ELSEVIER

PII: S0144-8617(96)00096-3

Carbohydrate Polymers **31** (1996) 253–261

0144-8617/96/$15.00 + .00

Preparative and analytical size-exclusion chromatography of chitosans

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(Received 1 February 1996; accepted 6 March 1996)

Two chitosan samples (fraction of acetylated units (F_A) 0.15 and 0.52) were fractionated by preparative size exclusion chromatography (SEC). The molecular weights and molecular weight distributions of the fractions were analyzed by analytical size exclusion chromatography coupled to an on-line low angle laser light scattering detector and a differential refractive index detector (SEC-LALLS-DRI), and their intrinsic viscosities were determined. The exponent (a) of the Mark–Houwink–Kuhn–Sakurada (MHKS) equation was found to be 0.92±0.07 and 1.1±0.1, respectively, at I = 0.1 and pH 4.5. No variation in F_A related to molecular weight was found. Reversible interaction between chitosans and different column packings strongly influenced the log M–V relationships. This interaction was generally most pronounced for the low-F_A chitosan, suggesting that the protonated amino groups are involved. Ammonium acetate buffer reduced this effect and the use of a new type of SEC-packing seemed to eliminate it. The more highly acetylated chitosan also had a more pronounced tendency towards concentration dependent self-association, which most probably involve intermolecular hydrophobic interactions between the acetyl groups.

INTRODUCTION

Chitin, a structural polysaccharide in the outer skeleton of arthropods, is a linear polysaccharide consisting of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose units and is insoluble in water. Chitosan is commercially prepared by (partial) *N*-deacetylation of chitin, resulting in a (partially) water-soluble and positively charged polysaccharide at low pH values.

A distinct compositional heterogeneity has been determined in heterogeneously (i.e. commercially) deacetylated chitosans as they can be fractionated into an acid-soluble fraction and a chitin-like acid-insoluble fraction. The acid-soluble fractions with F_A from 0.20 to 0.52 were found to be only negligible depolymerized during the deacetylation process under nitrogen purge at 75°C (Ottøy *et al.*, 1996), and the distribution of the two monomers along the chain of the acid-soluble fractions were shown to be consistent with a Bernoullian (random) distribution (Ottøy *et al.*, 1996). Chitosans are polydisperse with respect to molecular weight (Beri *et al.*, 1993; Yomota *et al.*, 1993). In order to investigate if any correlation between molecular weight and chemical composition exists in the population of chains in such chitosan samples, preparative size exclusion chromatography (SEC) with subsequent chemical characterization of the fractions with different molecular weights may be performed.

Preparations of well-characterized chitosans with a narrow molecular weight distribution (MWD) may also be important for some medical applications, for regulatory reasons. In addition, fractions of chitosans with a more narrow MWD may be used to determine the Mark–Houwink–Kuhn–Sakurada parameters as an alternative to estimates based on unfractionated chitosans (Anthonsen *et al.*, 1993).

Determination of molecular weights by high-performance size-exclusion chromatography (HPSEC) is claimed to be a rapid, reproducible and simple method relative to classical methods as static light scattering and osmometry. It is, however, crucial that the separation mechanisms in HPSEC are determined by the size of the molecules and not by charge, adsorption or exclusion. Such effects have been reported for fully deacetylated chitosans on cationic silica gels (Domard & Rinaudo, 1984), and for fully deacetylated oligomers prepared from chitosan sepa-

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rated on polyacrylamide gels (Domard & Cartier, 1989; Domard & Cartier, 1991).

Determination of the molecular weight and molecular weight distribution by HPSEC demands either proper molecular weight standards with a narrow MWD, application of universal calibration (Grubisic *et al.*, 1967; Hamielec & Ouano, 1978) or on-line detectors for determination of absolute molecular weight (e.g. light scattering) and concentration (Lecacheux *et al.*, 1986; Martin, 1982; Martinsen *et al.*, 1991; Yu & Rollings, 1987). Dextrans and pullulans, which are highly flexible and uncharged polysaccharides, have been used as standards for determination of molecular weights of more rigid polysaccharides, resulting in vigorously overestimated molecular weights (Arcidiacono & Kaplan, 1992; Beri *et al.*, 1993; Goto *et al.*, 1981; Hasagawa *et al.*, 1994; Wu *et al.*, 1976). A better way of calibrating the HPSEC-system is to use chitosan fractions with a narrow MWD obtained from preparative SEC. Chitosan has been characterized by HPSEC (TSK-gels) coupled to an on-line multiple angle laser light scattering (MALLS)detector (Beri *et al.*, 1993). However, Beri and co-workers only characterized molecular weight polydisperse chitosans with F_A-values varying from 0.01 to 0.24.

Conventional static light scattering has, in some cases, been shown to overestimate the weight average molecular weights (M_w) of polysaccharides, due to concentration dependent aggregate formation in solution (Anthonsen *et al.*, 1994; Jordan & Brant, 1978; Smidsrød & Grasdalen, 1984; Smidsrød & Haug, 1968; Vårum *et al.*, 1992). With aggregates present, a bimodal LALLS-signal can be expected when column materials which separate the entire molecular weight distribution are used. This has been shown for fully deacetylated chitosans (Domard & Rinaudo, 1986) and for a chitosan with $F_A = 0.6$ (Anthonsen *et al.*, 1994). The physical basis for the aggregation phenomena in chitosan solutions is poorly understood. One possibility is that chitosan self-associates through intermolecular hydrophobic interactions between the acetyl groups (Amiji, 1995). An investigation of the possible correlation between F_A and the tendency towards self-association would give information about the nature of the aggregation phenomena.

In this work we report on the fractionation of two chitosans with $F_A = 0.15$ and 0.52 by preparative SEC to obtain fractions of a more narrow molecular weight distribution. The intrinsic viscosities and the chemical compositions of the fractions were determined, and the MHKS-parameters were determined. The molecular weight and MWD of both fractionated and unfractionated chitosans are determined using HPSEC-LALLS-DRI. This method is further used to investigate chitosan–column interactions, and the tendency of chitosan to self-associate.

Table 1. Chitosan sample characteristics

Sample	$[\eta]$ (ml/g)	F_A	Reference
Chitosan 1	833	0.15	Vårum et al., 1991a
Chitosan 2	385	0.52	Ottøy *et al.*, 1995
Chitosan 3	439	0.15	Anthonsen *et al.*, 1993
Chitosan 4	1000	0.52	Ottøy *et al.*, 1995
Chitosan 5	1220	0.23	Ottøy *et al.*, 1995
Chitosan 6	1120	0.20	Ottøy *et al.*, 1995
Chitosan 7	502	0.60	Anthonsen *et al.*, 1993
Chitosan 8	564	0.15	Anthonsen *et al.*, 1993
Chitosan 9	665	0.0	Anthonsen *et al.*, 1993

MATERIALS AND METHODS

Materials

The intrinsic viscosities and fraction of acetylated units for Chitosan 1–9 are listed in Table 1. Chitosan 1 was deacetylated for 2 h. Chitosan 2 was depolymerized by ultrasonic irradiation of the acid-soluble fraction of the 2 h sample previously described (Ottøy *et al.*, 1996), using a Labsonic 2000 equipped with standard titanium probe (B. Braun, Melsungen, FRG). The tritium[^{3}H]-labelled chitosans, ChitR1 and ChitR2, were obtained by reacetylation of fully deacetylated chitosan (Kurita *et al.*, 1989). The specific activities of ChitR1 and ChitR2 were determined by a Scintillation counter (Wallac 1410, Wallac, Oslo, Norway) to 162 000 DPM/mg and 149 000 DPM/mg. All chitosans were dialyzed exhaustively, and freeze-dried in the chloride salt form. The samples were dried under vacuum over P_2O_5, and the water content of the samples were found, by DOC-analysis (Suzuki *et al.*, 1992), to be negligible. The alginates used are described elsewhere (Christensen *et al.*, 1996). The pullulan standard ($M_n = 139.10^4$ g/mol) used to determine adsorption to the TSK-columns was kindly supplied by Hayashibara Biochemical Lab. Inc., Okayama, Japan. The pullulan standards used for log M–V_e plots were from Polymer Lab. Ltd, Shropshire, UK.

Preparative size exclusion chromatography (SEC)

The chromatography system consisted of two columns, each 9×95 cm (Amicon Witten, Germany) attached in series, packed with Sepharose CL-6B and Sepharose CL-4B (Pharmacia, Uppsala, Sweden), respectively. Chitosans (1–1.3 mg/ml in 640 ml) were applied to the columns and eluted with 0.02 M Na acetate, pH 4.5, containing 0.1 M NaCl and 0.02% (w/v) NaN_3 at 250 ml/h. The relative chitosan concentration was monitored by a RID-detector (Shimadzu, RID-6A). Fractions of ~182 ml were collected, and the fractions containing chitosan were pooled to give 10 (Chitosan 1) and 9 (Chitosan 2) fractions. The fractions were concentrated on a rotary evaporator, dialyzed exhaustively against 0.2 M NaCl followed by distilled water

and freeze-dried. For the column system used, V_o was determined as 4.01 and V_t was determined as 13.01.

^{1}H-NMR spectroscopy

The F_A-values and the diad frequencies of fractionated and unfractionated chitosans were obtained by ^{1}H-NMR spectroscopy as previously described (Vårum *et al.*, 1991a).

Viscometry

The intrinsic viscosities $[\eta]$ were determined in a Schott-Gerate Ubbelohde viscosimeter as described by Draget *et al.* (1992). The solutions were filtered through Millipore AA-filters (0.8 μm) before determining $[\eta]$.

Determination of the Mark–Houwink–Kuhn–Sakurada (MHKS) parameters

The MHKS-equation gives the relation between the intrinsic viscosity $[\eta]$ and the molecular weight (M_v):

$$[\eta] = K \cdot M_v^a$$

where K and a are empirical constants. The viscosity average molecular weight (M_v) (Tanford, 1961) can be replaced by the weight or number average molecular weights (M_w or M_n), in that case the determined K-value must only be used on samples with the same polydispersity index (M_w/M_n).

Analytical HPSEC-LALLS-DRI

HP SEC combined with low angle laser light scattering (LALLS) detection was performed as described by Christensen et al. (1993). The dn/dc-value used in all experiments was 0.162 (Anthonsen et al., 1994). Two different column systems were used. In both cases a TSK Guard PWH column, ID = 7.5 mm, l = 75 mm, (LKB, Uppsala, Sweden) was used. The first system consisted of two serially connected columns (Ultropac TSK G6000-PW and TSK G5000-PW, ID = 7.5 mm, l = 600 mm). The second system consisted of columns (Pharmacia HR 10/30, ID = 10 mm, l = 300 mm) packed with macroporous, monodisperse (15 μm) and hydrophilic polymer particles with very large pores (Christensen *et al.*, 1996). The samples were eluted at 0.4 ml/min (Spectra-Physics IsoChrom LC-pump), at ambient temperature. The solvent used in all experiments was 0.2 M ammonium acetate, pH 4.5. The sample concentration was 0.125–0.250 mg/ml (1–2 mg/ml for detection of aggregates) and the amount of sample injected was 200–1750 μl depending on the sample concentration. Before injection, the solutions were filtered through Millipore HA-filters (0.45 μm). For all samples, 2–4 parallels were analyzed.

RESULTS AND DISCUSSION

Preparative SEC

A fully acid-soluble, heterogeneously deacetylated chitosan (Chitosan 1; F_A = 0.15) was separated into 10 fractions on Sepharose CL-6B–CL-4B. Fraction characteristics ($(K_{av})_i$, $(W)_i$,$[\eta]_i$, $(M_w)_i$, $(M_n)_i$) and $(M_w/M_n)_i$) are shown in Table 2. The M_w/M_n-value of unfractionated Chitosan 1 was 2.1, which is consistent with the Kuhn-distribution (Tanford, 1961), and in accordance with previous results obtained for heterogeneously deacetylated chitosans (Ottøy *et al.*, 1996). The polydispersity indices of the fractions vary between 1.2 and

Table 2. Fraction numbers (i), distribution coefficients ($(K_{av})_i$), weight fractions (W_i), intrinsic viscosities ($[\eta]_i$), molecular weights ($(M_w)_i$ and $(M_n)_i$) and polydispersity indices ($(M_w/M_n)_i$) of Chitosan 1 fractionated on preparative SEC with Sepharose CL 6B–CL 4B

i	$(K_{av})_i$[1]	W_i	$[\eta]_i$ (ml/g)[2]	$(M_w)_i \cdot 10^{-5}$ (g/mole)[2]	$(M_n)_i \cdot 10^{-5}$ (g/mole)[2]	$(M_w/M_n)_i$[2]
1	0.06	0.143	1310	7.2	4·9	1.5
2	0.18	0.161	1000	3.6	2·6	1.4
3	0.28	0.194	899	2.7	2·2	1.2
4	0.38	0.163	506	1.7	1·4	1.2
5	0.48	0.158	461	1.1	0·84	1.3
6	0.58	0.087	206	0.61	0·48	1.3
7	0.68	0.045	118	0.34	0·28	1.2
8	0.78	0.06	n.d.	0.26	0·20	1.3
9	0.88	0.017	n.d.	0.22	0·15	1.5
10	0.97	0.005	n.d.	n.d.	n.d.	n.d.
			$[\eta]_{calc} = \sum_{i=1}^{7}(W_i[\eta]_i)$ = 720	$(\overline{M}_w)_{calc} = \sum_{i=1}^{9}(W_i(\overline{M}_w)_i)$ = 2.7		
	Unfractionated sample		833	2.7	1.3	2.1

[1] $K_{av} = (V_e - V_o)/(V_t - V_o)$.
[2] n.d. = not determined.

Table 3. Fraction numbers (i), distribution coefficients ($(K_{av})_i$), weight fractions (W_i), intrinsic viscosities ($[\eta]_i$), molecular weights ($(M_w)_i$ and $(M_n)_i$) and polydispersity indices ($(M_w/M_n)_i$) of chitosan 2 fractionated on preparative SEC on Sepharose CL 6B–CL 4B

i	$(K_{av})_i$[1]	W_i	$[\eta]_i$ (ml/g)[2]	$(M_w)_i \cdot 10^{-5}$ (g/mole)[2]	$(M_n)_i \cdot 10^{-5}$ (g/mole)[2]	$(M_w/M_n)_i$[2]
1	0.01	0.206	621	1.6	1.0	1.6
2	0.10	0.247	487	1.4	1.0	1.4
3	0.20	0.217	341	1.1	0.8	1.5
4	0.31	0.162	257	0.73	0.54	1.4
5	0.41	0.095	171	0.55	0.44	1.3
6	0.51	0.025	n.d	0.29	0.20	1.3
7	0.61	0.022	n.d	n.d.	n.d.	n.d.
8	0.73	0.015	n.d.	n.d.	n.d.	n.d.
9	0.87	0.011	n.d.	n.d.	n.d.	n.d.
			$[\eta]_{calc} = \Sigma_{i=1}^{5}(W_i[\eta]_i)$	$(\bar{M}_w)_{calc} = \Sigma_{i=1}^{6}(W_i(\bar{M}_w)_i)$		
			= 380	= 1.1		
	Unfractionated sample		385	1.1	0.67	1.7

[1] $K_{av} = (V_e - V_o)/(V_t - V_o)$.
[2] n.d. = not determined.

1.5. The average intrinsic viscosity ($[\eta]_{calc}$) of the whole sample is slightly lower than the $[\eta]$-value obtained for the unfractionated sample, and the weight average molecular weight ($(M_w)_{calc}$) of the whole sample is very close to the M_w-value obtained for the unfractionated sample.

A fully acid-soluble chitosan with $F_A = 0.52$ (Chitosan 2) was first depolymerized by ultrasonic irradiation and then fractionated on the Sepharose columns (Table 3). The unfractionated sample has a lower polydispersity index ($M_w/M_n = 1.7$) than Chitosan 1, as expected for an ultrasonically degraded sample (Vårum *et al.*, 1991b; Yanaki *et al.*, 1983). The M_w/M_n-values of fraction 1 to 6 range from 1.3 to 1.6. The $[\eta]_{calc}$- and $(M_w)_{calc}$-values for Chitosan 2 are consistent with the $[\eta]$- and M_w-values obtained for the unfractionated sample.

The sample recovery for both fractionations was ~80%, which is acceptable for preparative SEC. The recoveries indicate no selective irreversible adsorption of the positively charged chitosan, as the combined $[\eta]_{calc}$- and $(M_w)_{calc}$-values are consistent with the $[\eta]$- and M_w-values determined for the unfractionated chitosans. Neither do the K_{av}-values between 0 and 1 for all fractions in both fractionations point to any major reversible adsorption effects. An attempt to separate more high-molecular weight chitosans on Sepharose CL-2B were unsuccessful due to complete adsorption of chitosan to this gel. Sepharose CL-2B is less crosslinked (larger pore size) than CL-4B and CL-6B, and the adsorption to this gel can be explained by varying contents of residual negatively charged groups (i.e. sulphate and carboxyl), interacting with the positively charged chitosans.

The universal calibration plots ($[\eta] \cdot M_n$ vs K_{av}) for both chitosans are given in Fig. 1. The number of average molecular weights (M_n) were used here, as this has been shown to be the correct type of molecular weight average for universal calibration of polydisperse

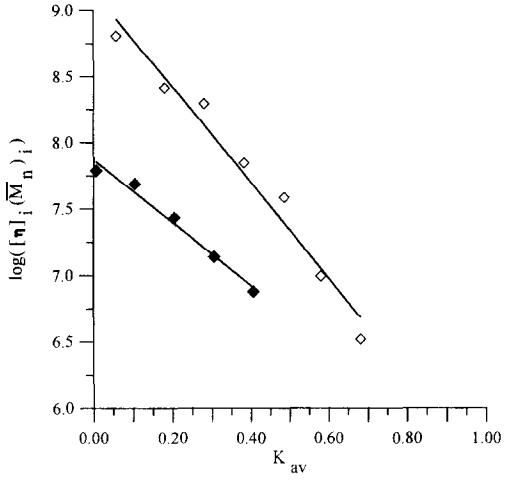

Fig. 1. Semi-logarithmic plots of the hydrodynamic volume vs K_{av} (universal calibration) for (◇) chitosan 1 ($F_A = 0.15$) and (◆) chitosan 2 ($F_A = 0.52$) fractionated on preparative Sepharose CL-6B–CL-4B columns.

samples (Hamielec & Ouano, 1978). If the separation is governed by the hydrodynamic volume only, one should obtain nearly identical plots for both chitosans, as shown previously for other polysaccharides (Martinsen *et al.*, 1991; Vårum *et al.*, 1991b). Linear plots were indeed obtained for both chitosans, but the lines differed both in the slope and the intercept with the ordinate. The shift of Chitosan 1, which has the highest charge density, towards higher K_{av}-values, may be explained by a more pronounced ionic interaction with negatively charged groups on the gel matrix. Estimation of molecular weights of chitosans with different F_A-values based upon a single universal calibration curve is, therefore, precluded with the column materials used here. However, the linearity in the plots indicates that reliable calibration plots for chitosans of a specific chemical composition (F_A) can be obtained.

Double logarithmic plots of $[\eta]$ vs M_w for the chitosan

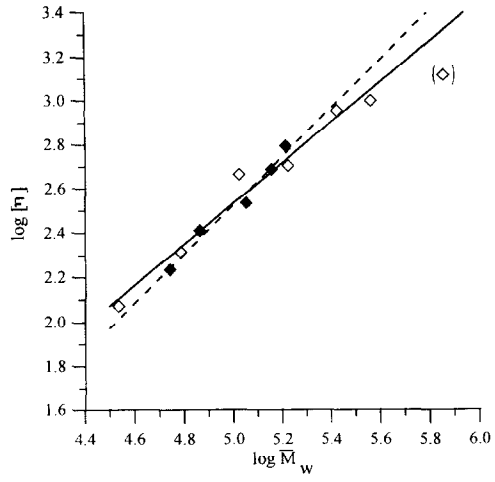

Fig. 2. Double-logarithmic plots of intrinsic viscosity vs molecular weight (M_w) (MHKS-plots) for (—◇—) chitosan 1 and (–◆–) chitosan 2 fractionated on preparative Sepharose CL-6B–CL-4B columns.

fractions are given in Fig. 2. We used the weight average molecular weights because the experimental error when obtaining the number average molecular weights from HPSEC-LALLS-DRI is higher (see below), and the weight average is closer to the viscosity average for a-values around 1. For Chitosan 1, with the highest molecular weight, we observed a tendency towards shear thinning (Bohdanecký & Kovár, 1982). Therefore we excluded fraction 1 from the linear regression used to obtain the MHKS-equation. The MHKS-equations for the chitosans, in 0.02 M NaAc/HAc, 0.1 M NaCl, pH 4.5 are:

$$F_A = 0.15 : [\eta](\text{ml/g}) = 8.5 \cdot 10^{-3} M_w^{0.92\pm0.07}, r = 0.9859$$

$$F_A = 0.52 : [\eta](\text{ml/g}) = 1.1 \cdot 10^{-3} \cdot M_w^{1.1\pm0.1}, r = 0.9750$$

The exponent in the MHKS-equations increases with increasing F_A, in reasonable accordance with previous results (Anthonsen *et al.*, 1993; Wang *et al.*, 1991). On the other hand, Rinaudo *et al.* (1993) found no effect on the stiffness of the chitosan chain with F_A, which may be explained by the limited range of F_A-values of their chitosans (0.02–0.21). The K-values depend on the molecular weight average used, and on the molecular weight distribution of the samples. The K_w-values we obtained are lower than the K_n-values previously determined (Anthonsen *et al.*, 1993), in accordance with theory. However, the samples prepared by Anthonsen and coworkers were randomly degraded, implying $M_w/M_n \approx 2$, whereas our samples were fractions with more narrow distribution, which means that the K-values obtained cannot be directly compared. The MHKS-parameters obtained here can be used to determine M_w for samples when $[\eta]$ has been determined in the same solvent as used herein. Since M_w is close to M_v when $a \approx 1$, variations in MWD between different samples may be tolerated.

The chemical composition and diad frequencies of the unfractionated chitosans and selected fractions were determined by ^{1}H-NMR spectroscopy (Table 4). The results show no systematic variation in chemical composition for the different samples. Thus, the distribution in chemical composition of fully acid-soluble chitosans previously described (Ottøy *et al.*, 1996; Vårum *et al.*, 1994) is apparently not correlated to the molecular weight of the chitosans. This result should be expected for undegraded chitosans and chitosans degraded without altering the average F_A. However, even though the average F_A is independent of the molecular weight, there may be a broadening in compositional distribution with decreasing molecular weight (Vårum *et al.*, 1994). Also, the adsorption effects discussed above are not large enough to cause any chemical fractionation inside the two different molecule populations contained in Chitosan 1 and Chitosan 2.

Analytical HPSEC of fractionated and unfractionated chitosan

Adsorption of chitosans on TSK HPSEC-columns

It is important that the separation mechanisms in HPSEC is determined by the size of the molecules and not by charge effects, adsorption or exclusion. With 0.02 M acetic acid/Na acetate and 0.1 M NaCl pH 4.5 as eluent, we observed irreversible adsorption of chitosans to the TSK-columns. The most extensive adsorption was observed with the fully deacetylated chitosan (data not shown). However, with 0.2 M ammonium acetate, pH 4.5 as eluent, the irreversible adsorption could be eliminated, as previously found for fully deacetylated chitosan oligomers separated on Biogel (Domard &

Table 4. Intrinsic viscosities, chemical composition and diad frequencies of chitosans fractionated on preparative SEC on Sepharose CL 6B–CL 4B

Sample	$[\eta]$ (ml/g)	F_A	F_{AA}	$F_{AD} = F_{DA}$	F_{DD}
Chitosan 1 (unfractionated)	833	0.15	0.05	0.12	0.71
Fraction 1	1310	0.18	0.05	0.13	0.69
Fraction 3	899	0.19	0.05	0.13	0.69
Fraction 6	206	0.16	0.04	0.12	0.71
Chitosan 2 (unfractionated)	385	0.52	0.28	0.24	0.24
Fraction 1	621	0.51	0.28	0.23	0.26
Fraction 4	257	0.54	0.31	0.23	0.23

Table 5. Recovery of ^{3}H-labelled chitosans on TSK G6000-PW G5000-PW columns

Sample	$[\eta]$ (ml/g)	F_A	Amount injected (mg)	Recovery (%)
ChitR1	660	0.37	0.2	90
			0.3	92
			0.4	85
ChitR2	250	0.20	0.3	90
			0.5	81

Cartier, 1989). This was shown by measuring the recoveries of different injected amounts of two tritium-labelled chitosans with different chemical composition and molecular weight (Table 5). As an independent approach, the relationship between the integrated area and the amount of injected sample was investigated for Chitosan 3 (Fig. 3). The linearity in the plot ($r = 0.9992$), and the fact that the intercept with the ordinate is 0, confirms that any irreversible adsorption to the columns was negligible. The integrated areas for the same injected amount of chitosans with different chemical composition ($F_A = 0.0$, $F_A = 0.15$ and $F_A = 0.6$) were also comparable (data not shown).

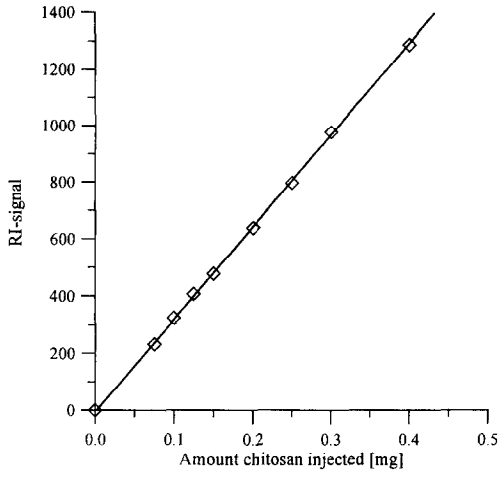

Fig. 3. Area of concentration-profile (RI-signal) plotted as a function of injected amount of chitosan 3 fractionated on analytical TSK G6000-PWXL–G5000-PWXL columns.

Determination of molecular weight and molecular weight distribution

The chosen experimental condition (0.2M ammonium acetate, pH 4.5) was used to characterize the chitosans and the corresponding fractions on the TSK HPSEC-columns (Fig. 4). The figure confirms that the polydisperse chitosans have been fractionated into fractions with different molecular weights and of more narrow molecular weight distributions. Both Chitosan 1 and Chitosan 2 are well separated on the TSK-columns according to the on-line determined molecular weights (Fig. 5a). The calculated molecular weights and polydispersity indices (M_w/M_n) are given in Tables 2 and 3. There is a scatter in the M-values in the low-molecular weight range, due to a low signal to noise ratio in the LALLS-signal (not shown). This primarily effects M_n. It appears from Fig. 5a that Chitosan 2 has a somewhat lower M_w than Chitosan 1, at the same elution volume. This may be explained by increased chain stiffness with increasing F_A and/or by an increased weak reversible adsorption to the gel with decreasing F_A. In an attempt to distinguish between the two mechanisms, we separated three chitosans with different F_A as well as two alginates on another column material (Christensen *et al.*, 1996). The results are given in Fig. 5b, showing that the on-line determined molecular weights of the two alginates and three chitosans are almost identical at the same elution volume. These results suggest that weak reversible adsorption can explain the results in Fig. 5a. A series of flexible pullulan standards with a narrow MWD were also analyzed, it can be seen from Fig. 5 that the pullulan standards elute at higher volumes, due to lower molar hydrodynamic volumes, compared to alginate and chitosan, at the same molecular weights.

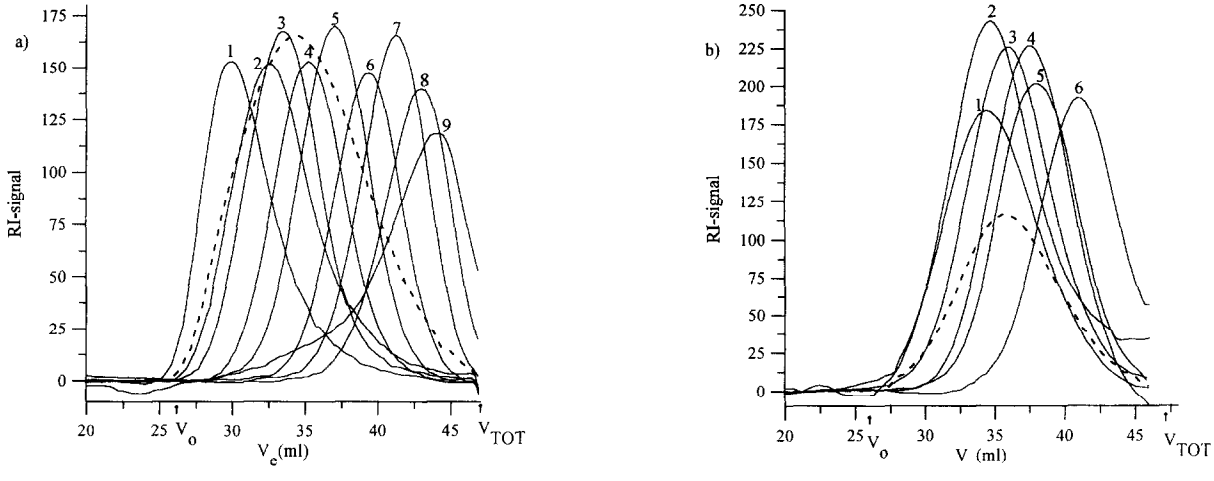

Fig. 4. HPSEC-chromatograms of chitosan fractionated on analytical TSK G6000-PWXL–G5000-PWXL (———) Unfractionated chitosan. (—) Fractions obtained from prep. SEC. (a) chitosan 1 ($F_A = 0.15$); (b) chitosan 2 ($F_A = 0.52$).

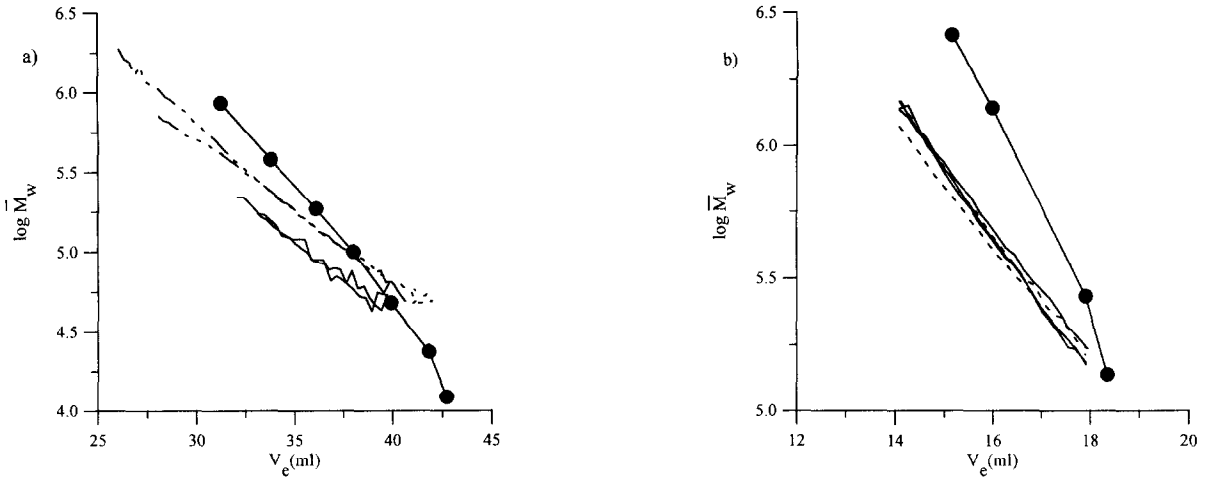

Fig. 5. (a) M_w determination of (—●—) pullulan standards, (— – – –) chitosan 1 ($F_A = 0.15$) and (—) chitosan 2 ($F_A = 0.52$) fractionated on analytical TSK G6000-PW–G5000-PW; (b) M_w determination of (—●—) pullulan standards, (– – –) alginate and (—) chitosan 4, chitosan 5 and chitosan 6 fractionated on column packing (Christensen *et al.*, 1996).

The difference in V_e between the pullulan standards and the chitosan/alginate samples is most pronounced in Fig. 5b, indicating less reversible adsorption to this column. It should also be noted that this column material is very well suited for characterizing MWD of alginates and chitosans with varying F_A, as the log M_w vs V_e calibration curves are almost identical.

The influence of F_A *and molecular weight on the tendency of chitosan to self-associate in solution*

It has been shown that chitosan may form concentration dependent aggregates in solution (Amiji, 1995; Anthonsen *et al.*, 1994), although it was not possible to reveal any differences in aggregate formation between chitosans of different chemical composition by use of static laser light scattering (Anthonsen *et al.*, 1994). When analyzing the chitosans by HPSEC-LALLS-DRI at concentrations above 1 mg/ml we frequently noticed a bimodal LALLS-signal. The first peak of the LALLS-signal can be ascribed to a small amount of high molecular weight aggregates (Domard & Rinaudo, 1986). The presence of the aggregate peak in the LALLS-signal was most pronounced for highly acetylated chitosans when comparing at the same concentration and at about the same molecular weight. In Fig. 6 the LALLS-signals of three different chitosans are shown at concentrations of: (a) $c = 1$ mg/ml; and (b) $c = 0.125$ mg/ml. At $c = 1$ mg/ml the LALLS-signal of Chitosan 7 is obviously bimodal, whereas for Chitosan 8 it is broad and somewhat flattened, and for Chitosan 9 it is unimodal. This indicates an increasing tendency of chitosans to self-associate with increasing acetyl content. Thus, chitosan is likely to aggregate through intermolecular, presumably hydrophobic, interactions between the acetyl groups for the solvent used in these experiments. Although ammonium acetate is known to interrupt

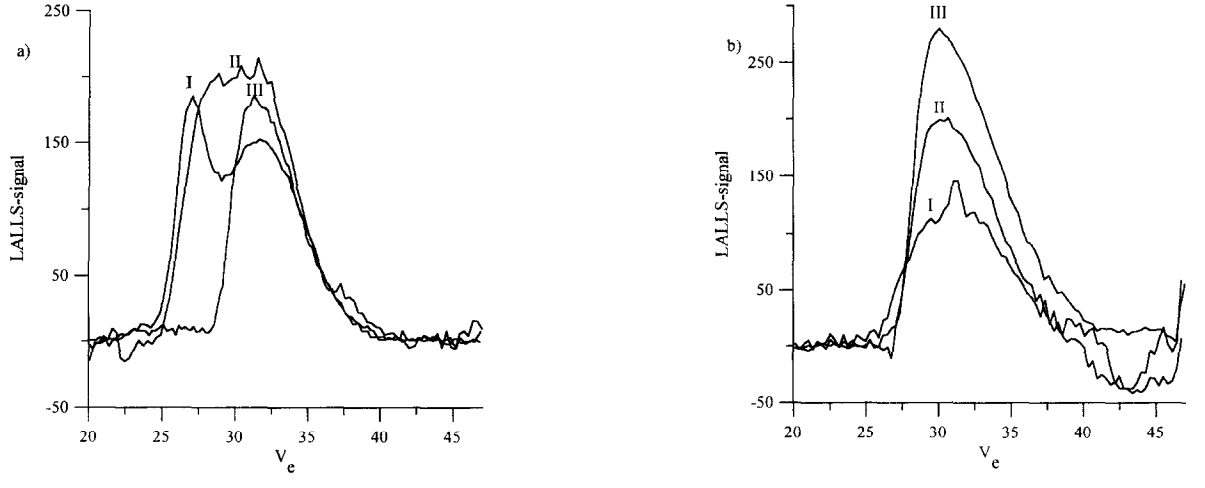

Fig. 6. Concentration normalized LALLS-signals of chitosans fractionated on analytical TSK G6000-PW–G5000-PW. I: chitosan 7 ($F_A = 0.6$); II: chitosan 8 ($F_A = 0.15$). III: chitosen 9 ($F_A = 0.0$) chitosan concentration before injection on the column system; (a) 1 mg/ml, (b) 0.125 mg/ml.

Table 6. The influence of F_A and molecular weight on the tendency of chitosan to self-associate in solution ($c = 1$ mg/ml)

Sample	$M_w \cdot 10^{-5}$ (g/mole)	% Aggregates
	Chitosan 1 ($F_A = 0.15$)	
Fraction 3	2.7	18
Fraction 4	1.7	7
Fraction 6	0.61	3
	Chitosan 2 ($F_A = 0.52$)	
Fraction 1	1.6	19
Fraction 3	1.1	5
Fraction 5	0.55	3

hydrogen-bonds (Domard & Rinaudo, 1984), changes in the solvent (i.e. increasing concentration of ammonium acetate or changing pH) had no influence on the tendency of chitosan to self-associate, implying that the self-association is not caused by hydrogen-bonding. From HPSEC-LALLS-DRI it is possible, by excluding the high molecular weight tail from the calculations, to make a *rough* estimate of the fraction of aggregates formed in solution (Anthonsen *et al.*, 1994). This was done for some of the fractions (Table 6). The relative content of aggregates in solution increased with increasing molecular weight, as expected. When comparing Chitosan 1, fraction 4, and Chitosan 2, fraction 1, which have about the same molecular weights and polydispersity indices, it can be seen that the more acetylated Chitosan 2 has a much higher tendency towards self-association, again suggesting that the aggregate formation at the present solvent conditions is caused by intermolecular hydrophobic interactions between the acetyl groups.

ACKNOWLEDGEMENTS

We thank Ms Mildrid Myhr for helpful discussions and skilful technical assistance. We thank the Norwegian Research Council and Pronova Biopolymers a.s. Drammen, for financial support.

REFERENCES

Amiji, M.M. (1995). Pyrene fluorescence study of chitosan self-association in aqueous solution. *Carbohydr. Polym.*, **26,** 211–213.

Anthonsen, M.W., Vårum, K.M. & Smidsrød, O. (1993). Solution properties of chitosans: conformation and chain stiffness of chitosans with different degrees of *N*-acetylation. *Carbohydr. Polym.*, **22,** 193–201.

Anthonsen, M.W., Vårum, K.M., Hermansson, A.M. & Smidsrød, O. (1994). Aggregates in acidic solutions of chitosans detected by static laser light scattering. *Carbohydr. Polym.*, **25,** 13–23.

Arcidiacono, S. & Kaplan, D.L. (1992). Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. *Biotechnol. Bioengin*, **39,** 281–286.

Beri, R.G., Walker, J., Reese, E.T. & Rollings, J.E. (1993). Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique. *Carbohydr. Res.*, **238,** 11–26.

Bohdanecký, W. & Kovár, J. (1982). *Viscosity of Polymer Solutions*. Elsevier Scientific Publishing Co., Amsterdam.

Christensen, B.E., Smidsrød, O., Elgsaeter, A. & Stokke, B.T. (1993). Depolymerization of double-stranded xanthan by acid hydrolysis: characterization of partially degraded double strands and single-stranded oligomers released from the ordered structures. *Macromolecules,* **2622,** 6111–6120.

Christensen, B.E., Myhr, M., Aune, O., Hagen, S., Berge, A. & Ugelstad, J. (1996). Macroporous monodisperse particles and their application in aqueous size exclusion chromatography of high molecular weight polysaccharides. *Carbohydr. Polym.*, **29,** 217–223.

Domard, A. & Rinaudo, M. (1984). Gel permeation chromatography of cationic polymer on cationic porous silica gels. *Polym. Commun.*, **25,** 55–58.

Domard, A. & Rinaudo, M. (1986). Fully deacetylated chitosan. *Chitin in Nature and Technology*, eds R. Muzzarelli, C. Jeuniaux & G.W. Gooday. Plenum Press, New York, pp. 315–316.

Domard, A. & Cartier, N. (1989). Glucosamine oligomers: 1. Preparation and characterization. *Int. J. Biol. Macromol.*, **11,** 297–302.

Domard, A. & Cartier, N. (1991). Glucosamine oligomers: 3. Study of the elution process on polyacrylamide gels, application to other polyamine oligomer series. *Polym. Commun.*, **324,** 116–119.

Draget, K.I., Vårum, K.M., Moen, E., Gynnild, H. & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions: a new gelling system. *Biomaterials,* **139,** 635–638.

Goto, A., Araki, C. & Izumi, Y. (1981). Molecular weight distribution of water-soluble pectins in citrus juices. *Proc. Int. Soc. Citricult.*, **2,** 931–934.

Grubisic, Z., Rempp, P. & Benoit, H. (1967). A universal calibration for gel permeation chromatography. *Polym. Lett.*, **5,** 753–759.

Hamielec, E. & Ouano, A.C. (1978). Generalized universal molecular weight calibration parameter in GPC. *J. Liq. Chromatogr.*, **1,** 111–120.

Hasagawa, M., Isogai, A. & Onabe, F. (1994). Molecular mass distribution of chitin and chitosan. *Carbohydr. Res.*, **262,** 161–166.

Jordan, R.C. & Brant, D.A. (1978). An investigation of pectin and pectin acid in dilute aqueous solutions. *Biopolymers,* **17,** 2885–2895.

Kurita, K., Koyama, Y., Nishimura, S. & Kamiya, M. (1989). Facile preparation of water-soluble chitin from chitosan. *Chem. Lett.*, 1597–1598.

Lecacheux, D., Mustiere, Y. & Panaras, R. (1986). Molecular weight of scleroglucan and other extracellular microbial polysaccharides by size-exclusion chromatography and low angle laser light scattering. *Carbohydr. Polym.*, **6,** 477–492.

Martin, M. (1982). Polymer analysis by fractionation with on-line light scattering detectors. *Chromatographia,* **157,** 426–432.

Martinsen, A., Skjåk-Bræk, G. & Smidsrød, O. (1991). Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates. *Carbohydr. Polym.*, **15,** 171–193.

Ottøy, M.H., Vårum, K.M. & Smidsrød, O. (1996). Compositional heterogeneity of heterogeneously deacetylated chitosans. *Carbohydr. Polym.*, **29**(1), 17–24.

Rinaudo, M., Milas, M. & Le Dung, P. (1993). Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. *Int. J. Biol. Macromol.*, **15,** 281–285.

Smidsrød, O. & Haug, A. (1968). A light scattering study of alginate. *Acta Chem. Scand.*, **22,** 797–810.

Smidsrød, O. & Grasdalen, H. (1984). Conformation of κ-carrageenan in solution. *Hydrobiologia*, **116/117,** 178–186.

Suzuki, Y., Tanoue, E. & Ito, H. (1992). A high-temperature catalytic method for the determination of dissolved organic carbon in seawater: analysis improvement. *Deep-Sea Res.*, **39,** 185–198.

Tanford, C. (1961). *Physical Chemistry of Macromolecules.* Wiley, New York.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. & Smidsrød, O. (1991). Determination of the degree of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr. Res.*, **211,** 17–23.

Vårum, K.M., Martinsen, A. & Smidsrød, O. (1991). Fractionation and viscosimetric characterization of a (1→3),(1→4)-β-D-glucan from oat, and universal calibration of a high-performance size-exclusion chromatographic system by the use of fractionated β-glucans, alginates and pullulans. *Food Hydrocoll.*, **54,** 363–374.

Vårum, K.M., Smidsrød, O. & Brant, D.A. (1992). Light scattering reveals micelle-like aggregation in the (1→3),(1→4)-β-D-glucans from oat aleurone. *Food Hydrocoll.*, **5,** 497–511.

Vårum, K.M., Ottøy, M.H. & Smidsrød, O. (1994). Water-solubility of partially *N*-acetylated chitosans as a function of pH: effect of chemical composition and depolymerization. *Carbohydr. Polym.*, **25,** 65–70.

Wang, W., Bo, S., Li, S. & Qin, W. (1991). Determination of the Mark–Houwink equation for chitosans with different degrees of deacetylation. *Int. J. Biol. Macromol.*, **13,** 281–285.

Wu, A.C.M., Bough, A., Conrad, E.C. & Alden, K.E. Jr (1976). Determination of molecular-weight distribution of chitosan by high-performance liquid chromatography. *J. Chromatogr.*, **128,** 87–99.

Yanaki, T., Nishi, K., Tabata, K. & Kojima, T. (1983). Ultrasonic degradation of schizophyllum commune polysaccharide in dilute aqueous solution. *J. Appl. Polym. Sci.*, **28,** 873–878.

Yomota, C., Miyazaki, T. & Okada, S. (1993). Determination of the viscometric constants for chitosan and the application of universal calibration procedure in its gel permeation chromatography. *Coll. Polym. Sci.*, **271,** 76–82.

Yu, L.-P. & Rollings, J.E. (1987). Low-angle laser light scattering—aqueous size exclusion chromatography of polysaccharides: molecular weight distribution and polymer branching determination. *J. Appl. Polym. Sci.*, **33,** 1909–1921.