested that this macromolecule adopts a highly expanded conformation in solution consistent with an asymmetric coiled structure. Further comment is made on the low values for $[\eta]$ in this and a previous study, in terms of the presence of compact supramolecular aggregates.

INTRODUCTION

Xylan is the second most abundant polysaccharide known to man and is found in plant cell walls. It belongs to the hemicellulose class of polysaccharides and is composed of a β-1,4-D-xylopyranan backbone to which single 4-O-methyl-α-D-glucuronosyl and/or α-L-arabinofuranosyl side chains are linked. Additional oligosaccharide side chains may also occur. The actual substitution pattern varies according to the source of the xylan (Stephen, 1983).

The heteroxylans from corn cob have found use in a number of applications ranging from use in textile printing to the pulp and paper industries (Naterova *et*

al., 1986; Bartos *et al.*, 1990; Lichneova *et al.*, 1991). Recently, the water-soluble corn cob xylan was shown to exhibit significant immunomodulating properties in mitogenic tests (Ebringerova *et al.*, 1995).

In a recent study by Ebringerova *et al.* (1992) it was shown that the water-soluble fractions of corn cob xylan (known as 'ws-AGX' and 'AGX-H') represent the arabinoglucuronoxylan type which contains, in contrast to the water-insoluble fraction, additional side chains comprising 2-O-β-D-xylopyranosyl-α-L-arabinofuranosyl moieties. Light scattering and viscometry were used as the primary analytical tools: analysis of the intrinsic viscosity $[\eta]$ and weight-average molar mass M_w data on subfractions of ws-AGX and AGX-H by

use of the Mark–Houwink (viscosity) relation revealed that there was a linear relation between $[\eta]$ and M_w up to a 'critical molar mass'. Above this value there was no further increase in $[\eta]$, indicating a change in conformation from a coil to a more compact spherical form. For the fraction ws-AGX this 'critical' M_w value was ~350,000 g/mol whereas for AGX-H it was approximately an order of magnitude greater. The reason for this behaviour was not fully understood but the suggestion was made that this could be attributed to aggregation or an increase in the degree of branching. Ebringerova *et al.* (1992) concluded that since the degree, and pattern, of arabinose substitution is related to the propensity for aggregation (Dea *et al.*, 1973; Morris *et al.*, 1977) and as the total amount of substituents was low for AGX (<30%) the M_w-independent behaviour of $[\eta]$ was due to aggregation. This concept of arabinoxylan self-association is not a novel one and has been observed in studies of xylan extracted from alternative sources such as wheat and sugar cane (Blake & Richards, 1971; Dea *et al.*, 1973; Andrewartha *et al.*, 1979).

The purpose of this present study was to try and shed further light on the solution properties of the water-soluble component of the corn cob heteroxylan, ws-AGX. By combining data from low-speed sedimentation equilibrium, sedimentation velocity, isopycnic density gradient analytical ultracentrifugation and viscometry, information regarding the purity/homogeneity, molar mass, polydispersity and gross conformation/ expansion of this polysaccharide, and the nature of any suspected self-association or supramolecular aggregate phenomena was obtained.

MATERIALS AND METHODS

Xylan

The raw heteroxylan, L-arabino-(4-*O*-methyl-D-glucurono)-D xylan (AGX) was isolated from an alkaline extract of the mill corn cob by ethanol precipitation (Ebringerova *et al.*, 1988) and its water-soluble fraction (ws-AGX) was separated by centrifugation from the insoluble fraction and freeze-dried.

Solutions

Xylan solutions were prepared by initially dissolving ws-AGX in deionized distilled water and allowing it to hydrate overnight at 4°C. The resulting solution was then dialysed exhautively against a phosphate chloride buffer, pH = 6.8, I = 0.10, prepared by dissolving 4.595 g $Na_2HPO_4.12H_2O$, 1.561 g KH_2PO_4 per litre of deionized distilled water and adding NaCl to give a combined ionic strength of 0.1, following Green (1933). Concentrations, c (g/ml) were corrected for moisture

content and in sedimentation velocity experiments, for radial dilution.

Sample purity

This was investigated using two independent methods:

(i) Spectrophotometrically. Measurement of the absorbance spectrum of a 0.6 mg/ml solution from 200 to 400 nm with a Beckman DU-50 spectrophotometer was used to check for the possible presence of protein or nucleic acid.

(ii) Analytical isopycnic density gradient ultracentrifugation (Ifft & Vinograd, 1966; Creeth & Horton, 1977). A summary of the usefulness of this technique for assaying the purity of polysaccharide solutions has been given in Harding (1992).

Macromolecular species, when centrifuged in an equilibrium density gradient of a dense salt (usually a caesium salt) attain equilibrium positions in the salt distribution according to their density. Proteins find an 'isopycnic' density of ~1.3 g/ml; glycoproteins 1.3–1.6 g/ml depending on the composition; polysaccharides ~1.6 g/ml and nucleic acids 1.6–1.7 g/ml.

In this study, a 2 mg/ml solution of xylan in 0.67 g/ml CsCl (corresponding to a loading density of 1.5 g/ml (Creeth & Horton, 1977)) was then run in an MSE (Crawley, UK) MkII analytical ultracentrifuge equipped with a Schlieren optical system, a mercury arc light scource and an on-line automatic photographic system and operated at a speed of 35,000 rpm at a temperature of 20.0°C until equilibrium was attained (after 48 h). The photographic record was analysed using a computer graphics tablet.

Viscometry

An Ostwald-type automatic Schott–Geräte automatic viscometer was used. The temperature was regulated by suspension of the viscometer in a thermostatically controlled Comark No. 2 water bath at (25.00 ±0.05)°C and recorded using an accurately calibrated platinum resistance thermometer. Relative kinematic viscosities were found from the solution:solvent flow time ratio: as the concentrations were sufficiently small (1.5–5.0 mg/ ml) to neglect density corrections, it was considered reasonable to assume that kinematic viscosity parameters approximate dynamic viscosity parameters (Tanford, 1955). The intrinsic viscosity $[\eta]$ (ml/g) was then found by plotting reduced specific viscosity η_{red} vs concentration, c (g/ml) and extrapolating to infinite dilution according to Huggins (1942):

$$\eta_{red} = [\eta](1 + K_{\eta}[\eta]c) \tag{1}$$

where K_{η} is the Huggins constant.

Sedimentation velocity

Sedimentation velocity measurements were performed using a Beckman Optima XL-A (Palo Alto, USA) analytical ultracentrifuge. Recently it has been shown that 'Toepler–Schlieren' traces may be obtained by certain XL-A instruments when the macromolecular solution is scanned at wavelengths away from any absorption maxima (Cölfen & Harding, 1995). In a further recent study (Dhami *et al.*, 1995) on a range of polysaccharides, sedimentation coefficients obtained by simultaneous 'Toepler–Schlieren' (Lloyd, 1974) images from the XL-A and conventional phase-plate or 'Philpott–Svennson' optics on a Beckman Model E ultracentrifuge have been shown to be comparable. Advantage is taken of this feature here. An initial wavelength scan (see Cölfen & Harding, 1995) at low speed in the XL-A revealed that a wavelength of 440 nm was appropriate for the Schlie-ren measurements. A speed of 60,000 rpm was then chosen at a scanning interval of 16 min. A total of eight scans was used for each concentration. Succes-sive radial scans were analysed via a computer graphics digitizing tablet using a routine known as XLAVEL to obtain sedimentation coefficient and radial dilution factors. Sedimentation coefficients were then corrected to standard conditions of density and viscosity (density and viscosity of water as solvent at 20°C) (see Tanford, 1961; Van Holde, 1985). The partial specific volume, $\bar{v}$ was (0.625 ± 0.006) ml/g. The 'infinite dilution' sedimenta-tion coefficient $s°_{20,w}$ (s) was found from the intercept of a plot of the reciprocal of the apparent sedi-mentation coefficient $s°_{20,w}$ against concentration, c (g/ml) (corrected for radial dilution), fitted to the relation (Schachman, 1959, p.91; Creeth & Knight, 1965):

$$1/s_{20,w} = (1/s°_{20,w}).(1 + k_s c) \qquad (2)$$

Low-speed sedimentation equilibrium

Molar mass parameters for the xylan preparations were determined using the technique of low-speed or 'inter-mediate speed' (Creeth & Harding, 1982) sedimentation equilibrium in the analytical ultracentrifuge. As with the viscometry and sedimentation velocity studies, measurements were made at a series of concentrations, and, by extrapolation to infinite dilution, the absolute weight-average, M_w and z-average, M_z molar masses found. The type of average molar mass parameter obtained directly is dependent upon the optical system used. The Rayleigh interference system yields directly weight-average molar masses, M_w, whereas the Schlie-ren optical system provides directly measurements of M_z. From these the highly useful polydispersity index (M_z/M_w) can then be obtained.

(a) M_w determination. Apparent weight-average molar masses, $M_{w,app}$ of xylan ws-AGX ranging in load-ing concentration from 0.5 to 3.0 mg/ml were measured using a Beckman Model E analytical ultracentrifuge equipped with a resistance/tempera-ture indicator control unit, a Rayleigh interference optical system, a 5 mW He–Ne laser light source and a photographic system: 30 or 12 mm (depend-ing on the concentration) optical path length double-sector cells were used with 100 μl in the solution channel and 110 μl in the solvent channel. The ultracentrifuge was run at 6000 rpm at 20°C for 48–64 h until equilibrium was attained. Solute distributions at equilibrium were captured photo-graphically and then digitized using an LKB (Bromma, Sweden) Ultroscan laser densitometer. The digitized data were then analysed using the Fourier-cosine series TURBO-PASCAL algorithm ANALYSER (Harding & Rowe, 1987; Rowe *et al.*, 1992) which gives an accurate record of concentra-tion (in relative fringe displacement units) vs radial distance. This distribution was interpreted using the routine MSTARI (Harding et al., 1992) which calculates amongst other things $M_{w,app}$ over the whole distribution using a function known as M^* (Creeth & Harding, 1982). M_w and the second thermodynamic virial coefficient, B (ml.mol/g^2) can both in principle be obtained from a plot of (1/ $M_{w,app}$) vs c (Tanford, 1961)

$$(1/M_{w,app}) = (1/M_w) + 2Bc \qquad (3)$$

although for polysaccharides which tend to give curved plots a more safer extrapolation procedure for obtain-ing M_w is to plot $M_{w,app}$ vs c directly and then graphi-cally extrapolate to $c = 0$ (see, e.g. Harding, 1992). Since B does not require an extrapolation, equation 3 is usually reliable for the estimation of B.

z-Average molar masses for the whole solute distri-bution can also be obtained in principle from Rayleigh optics (see Creeth & Pain, 1967) but this requires differentiation of the basic fringe data, leading to an amplification of any noise. A much better way is to use (phase-plate) Schlieren optical traces, which yield M_z directly, as the following describes.

(b) M_z determination. The apparent z-average molar masses $M_{z,app}$ at loading concentrations ranging from 1 to 5 mg/ml were measured using the MSE MkII analytical ultracentrifuge equipped with an Xenon light source, a phase-plate Schlieren optical system and on-line photography. A loading volume of 160 μl of each xylan solution was used. An oper-ating speed of 6000 rpm was used, and equilibrium was obtained in 48–64 h, and the distribution photographed. Solvent baselines were obtained by overspeeding at 30,000 rpm. Equilibrium and base-line photographs were analysed using the BASIC

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algorithm SEQMZSCH (written for PC by Dr. H. Cölfen of this laboratory) via an IBM personal computer interfaced to a graphics tablet, and M_z obtained from the Lamm equation (see, e.g: Creeth & Pain, 1967). Marler *et al.* (1964) have shown that the optimal way of extracting M_z from $M_{z,app}$ is from a plot of the inverse square root against concentration:

$$(1/M_{z,app})^{1/2} = (1/M_z)^{1/2}\{1 + 2BM_z^{1/2}c\} \tag{4}$$

and this procedure is followed here.

Table 1. Hydrodynamic parameters for ws-AGX xylan

Parameter	
M_w (g/mol) $\times 10^{-5}$	1.50 ± 0.15
M_z (g/mol) $\times 10^{-6}$	1.91 ± 0.20
M_z/M_w	1.3 ± 0.2
B (ml mol g^2) $\times 10^4$	2.28 ± 0.05
$[\eta]$ (ml/g)	41.6 ± 2.2
K_η	3.6 ± 0.6
$s^\circ_{20,w}$ (s) $\times 10^{13}$	0.99 ± 0.20
Π	1.7 ± 0.5
f/f_0	15 ± 5

RESULTS AND DISCUSSION

A summary of the hydrodynamic properties obtained in this study is given in Table 1.

Homogeneity

Absorbance spectra of a 0.6 mg/ml solution of xylan ws-AGX in a 1 cm path length cuvette revealed the presence of species absorbing at 280 nm which remained after dialysis. This led us to suspect the presence of protein in the preparation. Analytical isopycnic density gradient ultracentrifugation in CsCl, however, demonstrated this was not the case. The gradient was chosen so that the density at the meniscus was 1.466 g/ml and the base was 1.535 g/ml. Polysaccharide would accumulate at the base, and any protein at the meniscus. Although the polysaccharide is clearly present (Fig. 1), the absence of a significant trace at the meniscus implies the UV-

absorbance observed in the spectrophotometer is not protein but some other species: the most likely explanation is the presence of trace amounts of the strongly UV-absorbing lignin, known to be present in xylan preparations (Ebringerova, 1992). Confirmation of the absence of significant amounts of macromolecular impurity comes from the Toepler–Schlieren images from sedimentation velocity (Fig. 2). Despite the noise, (an unavoidable feature of velocity scans with the XL-A) only single shoulderless sedimenting boundaries were observed at the concentrations studied.

Molar mass parameters: sedimentation equilibrium

The weight-average molar mass from this study is in good agreement with the previous study by Ebringerova *et al.* (1992). From static light scattering measurements (and so-called 'Guinier plots') of ws-AGX after ultracentrifugation, a weight-average molar mass

↑
meniscus

↑
base

Fig. 1. Isopycnic density gradient sedimentation equilibrium profile in CsCl for ws-AGX. Temperature = 25.0°C. Solvent I = 0.10, pH = 6.8. Loading concentration of xylan ws-AGX = 2.90 mg/ml. Rotor speed = 35,000 rpm. Density at the meniscus = 1.466g/ml; density at the cell base = 1.535 g/ml. The presence of polysaccharide (cell base) can be clearly seen, but no evidence of significant amounts of protein (meniscus).
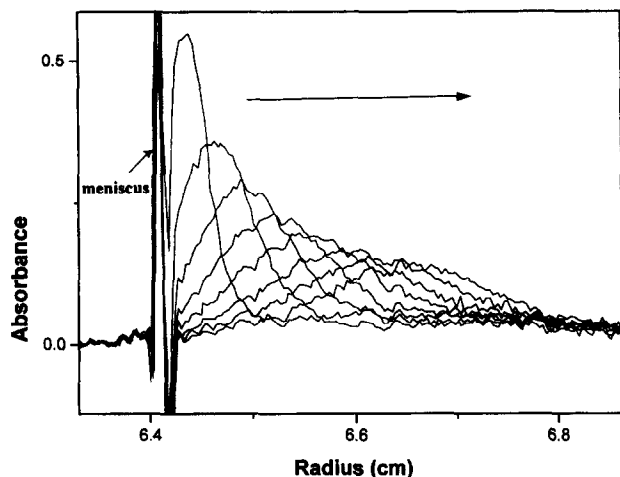
Fig. 2. Toepler–Schlieren sedimentation diagrams from radial scans at a wavelength of 440 nm of ws-AGX. Cell loading concentration = 2.0 mg/ml; rotor speed 60,000 rpm, temperature = 20.0°C; scan interval = 13 min. Direction of sedimentation (arrow) is from left to right.

Fig. 3. Plot of $M_{w,app}$ vs cell loading concentration c. From sedimentation equilibrium distributions recorded using Rayleigh interference optics. Graphical extrapolation procedure to $c = 0$. $M_w = (1.50 \pm 0.15) \times 10^5$ g/mol.

$M_w = (1.62 \pm 0.01) \times 10^5$ g/mol had previously been found. In the present study, direct extrapolation of the $M_{w,app}$ vs c data (Fig. 3) from sedimentation equilibrium yields a value of $(1.50 \pm 0.15) \times 10^5$ g/mol, in good agreement. A value of $(1.77 \pm 0.20) \times 10^5$ g/mol is obtained from the reciprocal plot data of Fig. 4, again in good agreement, together with an estimate of the second thermodynamic virial coefficient, B, of $(2.28 \pm 0.05) \times 10^{-4}$ ml.mol/g².

From the plot of $(1/M_{z,app})^{1/2}$ vs c (Fig. 5) an estimate for M_z of $(1.91 \pm 0.20) \times 10^5$ g/mol was obtained (together with an approximate estimate for B of ~2.6 ml.mol/g², supporting the value from the Rayleigh data). We thus estimate the polydispersity index (M_z/M_w) ~(1.3 ± 0.2) and this probably accounts for the broadness of the sedimenting boundaries observed during the sedimentation velocity experiments.

The form (namely, lack of a downward trend) of plots of both $1/M_{w,app}$ and $1/M_{z,app}$ vs concentration does not rule out the possible presence of supramolecular aggregates (which would accumulate at the cell base and be lost from optical registration); it would, however, appear to rule out the existence of a reversible self-association, at least of a simple type (monomer–dimer, monomer–dimer–trimer etc.).

Gross conformation, hydrodynamic expansion and aggregation phenomena

From the plot (Fig. 6) of the reciprocal (apparent) sedimentation coefficient vs concentration (corrected for radial dilution) and fitting the data to equation 2 a value of $s°_{20,w} = (0.99 \pm 0.20)$ S was obtained. Because of the limited range of concentrations studied, it was not possible to fix k_s with any precision, but there is
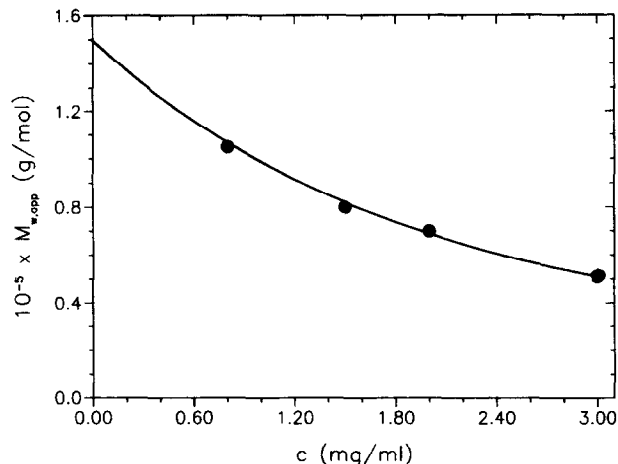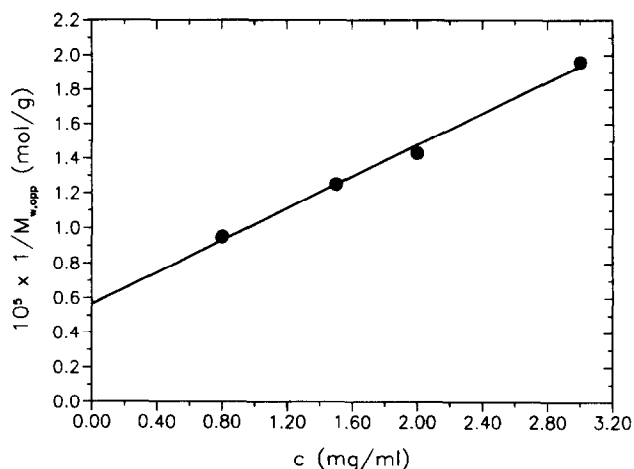

Fig. 4. Plot of $1/M_{w,app}$ vs cell loading concentration c. The line fitted is to $(1/M_{w,app}) = (1/M_w) + 2Bc$, where B is the second thermodynamic or osmotic pressure virial coefficient. $M_w = (1.77 \pm 0.20) \times 10^5$ g/mol; $B = (2.28 \pm 0.05) \times 10^{-4}$ ml.mol/g².

again no clear evidence of a simple self-association (which would have shown a clear decrease in the $(1/s_{20,w})$ data with concentration). This view is also confirmed from the form of the sedimenting boundaries as noted above. From the reduced viscosity vs concentration data of Fig. 7, a value for $[\eta]$ of (41.6 ± 2.2) ml/g was obtained, with an estimate for the Huggins constant K_η of (3.6 ± 0.6).

To grasp the conformation of the ws-AGX xylan we can use the function Π (Harding, 1981) which is independent of assumptions concerning the degree of hydration or swelling other than that it is the same in a sedimentation equilibrium experiment and during viscometry. The Π function is defined by

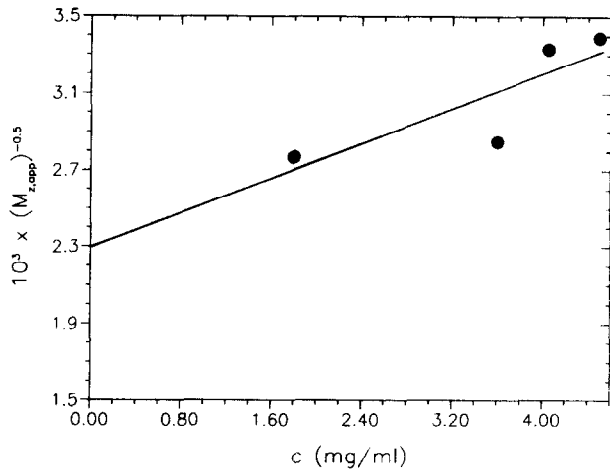$$\Pi = \{2BM/[\eta]\} + f(Z,I)/([\eta]M) \tag{5}$$

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Fig. 5. Plot of $1/M_{z,app}$ vs cell loading concentration data. From sedimentation equilibrium distributions recorded using (phase-plate) Schlieren optics. The line fitted is to $(1/M_{z,app})^{1/2} = (1/M_z)^{1/2}\{1 + 2BM_z^{1/2}c\}$. $M_z = (1.91 \pm 0.20) \times 10^5$ g/mol; $B = \sim 2.6 \times 10^{-4}$ ml.mol/g^2.
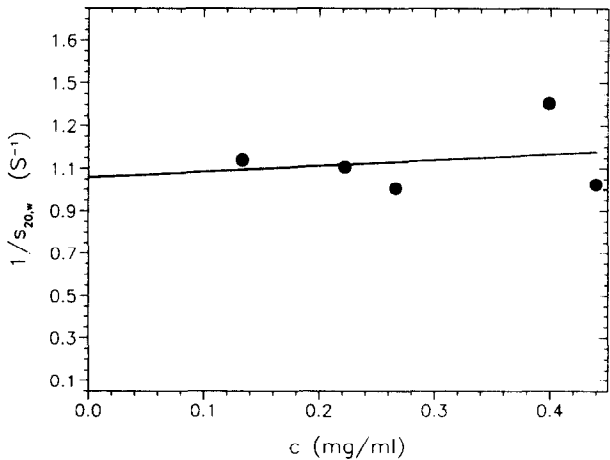

Fig. 6. Plot of $1/s_{20,w}$ vs concentration for xylan ws-AGX. Concentrations, c are true sedimenting concentrations (i.e. corrected for moisture content and radial dilution). Line fitted is $1/s_{20,w} = (1/s^\circ_{20,w})(1 + k_s c)$, with $s^\circ_{20,w} = (0.99 \pm 0.20) \times 10^{-13}$ s.

where $f(Z,I)$ is a function of molecular charge, valency (Z) and ionic strength (I). To a first approximation, if we assume that I is high enough to suppress charge effects, then $f(Z,I) \sim 0$ and if we further assume that $M = M_w$, a value for Π of $\sim(1.7 \pm 0.5)$ is obtained. For an equivalent prolate ellipsoid model this corresponds to an axial ratio of $\sim 13:1$. From this axial ratio it is possible to estimate the extent of expansion of the molecule through hydration from the frictional ratio. The frictional ratio (f/f_0) may be calculated from the infinite dilution sedimentation coefficient and the molar mass using the following equation (Squire & Himmel, 1979):

$$(f/f_0) = \{M(1 - \bar{v}\rho)/[N_A.(6\pi\eta_0 s^\circ_{20,w})]\}[(4\pi N_A/3\bar{v}M)]^{1/3}$$
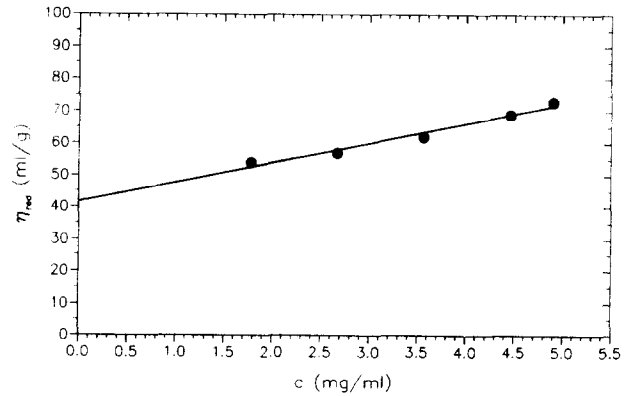
(6)

Fig. 7. Plot of reduced viscosity η_{red} vs concentration c for ws-AGX. Line fitted is $\eta_{red} = [\eta](1 + K_\eta [\eta]c)[\eta] = (41.6 + 2.2)$ ml/g. $K_\eta = 3.6 \pm 0.6$.

and from this (f/f_0) was found to be $\sim(15 \pm 5)$. Combination of the Perrin function, P (Perrin, 1936; Rowe, 1977), often referred as the 'frictional ratio due to shape' (Squire & Himmel, 1979) $\{\sim 1.7$ for an axial ratio of $13:1\}$ with the frictional ratio (f/f_0) enables the degree of expansion of the molecule $(\bar{v}_s/\bar{v})$ to be estimated, where $\bar{v}_s$ (ml/g) is the volume of the swollen molecule (polysaccharide + associated solvent) per unit mass of polysaccharide and $\bar{v}$ is the partial specific volume (esentially the anhydrous molecule):

$$(f/f_0) = P(\bar{v}_s/\bar{v})^{1/3}$$

(7)

From equation (7) we can estimate $(\bar{v}_s/\bar{v})$ as $\sim(700 \pm 400)$. Although, because of the approximations we have made, the actual numerical value has no real meaning, this treatment does suggest that the molecule is highly expanded, consistent with the coil-like model obtained from the Mark–Houwink relation (Ebringerova, 1992). There is, however, an apparent inconsistency involving the intrinsic viscosity data in that $[\eta]$ is too small for a macromolecule of this molar mass and expansion. A similar problem involving $[\eta]$ with the Mark–Houwink data was noted earlier by Ebringerova et al. (1992) where the explanation was given in terms of the presence of a significant proportion of compact spherical aggregates. In the present study these compact aggregates would lower $[\eta]$ in two ways: tight spheres have a lower $[\eta]$ and the concentration of the 'macromolecular' component would be overestimated in the evaluation of the η_{red} values. These aggregates would not affect the sedimentation velocity or sedimentation equilibrium data here as they would be rapidly sedimented out and lost from optical registration. Our data would, therefore, appear to support the presence of these supramolecular components in preparations of ws-AGX. Similar problems have been reported for alginates and pectins (Berth et al., 1994).

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