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Structural and molecular properties of a water-soluble arabinoxylan–protein complex isolated from rye bran

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Abstract

The main structural features of the water-soluble arabinoxylan–protein complex isolated from rye bran were evaluated by means of methylation analysis and ^{13}C NMR spectroscopy. A highly branched arabinoxylan with Ara*f* residues in mono- and di-substitution positions and side chains terminated with Xyl*p* groups represents the main component associated with a serine- and glycine-rich cell-wall protein. More than 30% of the arabinose is linked in various positions probably as oligosaccharide side-chains and/or branched (1 → 5)-arabinan. The molecular parameters were estimated by HPGPC as well as by gel chromatography combined with viscometry and light-scattering. The high molecular weight fraction close to the exclusion volume ($\overline{M}_w > 10^6$), which is shifted after treatment with Pronase to the region $10^4 < \overline{M}_w < 10^6$, is considered to consist of fragments of the native cell-wall matrix with arabinoxylan chains probably linked to a protein core.

1. Introduction

The water-extractable arabinoxylans play an important role in cereal processing, the baking performance of flours, and the digestibility of grain [1–4]. Their effects are related mainly to the highly viscous character of the polysaccharide in aqueous medium. The gel-forming ability of wheat flour pentosans was ascribed to the oxidative coupling reaction of the ester-bound ferulic acid [5–7] and not to the protein component [8]. Minor attention has been paid to water-unextractable

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heteroxylans, particularly to those present in bran fractions, and their associations with cell-wall proteins [9–13].

Fractional extraction studies of delignified cell-wall material of rye bran [14] have shown the presence of arabinoxylans differing in the degree of branching and containing various amounts of protein. Whereas most of the highly branched arabinoxylan component of rye flour is extractable with water [15,16], that of bran cell walls is only gradually released after delignification by solvents with increasing alkalinity [14,17,18]. It has been suggested [19] that the differences in extractability and solubility of the cereal grain heteroxylans are connected with cross-linked polymers in the cell-wall network.

The present study was undertaken to investigate the water-soluble arabinoxylan–protein complex (AXP) isolated by extraction of delignified rye bran with dilute aqueous sodium hydroxide.

2. Experimental

General methods.—Unless otherwise specified, general methods are those described in previous papers [14,17]. Amino acid composition was determined, after hydrolysis by 6 M HCl (20 h, 105°C, under N_2), with an automatic amino acid analyser T339 (Mikrotechna, Prague, Czechoslovakia). Hydroxyproline was determined by the colorimetric procedure described by Schwitzen and Summen [20]. Phenolic acid content was determined by the UV absorption of the alkaline extract of AXP at 320 nm according to Fausch et al. [21] using ferulic acid as standard. The methylation analysis of AXP was performed by a slightly modified [22] Ciucanu and Kerek method [23]. Permethylation of the product was checked by the absence of IR absorption bands of hydroxyl groups. ^{13}C NMR spectra of the samples (3% in D_2O) were recorded with a Bruker AM-300 (75 MHz) spectrometer at 40°C in the inverse gated decoupling mode. Chemical shifts are reported relative to internal MeOH ($\delta_{Me_4Si} = 50.15$). ^1H NMR spectra (300 MHz) were measured under the same conditions. The intrinsic viscosity [η] in water was measured at 21.0 ± 0.05°C using a Ubbelohde viscometer.

Isolation and purification of the xylan–protein complex.—The water-soluble fraction (R_2-B/L) obtained by extraction with aq 4.5% NaOH from sodium chlorite-delignified rye bran [14] was the crude xylan–protein complex. The solution of the complex in aq 4% NaOH was acidified with aq 5% HCl and precipitated with 4 vol of EtOH, and the precipitate was dialysed against distilled water. The non-dialysable water-soluble portion (AXP) was centrifuged at 10 000g for 15 min and recovered by lyophilisation.

Gel filtration.—HPGPC was performed using a commercial instrument (Laboratorni pristroje, Prague, Czechoslovakia) equipped with two Tessek Separon HEMA BIO-1000 exclusion columns, and aq 0.1 M $NaNO_3$ as solvent and eluent. The eluate was monitored by refractometry and UV absortion at 280 nm (protein detection). A set of pullulans P-100, P-200, P-400, and P-800 (Shodex Standard P-82, Macherey-Nagel) was used for the calibration of the columns. Therefore the

molecular weight parameters shown are relative to the pullulan reference material. A computing procedure [24] based on the linear effective calibration curve was applied to obtain the molecular weight distribution.

GPC on Sepharose CL-2B/Sepharose CL-4B was performed on two coupled columns (~ 380 mL; flow rate, ~ 10 mL/h) loaded with 45 mg of polysaccharide/15 mL per run. 37 mM Phosphate buffer (pH 6.5) with the addition of Na_2EDTA (1 μmol/L) was used as solvent and eluent. The concentration was monitored continuously with a differential refractometer (Knauer, FRG). Fractions (~ 10 mL) were taken for subsequent viscosity (Viscomatic, Fica, France) and molecular weight (M_w) measurements by light-scattering (Sofica, Fica, France), using $\delta n/\delta c = 0.15$ mL/g. Further details are given elsewhere [25]. For preparative purposes, GPC runs were repeated four times and the eluate was separated into three parts by collecting fractions Nos. 1–10 (AXP-a), 11–20 (AXP-b), and 21–30 (AXP-c), which represent 24, 47, and 28% of the fractionated sample, respectively.

Treatment of AXP with alkaline $NaBH_4$.—The polysaccharide (100 mg) was treated with 0.25 M NaOH (50 mL) containing 0.25 M $NaBH_4$ at 45°C for 5 h [26]. The alkali-treated sample was recovered by lyophilisation after neutralisation and dialysis (AXP-E).

Enzymatic treatment of AXP.—The polysaccharide (50 mg) was dissolved in distilled water (5 mL) and the pH was adjusted to 7.5 with 1 M NaOH. Pronase ex *Streptomyces griseus* (Koch–Light Laboratories, UK, 2.5 mg) was added; part (2.5 mL) of the solution was incubated at 37°C for 40 h (AXP-P1) and the remainder at 24°C for 72 h (AXP-P2). On a larger scale, AXP (150 mg) was treated with Pronase (9 mg) at 37°C for 48 h (AXP-P). After digestion, the mixture was heated at 95°C for 15 min, centrifuged (10 000*g*, 15 min), dialysed, and freeze-dried.

3. Results and discussion

Table 1 shows the analytical characteristics of the water-soluble arabinoxylan complex (AXP), isolated with aq 5% NaOH from the delipidated, destarched,

Table 1
General characteristics of AXP, the Pronase-treated sample (AXP-P), and fractions AXP-a, AXP-b, and AXP-c obtained by gel filtration [a]

Sample	Sugar composition (mol%) [b]					Uronic acid (%)	Protein (%) [c]	$[\alpha]_D$ (degrees)
	Ara	Xyl	Man	Glc	Gal			
AXP	45.1	46.1	tr	4.5	4.3	3.1	1.1	−96.5
AXP-a	43.9	49.9	0	5.6	0.6	+	+ [d]	−92.6
AXP-b	41.1	42.4	0.9	7.8	7.8	+	0 [d]	−94.1
AXP-c	64.6	33.1	0	2.3	tr	0	0 [d]	−202.0
AXP-P	44.7	47.4	0	4.0	3.9	2.4	< 0.1	−94.1

[a] Fraction AXP-a represents Nos. 1–10 as designated in Fig. 2, fraction AXP-b corresponds to Nos. 11–20, and fraction AXP-c corresponds to Nos. 21–30.
[b] Determined as alditol trifluoroacetates on OV 225.
[c] % Nitrogen ×6.25.
[d] Detected by UV absorption at 280 nm.

Table 2
Amino acid composition of intact (AXP), β-eliminated (AXP-E), and Pronase-treated (AXP-P) rye bran arabinoxylan samples and the aleurone intracellular protein fraction (IP) [19]

Amino acid [a]	AXP	AXP-E	AXP-P	IP
Asp	8.8	9.5	59.7	8.9
Thr	4.8	6.5	traces	4.2
Ser	9.6	6.6	17.8	6.3
Glu	8.2	13.4	23.5	15.1
Pro	traces	5.0	[b]	4.8
Gly	12.9	13.2	[b]	11.5
Ala	9.1	11.1	[b]	8.9
Val	4.5	7.3	[b]	5.6
Met	2.7	0	[b]	1.3
Ile	4.6	4.6	[b]	2.9
Leu	7.5	12.8	[b]	6.4
Tyr	2.7	1.1	[b]	2.3
Phe	5.1	3.3	[b]	3.2
His	4.1	0.8	[b]	3.3
Lys	2.4	1.6	[b]	4.6
Arg	9.1	0.7	[b]	9.0
Hyp	3.9	2.5	[b]	

[a] Expressed as rel. mol%.
[b] Not detectable.

depectinated, and delignified rye bran and purified by reprecipitation with EtOH, as previously described [14]. AXP contains 1.1% of protein and consists of arabinose and xylose in the molar ratio 97 : 100 with minor amounts of glucose and galactose. The uronic acid, which represents 3.1% of the polysaccharide, was present mainly as D-glucuronic acid and its 4-*O*-methyl derivative which are indicative of acidic xylans known to occur in bran tissues [10,27]. No phenolic substances detectable as ferulic acid were found, probably because of cleavage of the ester bond during the alkaline extraction step.

The amino acid composition of AXP (Table 2), when compared with that of aleurone intracellular protein [19], shows a marked similarity for most amino acids. However, there is less glutamic acid and proline, and more glycine and serine in AXP. In agreement with the literature [12,26], the data suggest the protein to be a hydroxyproline-poor structural cell-wall component. Alkaline sodium borohydride treatment of AXP caused a decrease of serine residues with a simultaneous increase of alanine residues, indicating that serine–carbohydrate linkages were cleaved by β-elimination [13].

Methylation analysis data of AXP are summarised in Table 3. The proportion of all terminal residues to branched ones confirms complete methylation, as had been indicated by IR spectroscopy. Evidently, the (1 → 4)-linked Xyl*p* backbone of AXP has a higher degree of branching (~ 0.64), expressed as the ratio of [Xyl + 2(3)-OMe-Xyl]/[Xyl + 2(3)-OMe-Xyl + 2,3-OMe_2-Xyl], compared with that (~ 0.59) of the nitrogen-free, water-soluble arabinoxylan fraction AX-III [17] isolated from rye bran in the previous ammoniacal extraction step. Also, the proportions of fully

Table 3
Methylation analysis of AXP

Methylated sugar [a]	Mode of linkage	Molar ratio [b]
2,3,5-Me_3-Ara	Ara*f*-(1 →	27.6
3,5-Me_2-Ara	→ 2)-Ara*f*-(1 →	2.0
2,5-Me_2-Ara	→ 3)-Ara*f*-(1 →	3.7
2,3-Me_2-Ara	→ 5)-Ara*f*-(1 →	4.3
3-Me-Ara	→ 2,5-Ara*f*-(1 →	6.7
		Total 44.3
2,3,4-Me_3-Xyl	Xyl*p*-(1 →	10.2
2,3-Me_2-Xyl	→ 4)-Xyl*p*-(1 →	13.4
2-Me-Xyl	→ 3,4)-Xyl*p*-(1 →	8.7
3-Me-Xyl	→ 2,4)-Xyl*p*-(1 →	2.3
Xyl	→ 2,3,4)-Xyl*p*-(1 →	12.7
		Total 47.3
2,3,4,6-Me_4-Gal	Gal*p*-(1 →	3.6
		Total 3.6
2,3,6-Me_4-Glc	→ 4)-Glc*p*-(1 →	1.7
2,4,6-Me_3-Glc	→ 3)-Glc*p*-(1 →	1.7
2,3-Me_2-Glc	→ 4,6)-Glc*p*-(1 →	1.4
		Total 4.8

[a] 2,3,5-Me_3-Ara=2,3,5-tri-*O*-methylarabinose, etc.; determined as partially methylated alditol acetates on SP 2340.
[b] Values were corrected by use of the effective carbon response factors [51].

substituted and non-reducing terminal Xyl*p* units are higher in AXP than in fraction AX-III. A higher occurrence of non-reducing terminal Xyl*p* units was reported for wheat [9] and rice bran [10] arabinoxylans as well as for arabinoxylans prepared from a milling by-product of wheat [29]. This structural feature is less pronounced or nearly absent in endospermic arabinoxylans [6,15]. Highly branched heteroxylans with terminal Xyl*p* have been isolated also from sugar cane [30] and marsh mallow leaves [31].

The absence of branched Gal*p* residues (Table 3) suggests that contamination with arabinogalactan, which occurs in cereal hemicelluloses [27], is unimportant. Arabinose was not only found at terminal non-reducing positions; more than 30% was branched. This is in contrast to data for endospermic arabinoxylans, which indicated only small amounts of branched Ara*f* residues [6,7,15,32]. Terminally linked Gal*p* and 1,2- and 1,3-linked Ara*f* residues are found in cell-wall glycoproteins [13,33] and 5-linked Ara*f* often occurs esterified to phenolic acids [34]. Various oligosaccharide chains terminated by Xyl*p* and Gal*p* residues linked to O-2, O-3, or O-5 of Ara*f* residues were reported to be attached as side-chains to heteroxylans [27,35]. Since no ferulic acid was detectable in AXP, the 5-linked Ara*f* may come from arabinoxylan side-chains and/or arabinan-like structures. The linked Glc*p* residues indicate the presence of β-glucans which are known to accompany xylan fractions isolated from cereal grain tissues [19,36].

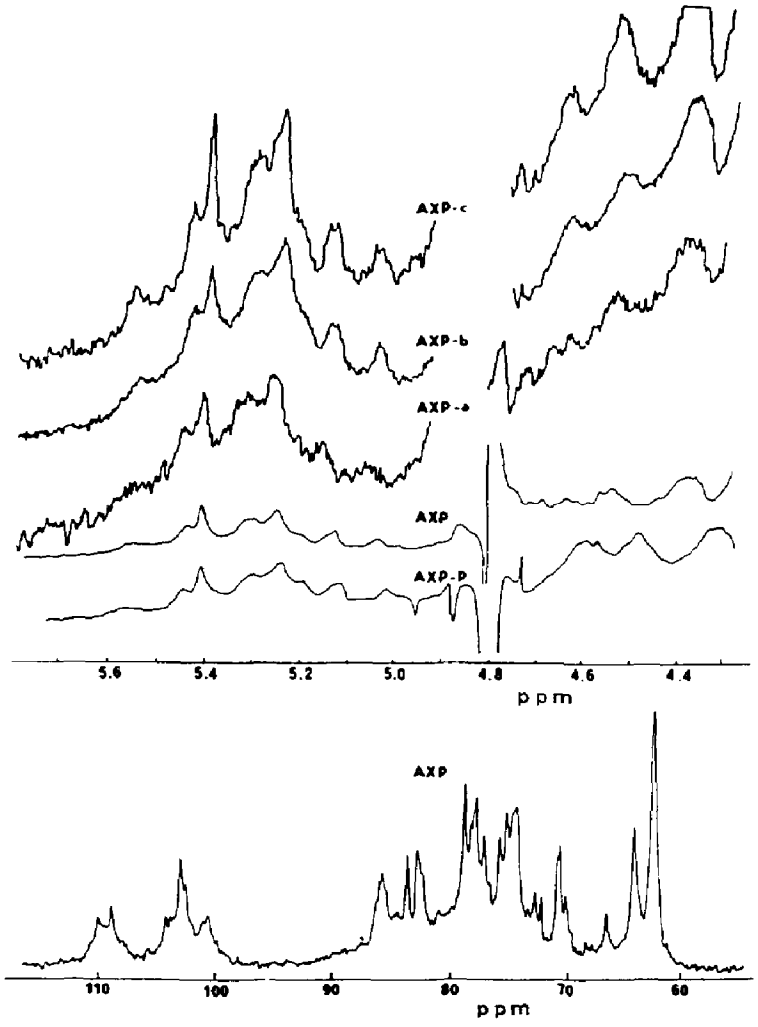

Fig. 1. ^{13}C NMR spectrum of AXP and partial ^{1}H NMR spectra of AXP, its fractions AXP-a, AXP and AXP-c, and the Pronase-treated sample AXP-P (in D_2O).

The ^{1}H and ^{13}C NMR spectra (Fig. 1) reflect the complex character of AXP ar are in accordance with the methylation analysis data. Partial assignment of th signals was made by comparison with our previous studies of heteroxylans [17,3 39] and data in the recent literature [6,15,40]. The signals at δ 102.7 and 102 belong to C-1 of internal unbranched and 3-*O*-branched Xyl*p* residues, respe tively. The presence of terminal non-reducing β-D-Xyl*p* units was confirmed b signals at δ 103.4 (C-1), 66.4 (C-5), and 70.4 (C-4), the last being overlapped b shifts of the hexopyranose residues [41]. A ^{13}C NMR study [42] of synthet oligosaccharides composed of methyl β-D-xylopyranoside substituted at O-2, O- and O-2,3 with α-L-Ara*f* residues has shown that only O-2 substitution in bo cases led to a significant upfield shift. Based on these data and methylatic analysis, the signal at $\delta \sim 101$ (Fig. 1) was assigned to C-1 of the Xyl*p* residu

Table 4
Molecular weight parameters of AXP and the Pronase-treated samples AXP-P1 [a] and AXP-P2 [b] estimated by HPGPC on Separon BIO-S 1000

Sample	$\overline{M}_w$	D [c]	MWD [d]		
			$<10^4$	10^4–10^6	$>10^6$
AXP	315000	2.33	5	69	26
AXP-P1	231000	2.26	6	79	15
AXP-P2	235000	2.04	6	82	12

[a] Pronase treatment: 37°C, 40 h; [b] 24°C, 72 h.
[c] Polydispersity.
[d] Molecular weight distribution (in vol%).

branched at O-2,3. The proportion of single and double substitution of the Xyl*p* residues by Ara*f* units can be deduced from the C-1 resonances at δ 108.7 and 109.7 and the H-1 resonances at δ ~5.40 and ~5.25 assigned to terminal Ara*f* units located in various positions [17]. Many signals are present between the above-mentioned peaks which we suggest belong to branched Ara*f* units in the previously reported rye bran arabinoxylan fraction AX-III [17]. This agrees with the high proportion of branched Ara*f* units found by methylation analysis (Table 3). However, this region is very susceptible to the environment of the Ara*f* substituents as was demonstrated for arabinoxylan oligomers with mono- and di-arabinosylated Xyl*p* residues [6,40]. The groups of chemical shifts at δ 107.4–108.6 (C-1) and 67–68 (C-5) are indicative of branched (1 → 5)-arabinans [43], as are the H-1 signals [44] which appeared at δ 5.0–5.2.

High performance GPC on Separon HEMA BIO-S 1000 columns calibrated with pullulan standards revealed the molecular heterogeneity of AXP (Table 4). About 70% of AXP had an apparent molecular weight ($\overline{M}_w$) between 10^4–10^6 and ~26% had $\overline{M}_w$ higher than 10^6; the low-M_w tail (~5%) had M_w ~ 10^4.

To obtain more information about the molecular and chemical heterogeneity of AXP, gel filtration on Sepharose CL-2B/Sepharose CL-4B columns coupled with intrinsic viscosity and light-scattering measurements were performed. The elution profile of AXP (Fig. 2, curve A) was similar to that obtained by HPGPC. From four runs, fractions AXP-a, AXP-b, and AXP-c were collected as designated in Fig. 2, and analysed (Table 1). The first two fractions showed similar Ara : Xyl ratios and optical rotation values. Fraction AXP-c, which contained the low-M_w portion, was rich in Ara, suggesting the presence of arabinan, in agreement with methylation analysis and NMR spectroscopy. However, only qualitative differences are seen in the anomeric region of the ^{1}H NMR spectra of AXP and its fractions (Fig. 1) where signals of Ara*f* are located [41,44].

The protein component of AXP was detected by UV absorption at 280 nm in fraction AXP-a only. After incubation with Pronase, the treated samples showed a decrease of the average-$\overline{M}_w$ and of the proportion of the high-M_w fraction (Table 4), depending on the treatment conditions. Simultaneously, the UV absorption at 280 nm in the high-M_w region was reduced, indicating cleavage of associated

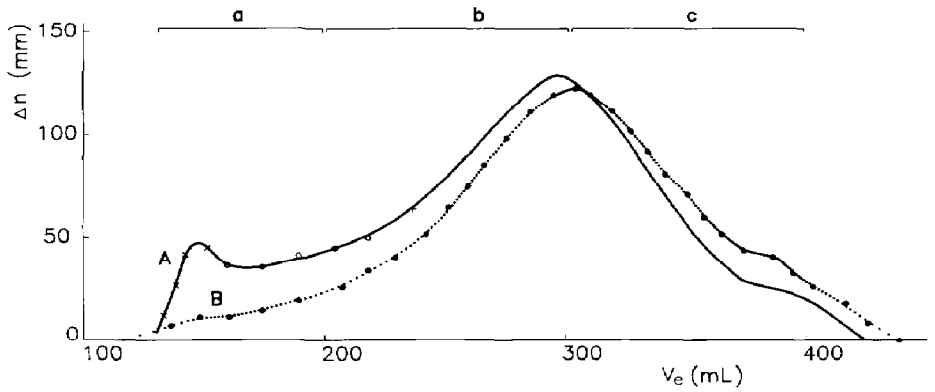

Fig. 2. Elution lines of AXP (A) and the Pronase-treated sample AXP-P (B) on Sepharose CL-2B/Sepharose CL-4B: a, b, and c are fractions of AXP collected as described (see Footnote of Table 1).

protein. Proteolytic treatment of plant gums [45,46] has been shown to affect their molecular weight distribution (MWD), particularly the high-M_w fraction, suggesting an association of carbohydrate with protein. After exhaustive treatment of AXP with Pronase, the non-dialysable portion (AXP-P) showed no UV absorption but still contained traces of amino acids (Tables 1 and 2); only aspartic acid, serine, glutamic acid, and traces of threonine could be unambiguously estimated. A similar composition of amino acids except serine has been reported for a proteoglycan fraction of runner-bean cell walls [28]. Sugar composition, optical rotation, and the anomeric region pattern of the ^{1}H NMR spectra (Fig. 1) of AXP and AXP-P suggest that treatment with Pronase involved no substantial structural changes in the polysaccharide. Pronase hydrolysed only the protein as documented by the absence of carbohydrate signals in the ^{13}C NMR spectrum of the dialysate obtained from the Pronase-treated sample. As seen in Fig. 2 (curve B), the high-M_w tail in the gel chromatogram of AXP was diminished strongly after the enzymatic treatment. However, the $[\eta]$ of AXP decreased only from 1.02 to 0.95 dL/g, i.e., to ca. 90% of its original value.

These results suggest that such glycoproteins as extensin and arabinogalactan–proteins which occur in plant cell walls [33,47] are unlikely to be present in AXP. Probably, the high-M_w fraction of AXP ($\overline{M}_w > 10^6$, AXP-a) consists of arabinoxylan chains from the cell-wall matrix covalently linked to a protein core, a structure which has been suggested for arabinogalactan–protein complexes isolated from plant gums [45,46] and wheat [48]. The restriction of protein to high molecular weight fragments may lead to misinterpretations in evaluation of the molecular weight parameters of native polysaccharide–protein complexes, and particularly to overestimation of $\overline{M}_w$ by light-scattering measurements.

In order to characterise the MWD of AXP-P and to establish a Mark–Houwink relationship, GPC analysis coupled with viscosity and light-scattering between 30 and 150° was applied. As seen in Fig. 2, the polysaccharide was eluted over the entire range from exclusion volume (V_o) to total volume (V_t), indicating a broad size distribution with a small shoulder on the high elution volume end. The plot of

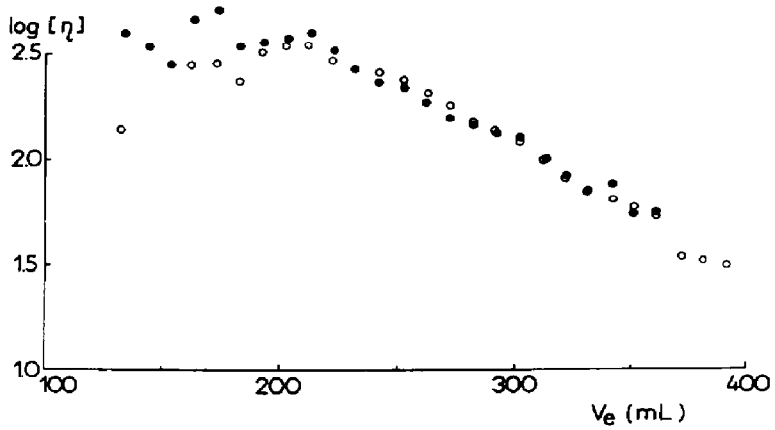

Fig. 3. Dependence of $[\eta]$ on the elution volume for AXP-P (●) and AGX-H [39] (○).

intrinsic viscosities of the fractions against elution volume V_e (Fig. 3) shows a similar non-linear trend, as previously reported for corn cob arabinoglucuronoxylan AGX-H [39] and pectin [25]. The plateau at lower V_e indicates the presence of a particulate component.

The angular dependencies of the scattering light are given as Guinier plots for the fractions passed, before light-scattering measurement, through a 0.45-μm

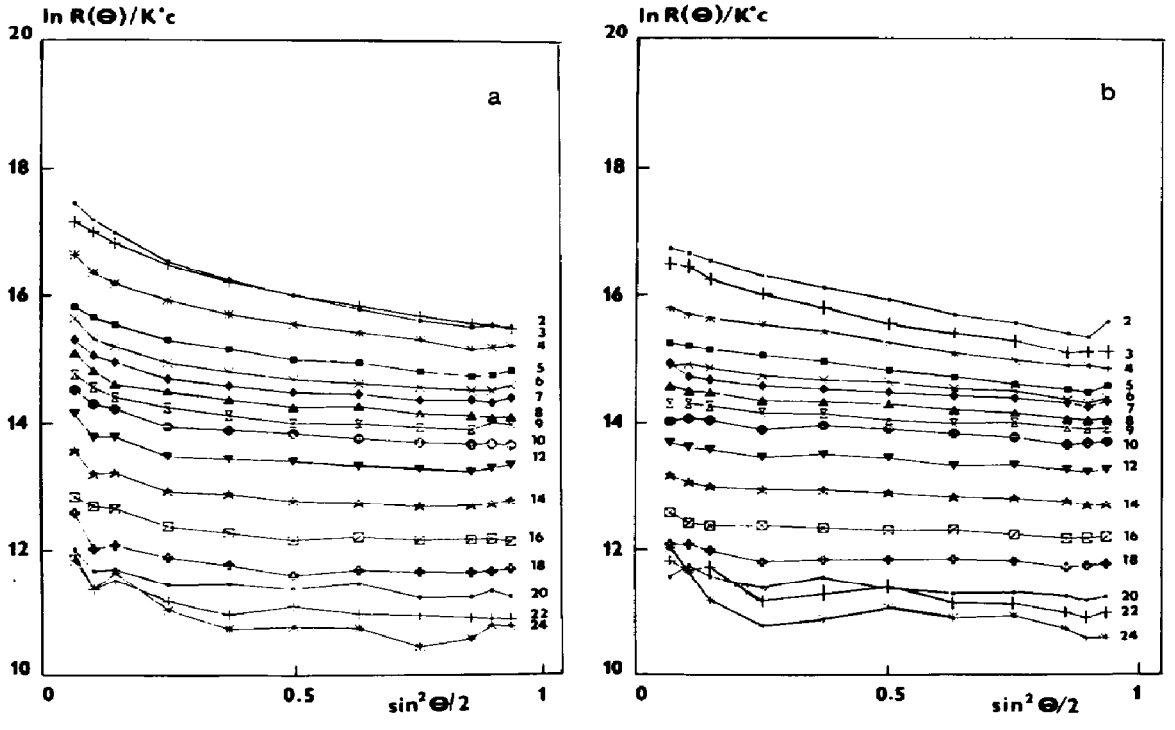

Fig. 4. Guinier plot of AXP-P fractions Nos. 2–24: (a) after filtration through a 0.45-μm pore-size membrane filter; (b) after subsequent filtration through 0.2-μm filter.

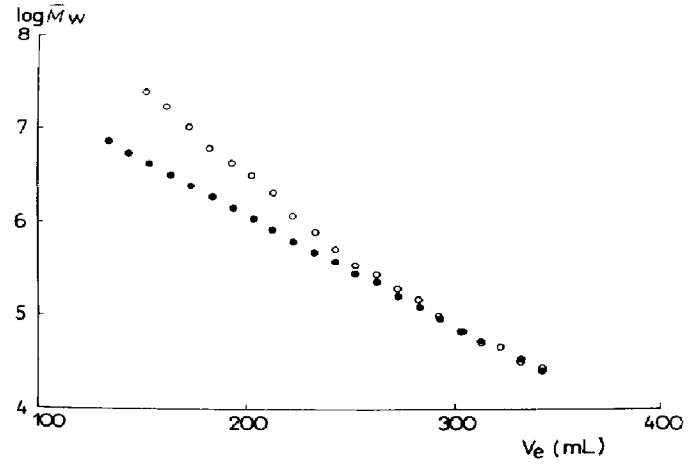

Fig. 5. Dependence of molecular weight M_w on the elution volume for AXP-P (●) and AGX-H [39] (○).

pore-size membrane filter (Fig. 4a) and subsequently through a 0.2-μm filter (Fig. 4b). It is evident that, by diminishing the pore size, the scattering curves became flatter in the low angle range ($\sin^2\theta/2 < 0.25$). Although the scattering level was reduced, particularly in the wide angle range for fractions 2–10, the plots still indicate non-ideal conditions, e.g., broad distribution or residual particulate component. Therefore, a two-component interpretation [25] was applied, taking fraction 2 as the model curve for the pure particulate component. The molecular weights obtained for fractions 11–24 corresponding to the molecularly dispersed major component are given in Fig. 5 and the curve was linearly extended over the whole elution-volume range. The data from Figs. 3 and 5 were used to establish the universal calibration line, which showed a constant positive deviation from that of dextran sulfate (Fig. 6). Although there are differences in the universal calibra-

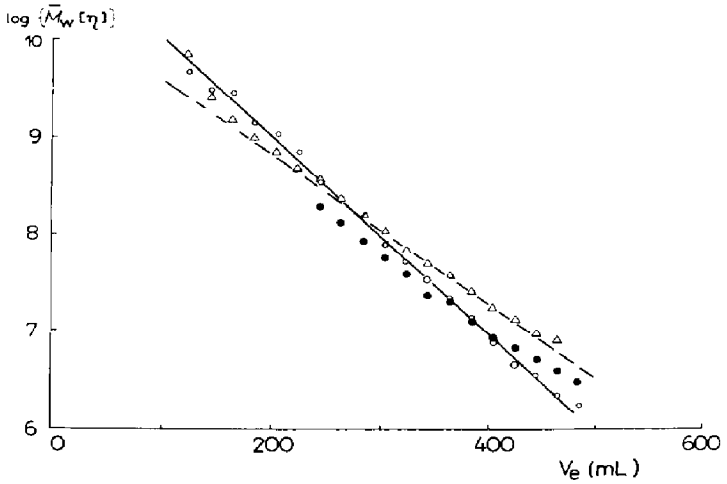

Fig. 6. Universal calibration plot of AXP-P (●), on Sepharose CL-2B/Sepharose CL-4B; for comparison, corresponding data for AGX-H [39] (○) and dextran sulphate (△) are included.

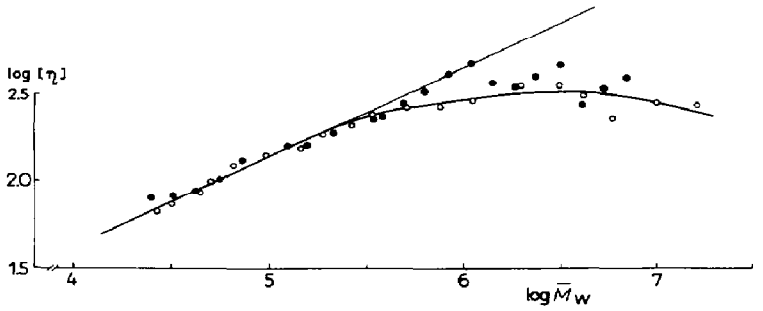

Fig. 7. Mark–Houwink plot for AXP-P (•) and AGX-H [39] (○); data for AXP-P were taken from Figs. 3 and 5.

tion lines of AXP-P and the corn cob xylan [39], their Mark–Houwink plots (Fig. 7) exhibit the same shape with a linear part between $\bar{M}_w$ 10^4–10^6, corresponding to the average-$\bar{M}_w$ of the main arabinoxylan component of AXP-P. The estimated exponent a = 0.5 is characteristic of molecules in unperturbed coil-shaped structures. In contrast, for rye flour pentosan, the reported [49] exponent a = 0.98 indicates a lower flexibility of the arabinoxylan chain because of a more extended conformation in solution. Structural differences in side-chain composition of the compared heteroxylans may contribute to their different hydrodynamic behaviour.

The MWD of AXP-P (Fig. 8) is rather broad and still displays a minor amount of the high-M_w fraction with exponent a = ca. 0 (Fig. 7), corresponding to globular components of either highly branched molecules or cross-linked native complexes which were alkali-stable. The calculated "corrected" average molecular weight of AXP-P was found to be ca. 350 000. The corresponding $\bar{M}_w$ value derived from the universal calibration plot (Fig. 6) is lower (~ 280 000), but lies in the range of the HPGPC-derived $\bar{M}_w$-values estimated for the Pronase-treated samples (Table 4). However, the correlation between number-average molecular weight and data from universal calibration plots is more reliable [50].

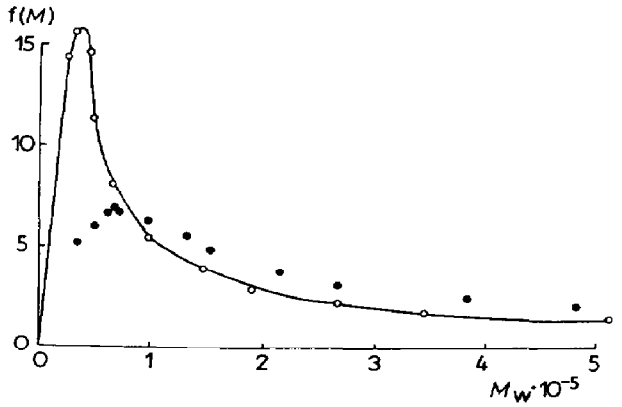

Fig. 8. MWD-curves for AXP-P (•) and AGX-H [39] (○); f(M) is given in percent and calculated for AXP-P from Fig. 2; for molecular weights (M), see Fig. 5.

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