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Carbohydrate Polymers 47 (2002) 39–51

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

The degree of acetylation of chitosans and its effect on the chain conformation in aqueous solution

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Received 1 November 1999; revised 26 October 2000; accepted 27 November 2000

Abstract

The average degree of acetylation (DA) of chitosans from several sources as well as the distribution of the DA across the molecular weight distribution was measured by titration with poly(styrene sulphonate) as counter polyanion and toluidine blue as metachromatic indicator. The method was shown to be useful within a broad range of DAs. There was no compositional heterogeneity for more than 90% of the population in any sample. Parallel macromolecular characterisation by static light scattering and viscometry standing alone or connected with gel permeation chromatography (GPC) revealed no measurable effect of the DA on the chain conformation where the DA varied between a few percent and ~60%. Relationships between intrinsic viscosity and radius of gyration with molecular mass were established. The data when considered with previous work suggest no evident differences between chitosans from different sources and no significant effect of the ionic strength between ~0.1 and 0.3 M on the conformation. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosan; Degree of acetylation; Molecular mass; Conformation; Wormlike chain; Colloid titration; GPC; Static light scattering; Viscometry

1. Introduction

Chitosans are copolymers of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc). They are soluble in aqueous acidic solution. At a pH $\ll$ pK_a ~ 6–7, the macromolecules are polycations and at pH 4.5 and below, they are completely protonated (Rinaudo, Milas & Dung, 1993). Since only the non-acetylated amino groups bind protons, the charge density of the polyelectrolyte depends on the ratio of the two monomers in a chain. A low degree of acetylation (DA) means a highly charged polyion in acidic solution.

Nowadays chitosans are used in low-calorie diets and pharmaceuticals as well as cosmetic formulae. Both the DA and the molecular weight are key parameters determining the properties relevant to these applications. The majority of commercial chitosans have average DAs as high as 25–30%. Low-molecular weight (LM) and high-molecular weight (HM) products are on the market.

For a better understanding of the functions these species have in their natural environment or in man-made composites, a solid knowledge of the molecular structure and solution behaviour is useful. In previous work, we have found that a single-stranded worm-like chain with a persistence length of ~6 nm described adequately the conformation of macromolecules in aqueous solutions using static light scattering (SLS) (Berth, Dautzenberg & Peter, 1998) and hydrodynamic methods (Cölfen, Berth & Dautzenberg, 2001). The ionic strength I ~ 0.12 M was used to suppress charge effects. As pointed out in these papers, two items were not addressed sufficiently and are the subject of the present work. These are: (i) the chemical homogeneity of the materials in terms of the distribution of DA across the molecular weight distribution within a given sample, and (ii) the steric effect of amino groups in terms of the influence of DA on the chain conformation over a broad range of DAs.

To achieve these objectives, we have built on previous work by our group:

(i) The molecular weight distribution of the samples could be analysed by semi-preparative gel permeation chromatography (GPC) on Sepharose gels combined with off-line SLS on individual fractions. To determine the respective DA data, we were interested in a quick and easy to handle method for the determination of DA which, like SLS and viscosity measurements, worked reliably in the eluant at the low polymer concentrations given by GPC. As such, colloid titration with toluidine

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0144-8617/02/$ - see front matter
PII: S0144-8617(00)00343-X

blue as metachromatic indicator (Wassmer, Schroeder & Horn, 1991) seemed promising. The method has been established for chitosans (see, e.g. Aiba, 1986, 1991; Roberts, 1992) and was recently applied to chitosan oligomers (Hattori, Katai, Kato, Izume & Mizuta, 1999). (ii) Any possible effect of the DA on the chain conformation has been discussed previously (Berth et al., 1998). As will be shown, this point needed reconsideration in the context of the findings from (i) because of the importance of the subject. We think there is still a lot of contradiction in the literature. For this reason, we will discuss our results in this context with some data from the literature. In addition to the three previously used commercial chitosans, we have used five more samples that were prepared by homogeneous re-acetylation of a widely de-acetylated commercial chitosan sample. The procedure was expected to produce largely homogeneous materials in terms of the DA distribution without depolymerisation (Roberts, 1997a). The code for each sample used in the following applies the target DA; Chit 10 means, e.g. chitosan of an intended average DA of 10%, etc. Their macromolecular characterisation using SLS and capillary viscometry as major tools was as described previously (Berth et al., 1998).

2. Experimental

2.1. Chitosan samples

The three commercial chitosan samples referred to as Chit A, Chit B and Chit C have been described already elsewhere — for details, see Berth et al. (1998) and Cölfen et al. (2001).

The samples of various DAs were a highly appreciated gift from Professor G.A.F. Roberts. They were prepared and analysed in terms of DA in his department at the Trent University of Nottingham, UK. A commercial sample of chitosan (DA $\cong$ 2%) prepared by heterogeneous deacetylation was dissolved in 0.1 M CH_3COOH, filtered through two layers of monofilament polyester print screen fabric to remove insoluble material and gel particles, then precipitated by addition of NaOH solution ($\sim$1 M). The precipitate was washed with distilled water until neutral, then dried at 60°C under vacuum.

Portions (2 g) of the product were redissolved in 0.1 M CH_3COOH (200 ml) and diluted with methanol (350 ml). Then to each solution was added, with vigorous stirring, a further 50 ml of methanol containing the calculated amount of acetic anhydride required to give the target level of *N*-acetylation (assuming a reaction efficiency of 100%). After standing at 20°C for 24 h, the *N*-acetylated products were precipitated by addition of concentrated NH_4OH solution, filtered off, washed to neutral with 75% aqueous methanol, dried at 60°C under vacuum. The DA was analysed by dye adsorption as described in the literature (Maghami & Roberts, 1988; Roberts, 1997b) using a 5×10^{-3} M stock solution of C.I. Acid Orange 7 as reagent.

2.2. Solution preparation

Solvent: 0.02 M acetate buffer/0.1 M NaCl, pH 4.5, was consistently used as solvent.

For subsequent GPC, the samples were dissolved at room temperature ($\sim$2 mg/ml) overnight under slight shaking. Then the solution was ultracentrifuged for 90 min at 40,000 rpm and 25°C using a Beckman preparative ultracentrifuge L-70 and a fixed-angle rotor Ti 70.1. The supernatant was filtered through membrane filters in the order of decreasing pore size (5.0, 0.8, 0.45, 0.2 μm). About 16 ml (weight control) were injected into the GPC column.

To study the unfractionated parent samples by light scattering, viscometry and colloid titration, the samples were dissolved overnight to give solutions of $\sim$2 mg/ml at the maximum. The solutions (or suspensions) were then dialysed against a buffer in excess (30–50 ml solution against $\sim$250 ml buffer; membrane cut-off: 1 kDa). After dialysis, the pH of the solution reduced to a value between 4.6 and 4.5 (identical pH values in the polymer solution and dialysate). The polymer solution was ultracentrifuged as described above. The supernatant was separated carefully from the precipitate and filtered through a set of membrane filters (minisart, Sartorius-Membranfilter GmbH, Germany) with pore sizes of 5.0, 1.2, 0.65, and finally 0.2 μm (all at once) to give 'clarified stock solutions'. Series of normally five concentrations were prepared from this *stock* solution using the dialysate for dilution. The resultant concentration was measured by means of an interferometric differential refractometer (ScanRef; Nanofilm Technologie, Germany) using $\delta n/\delta c = 0.203$ ml/g at 25.0°C. To keep the error small, at least three concentrations (for linear regression) out of the series above were measured against the dialysate as blank. About 75–92% of the original matter was retrieved. The brownish re-acetylated samples showed a poorer solubility than their slightly coloured industrial relatives (see Berth et al., 1998).

2.3. Gel permeation chromatography (semi-preparative)

Three Pharmacia columns (Pharmacia, Sweden) were used alone or combined in an ascendant flow stream: 2.5 cm diameter × 90 cm length column filled with (i) Sepharose CL-2B or (ii) Sepharose CL-6B and (iii) 2.5 cm diameter × 45 cm length column filled with Sephadex G-75. Degassed 0.02 M acetate buffer, pH 4.5, containing 0.1 M sodium chloride, was used consistently as eluant. All other instrumental details have been given already (Berth et al., 1998). The concentration was monitored by means of a differential refractometer RID-6 (Shimadzu, Japan) and checked in an interferometer using $\delta n/\delta c = 0.203$ ml/g (ScanRef, NFT, Germany). The fractions of 16–17 ml

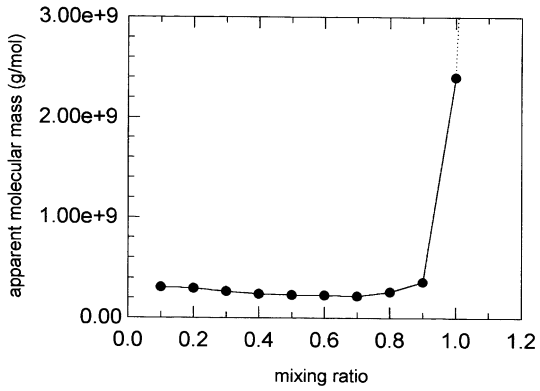

Fig. 1. Titration curve for Chit A with poly(styrene sulphonate) followed by light scattering; the mixing ratio is related to the number of charges showing 1:1 stoichiometry.

each (checked by weight) were used for colloid titration, light scattering and, finally, viscosity measurements. Prior to the light scattering measurements, the fractions were filtered through a 0.2-μm pore size filter into the dust-free measuring cell. For data handling, see Berth et al. (1998).

2.4. Colloid titration

All spectra were measured in a UV/VIS spectrometer, type Lambda 2 (Perkin–Elmer, USA). Titration was carried out directly in the measuring cells (1.0 cm optical path length). In the case of GPC fractions, to the chitosan solutions of about $1–2 \times 10^{-4}$ mol 'chitosan monomer' per 2.0 ml was added, under slight stirring, 1.00×10^{-3} M monoM **p**oly(**s**tyrene **s**ulfonate) in acetate buffer (PSS, desalted before use) from Polymer Standards Service, Germany, in steps of 5 or 10 μl (Eppendorf pipettes, Germany). The concentration of toluidine blue was about 5×10^{-6} M. The difference of absorption at two wavelengths, mostly $A_{635} - A_{600}$, was plotted against the volume of PSS solution added.

To titrate about 10 times higher concentrated solutions of the parent samples, the PSS concentration was increased to 1.00×10^{-2} M and the concentration of toluidine blue was kept constant.

The total polymer concentration (GlcNAc + GlcN) was taken from the DRI records (GPC) or measured refractometrically (parent samples) using uniquely $\delta n/\delta c = 0.203$ ml/g.

The DA was calculated using M_0(GlcN) = 161 g/mol and M_0(GlcNAc) = 203 g/mol for the monomeric units.

2.5. Static light scattering measurements

The light scattering measurements on the concentration series were performed at 22 or 25°C in different instruments against the dialysate as blank: (i) Sofica goniometer, model 42000, FICA, France; (operating wavelength of the laser light source: $\lambda_0 = 632$ nm) and (ii) ALV instrument inclusive software from Langen, Germany, at $\lambda_0 = 532$ nm. Angles of observation between 30 and 150° in steps of 5° or less were measured and taken into account. The Zimm procedure was used for data evaluation with $\delta n/\delta c = 0.203$ ml/g (Berth et al., 1998).

The light scattering measurements in the fractions from GPC were carried out off-line in a Sofica goniometer, model 42000 (FICA, France), equipped with a 1-mW helium/neon laser (wavelength: 632.8 nm; SpectraPhysics, USA) at 31° between 30 and 150° against the blank eluant. Further details are given elsewhere (Berth et al., 1998).

2.6. Capillary viscosity measurements

Capillary viscometry was carried out in an Ubbelohde-type

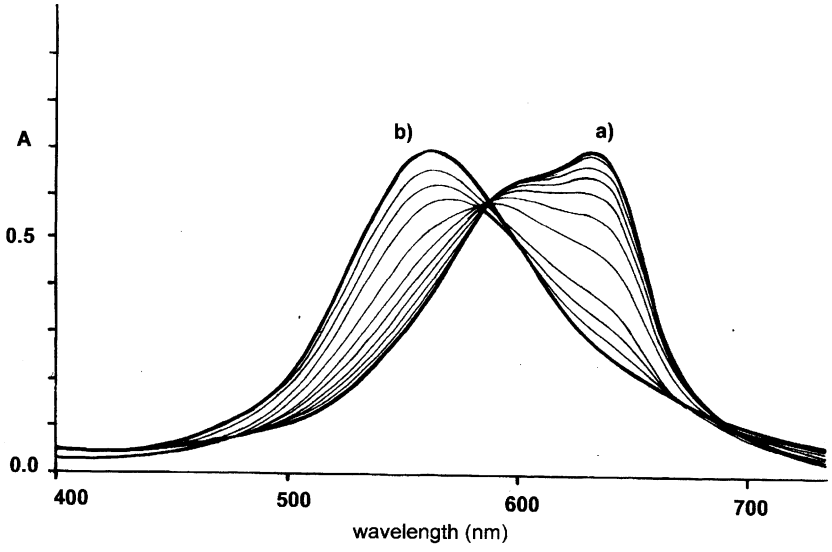

Fig. 2. Spectra of toluidine blue: (a) free in acetate buffer; and (b) bound to poly(styrene sulphonate). The other curves represent intermediate stages of titration.

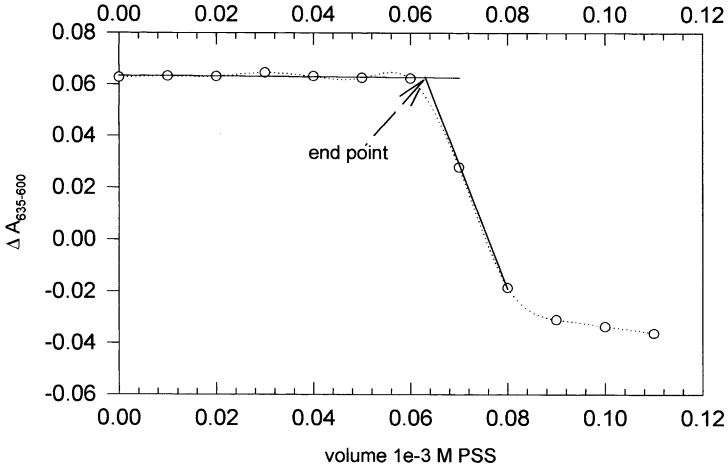

Fig. 3. Colloid titration with toluidine blue as metachromatic indicator; representative titration curve for the titration of chitosan with poly(styrene sulphonate).

viscometer at 25.0°C on the concentration series (dialysate as blank). The intrinsic viscosity $[\eta]$ in millilitres per gram as well as Huggins constants were calculated in the common way from a plot η_{sp}/c versus c.

3. Results and discussion

3.1. Colloid titration

3.1.1. Determination of the average DA of the parent samples

In our experiments, sodium salt of PSS was used as polyanion. To make sure that the polyelectrolyte complex formation between chitosan and PSS followed 1:1 stoichiometry in our solvent, separate SLS experiments (see, e.g. Dautzenberg, Hartmann, Grunewald & Brand, 1996) were performed. Fig. 1 shows the plot for the titration of Chit A with equimolar PSS solution (related to charge units and based on DA = 25% by potentiometric titration). Exactly at the mixing ratio of 1.0, the average molecular mass increases considerably and further addition of PSS initiated flocculation. This marks the end point of titration. The result is in good agreement with the findings of others (Hugerth, Caram-Lelham & Sundelöf, 1997) who observed a 1:1 charge stoichiometry for the reaction between carrageenan and chitosan.

Metachromatic indicators such as toluidine blue change their colour when they are bound to a polyanion (for details see, e.g. Wassmer et al., 1991). Colloid titration with metachromatic indicators makes use of the preferential and stoichiometric polyelectrolyte complex formation between polyanions and polycations. Titration of polycations can be carried out in different ways, e.g. by adding a polyanionic component to the indicator containing polycationic solution. Not until the polyanion is added in excess is the indicator bound. The bound indicator can be displaced by the added polycation.

Fig. 2 shows the spectra of toluidine blue (a) free in the presence of chitosan in acetate buffer at the beginning of titration and (b) bound to PSS beyond the end point of titration. The other curves represent intermediates. Under optimum conditions, a *sudden* transition from (a) to (b) can be achieved giving the titration curve shown in Fig. 3. The decrease of absorption at 635 nm together with the increase of absorption at 600 nm marks the beginning of the reaction between PSS and toluidine blue as soon as all chitosan has been consumed. Thus the added volume of PSS solution of known molarity gives directly the number of cationic charges (in moles) in the test solution. Titration in reverse order (adding chitosan to a known amount of PSS plus dye in solution) led to the same result.

Table 1
Characteristic parameters of the parent samples

Sample	Recovery rate[a] (%)	DA[b] (%)	M_w (g/mol)	$R_{G,z}$ (nm)	$B \times 10^3$ (ml mol/g^2)	$[\eta]$ (ml/g)	Huggins constant
Chit 10	75	3	178,000	70	6.18	418	0.62
Chit 20	92	31	180,000	67	3.57	461	0.59
Chit 30	78	24	144,000	55	5.22	420	0.52
Chit 45	84	45	141,000	63	4.68	357	0.76
Chit 60	77	53	95,000	37	2.6/3.2	358	0.59

[a] Determined in the clarified solution by means of the ScanRef interferometric refractometer and $\delta n/\delta c = 0.203$ ml/g with the dialysate as blank and related to the initial concentration expressed as milligrams air-dried original matter per millilitre buffer.
[b] Determined by colloid titration coupled with refractometrical concentration determination.

First of all, the method was applied to the commercial samples Chit A, B and C. Good reproducibility was achieved when the sample solution had been dialysed (equilibrium dialysis) against the solvent in excess. At the end of this operation, all the material was completely suspended exactly at pH 4.5. Using membranes with a cut-off of 1 kDa, no loss in carbohydrate but some dilution during dialysis was noticed. The DA values determined in this way ranged between 25 and 31% for both Chit A and Chit C for at least five parallel experiments on each sample and 22–27% for Chit B. This illustrates the experimental error, which is about ±3% on the DA scale. Within these limits, the potentiometrically titrated DA of 25% for Chit A and also the producer's DA for Chit B were confirmed. In contrast to the manufacturer's information, there was no significant difference between Chit B and C. The similar DAs of the three commercial samples invalidate the preceding part of our discussion in Berth et al. (1998) dealing with DA effects on the chain stiffness.

Results for the *re-acetylated* sample series with DAs between 10 and 60% are compiled in Table 1. The retrieved soluble proportion in the clarified solution (column 2) was distinctly lower than in the case of the commercial samples (see Berth et al., 1998) and the relatively poor solubility made these samples even more difficult to handle than the commercial ones. All data given here are related solely to the soluble proportion that remained after clarification. Major loss in material occurred during ultracentrifugation as part of the clarification procedure where visible amounts of brownish pigments and colourless gel-like material settled down. Some material was lost upon filtration (occasionally forming 'slime' on the filter membrane). A small amount of material might have been lost also during dialysis. This, however, would not restrict the use of the dialysate for dilution in any of our methods because of the low-molecular weight character of such permeating fractions and the resultant low concentration in the large volume of dialysate. Colloid titration and refractive index measurements in the dialysate provided blanks only and so did SLS and viscosity measurements.

The DA values (column 3 in Table 1) differ from the target values: 3% was found instead of 10% and 53% instead of 60%. The positions of Chit 20 with DA = 31% and Chit 30 with DA = 24% are swapped (as if, by accident, the samples in their bags had been mixed up) whereas in the case of Chit 45, the target value was met perfectly. So at least the main course and the difference of 50% between Chit 10 and Chit 60 appear correctly and illustrate the usefulness of the colloid titration method when applied to samples with DAs in this range of practical interest.

3.1.2. Distribution of the DA in GPC fractions

In order to get information on the distribution of the DA across the molecular weight distribution, the samples were fractionated by GPC. To achieve optimum separation for the samples of various molecular masses, different separation media were used. This was a combination of Sepharose CL-2B/Sephadex G-75 for the high-molecular weight Chit C whereas the low-molecular weight Chit B was fractionated satisfactorily on Sepharose CL-6B. Chit A was fractionated on Sepharose CL-2B.

The elution line (DRI response) of Chit C along with the DA level is shown in Fig. 4a. The DA values move around 25% for the fractions eluted between ~230 and ~600 ml and rise up to almost 100% for the fractions between about 600 and 780 ml, which make up about 7% of the total area under the elution line. The weighted-average DA over all fractions is ~29%, which is in good agreement with that of the parent sample (see above).

To illustrate the quality of fractionation achieved, the apparent molecular masses $M_{w,app}$ as well as the reduced viscosities η_{red} (measured at the low but finite concentrations given by GPC) are plotted against the elution volume V_e in Fig. 4b and c. The almost straight line in the Universal Calibration Plot ($\{M_w \times \eta_{red}\}$ versus V_e (see Grubisic, Rempp & Benoit, 1967) in Fig. 4d indicates a good separation according to the hydrodynamic volume. No significant viscosity or light scattering response was obtained in the region of the high-acetylated fractions thus indicating their low-molecular weight nature.

Fig. 5 shows the DAs plotted against the apparent molecular mass $M_{w,app}$ for fractions from the three commercial chitosans. Apart from the low-molecular weight peak, there is no significant variation of the DA across the molecular weight distribution. This is in good agreement with the findings of Ottøy, Vårum, Christensen, Anthonsen and Smidsrød (1996) by NMR experiments.

When fractionated on Sepharose CL-2B/Sephadex G-75 columns, the re-acetylated samples of various DAs follow the same pattern as shown in Fig 4a. All of them contain a few percent of a low-molecular weight fraction with high DA whereas the major proportion eluted first reveals a homogeneous distribution of the DA corresponding to the level of each parent sample. When the fractionation was done on Sepharose CL-2B alone, the low-molecular weight fraction appears only as a shoulder on the major fraction. This is shown in Fig. 6 using Chit 60 as an example. The DAs increase for the polymer eluted at the end but do no longer reach the high levels shown in Fig. 4a. In other words, the poorer separation in the low-molecular weight area gives the samples a more homogeneous appearance. Omitting the Sephadex G-75 column does not necessarily give a reduced quality of separation in the high-molecular weight area or falsified average data over all fractions. The re-calculated overall DA of 57% agrees reasonably well with that in Table 1. The same pattern applies to the other four chitosans. So it is obvious that no compositional heterogeneity of the materials can explain the somewhat curious findings such as the levelling-off of the $[\eta]$–M_w and $R_{g,z}$–M_w relationship in the *upper* part of the molecular weight distribution (see next paragraph).

It is appropriate to comment on the reliability of the DAs

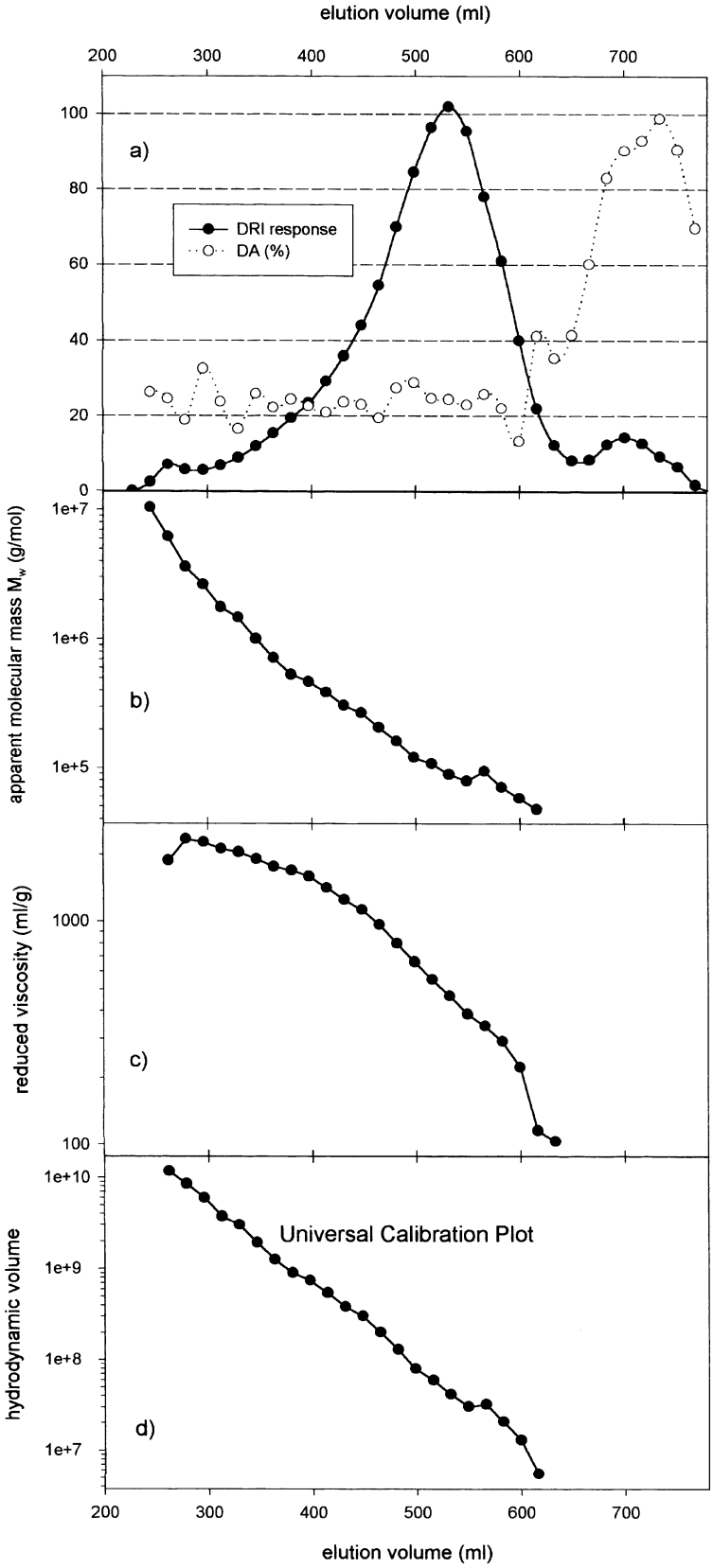

Fig. 4. Chit C fractionated on Sepharose CL-2B/Sephadex G-75: (a) elution line (filled circles — DRI response) and distribution of the degree of acetylation (open circles) in the fractions; (b) apparent molecular masses $M_{w,app}$ by static light scattering versus elution volume V_e; (c) reduced viscosities η_{red} versus elution volume V_e ; (d) universal calibration plot $\{(M_{w,app} \times \eta_{red})\}$ versus V_e.

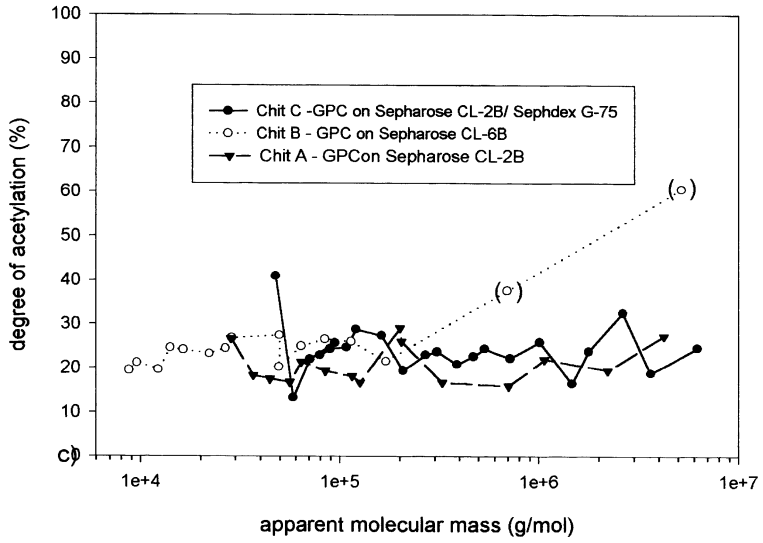

Fig. 5. Distribution of the degree of acetylation along the apparent molecular masses for the three industrial chitosans.

measured for the individual GPC fractions. To calculate them, we have assumed the refractive index increment to be constant along the elution volume axis so that the differential refractometer response supplies exactly the total polymer concentration ($c_{GlcN+GlcNAc}$). This assumption may not hold perfectly because of any possible effect of the DA on the specific refractive index increment (see, e.g. Anthonsen, 1993) but the resultant recovery of, e.g. 93% in the case of Chit C seems reasonable in the context with previous work (Berth et al., 1998).

Colloid titration estimates the proportion of *N*-glucosamine (c_{GlcN}). Based on our extensive experience with polyelectrolyte complexes (Dautzenberg, 2001), complex formation with 1:1 stoichiometry was considered reasonable as long as the DA of individual species does not exceed a value of, say, 90%. Otherwise the distance between charges (about 5 Å per monomer unit) might become too large to bring about complex formation. For this reason, the DAs for the last few low-molecular weight fractions might be somewhat overestimated (see also Hattori et al., 1999). We tend to assume highly acetylated oligomers in this area since the peak remained even after dialysis. Salt peaks (acetic acid, sodium acetate) would appear at the same elution volume and produce the same effect on DA but would have disappeared after dialysis. In view of the small amounts present, we think it is of minor importance to argue over a few DA percent more or less within that proportion.

3.2. Macromolecular characterisation of the samples of various DA

3.2.1. Studies on fractions from GPC

The fractions from GPC were taken to measure the apparent molecular mass $M_{w,app}$, the radii of gyration $R_{G,z}$ and the reduced viscosities η_{red} (single-concentration measurements). The resultant plots η_{red} and $R_{G,z}$, respectively, versus $M_{w,app}$ were expected to show systematic effects if the chain conformation was dependent on the DA. The elution lines and 'Universal Calibration Plots' for Chit 10, Chit 30, and Chit 60 on Sepharose CL-2B are shown in Fig. 7a and b. Chit 10 is eluted late (see the M_w column in Table 1). This, along with the shifted Universal Calibration Line, suggests that the normal size exclusion mechanism of GPC is perturbed by electrostatic interactions between some anionic groups of the Sepharose gel and the highly charged solute as has been proposed by Ottøy et al. (1996). Fig. 8 presents the plots of (a) η_{red} versus $M_{w,app}$ and (b) $R_{G,z}$ versus $M_{w,app}$ where the three commercial samples are included. (M_w was *not* replaced by the degree of polymerisation because of the small effect that correction would have on the logarithmic plots.) The levelling-off behaviour at the upper end of the molecular weight distribution is found again. Taking the experimental error of the individual data into account, no significant differences amongst the three samples and also their industrial relatives can be seen. The sets of η_{red}–$M_{w,app}$ curves coincide and can be represented by a straight line with a slope $a_\eta = 0.91$ in the molecular weight range between 40,000 and 300,000 g/mol. The corresponding radii of gyration do not differ by more than 10 nm at a given molecular mass where the curves for Chit 10 and Chit 60 practically coincide. Again one joint straight line with a slope of $a_R = 0.40$ might adequately describe the measured dependencies regardless of the DA. Superficially, this scaling exponent seems unacceptably low. Model calculations in terms of the worm-like chain model (Berth & Dautzenberg, 1998) have shown that, for monodisperse chitosan fractions under θ conditions, the scaling exponent a_R would be anticipated to be as high as

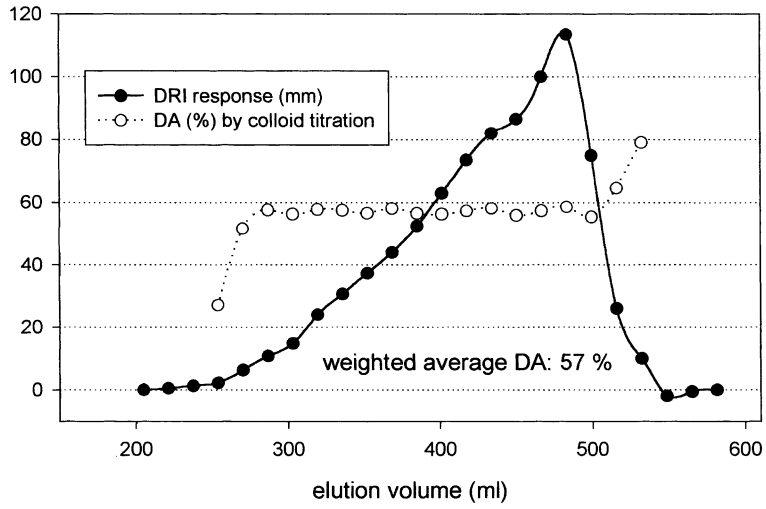

Fig. 6. Elution line and DA distribution for Chit 60 fractionated on Sepharose CL-2B.

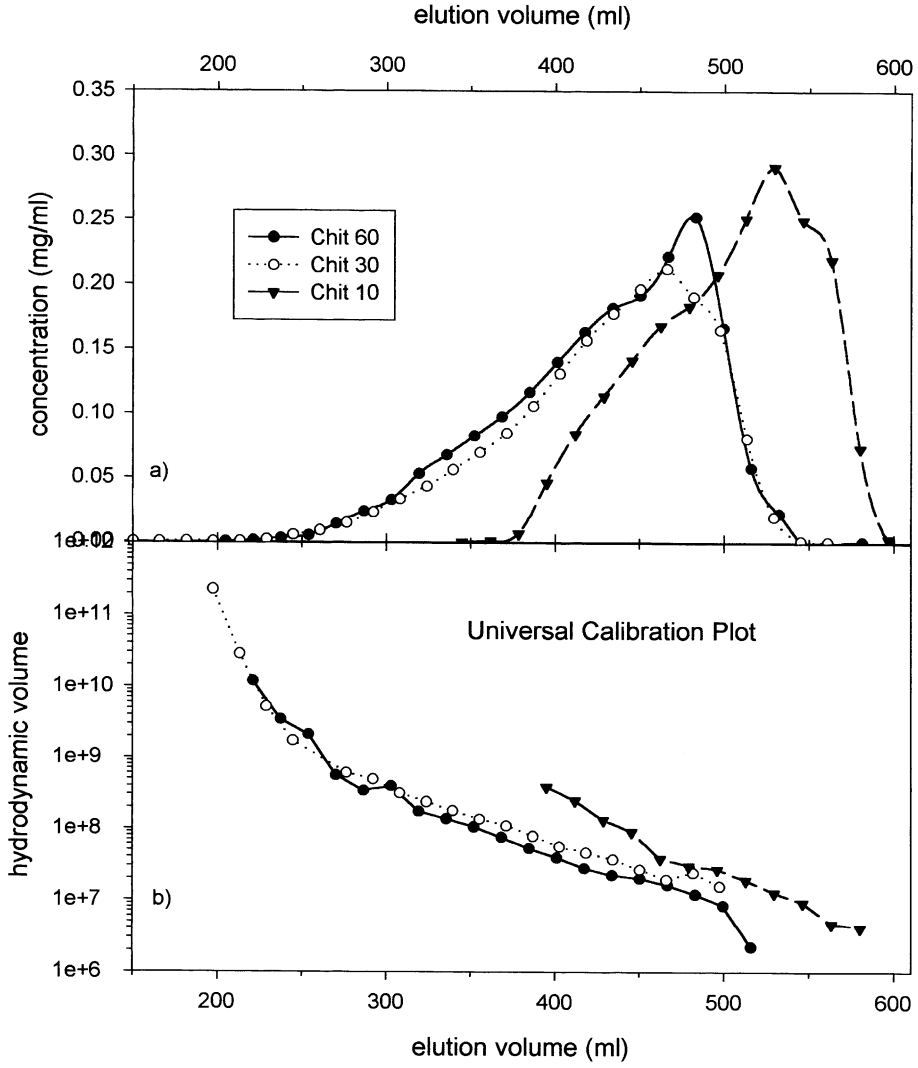

Fig. 7. Elution lines (a) and universal calibration plots (b) for Chit 10, Chit 30, and Chit 60 on Sepharose CL-2B.

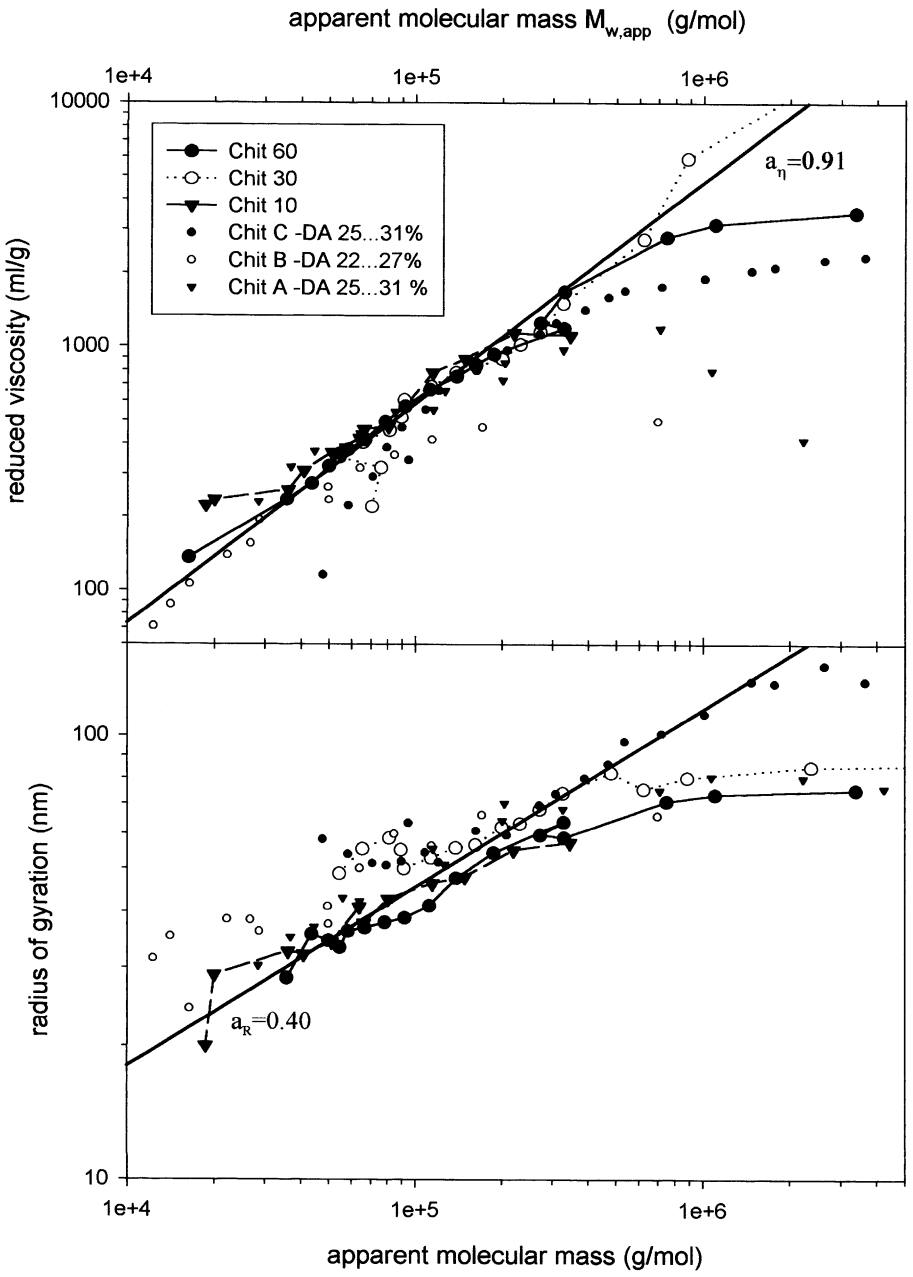

Fig. 8. Plots of the reduced viscosity η_{red} (top) and radius of gyration $R_{G,z}$ (bottom) versus apparent molecular mass $M_{w,app}$ measured on fractions from GPC.

0.52. According to the excluded volume concept (see, e.g. Tanford, 1965), measurements in a good solvent are expected to produce somewhat higher a_R values (monodispersity provided). The low value found can be explained simply by the increasing polydispersity of the fractions with decreasing average molecular mass. This conforms to a general experience ('peak broadening') in the field of chromatography and has been confirmed by HPSEC/MALLS experiments on our chitosan fractions from GPC (re-chromatography on TSK columns, unpublished). The *z*-average of R_G 'overemphasises' the largest species within a distribution compared with the weight-average of M. That is why rising polydispersities with increasing elution volume (inversely related to the average molecular mass) lead to lowered a_R values and a_R taken from GPC runs varies with the quality of separation. In contrast, the viscosity of any solution is related closely to the weight-average molecular mass so that varying polydispersities have only a small effect on the measured $[\eta]$–M_w relationship. So, when Fig. 8 is taken to conclude there are no effects of the DA on the chain conformation, this holds without reservation to the viscosity plot a. The same conclusion drawn from the radius of gyration b implies similar polydispersities for the fractions.

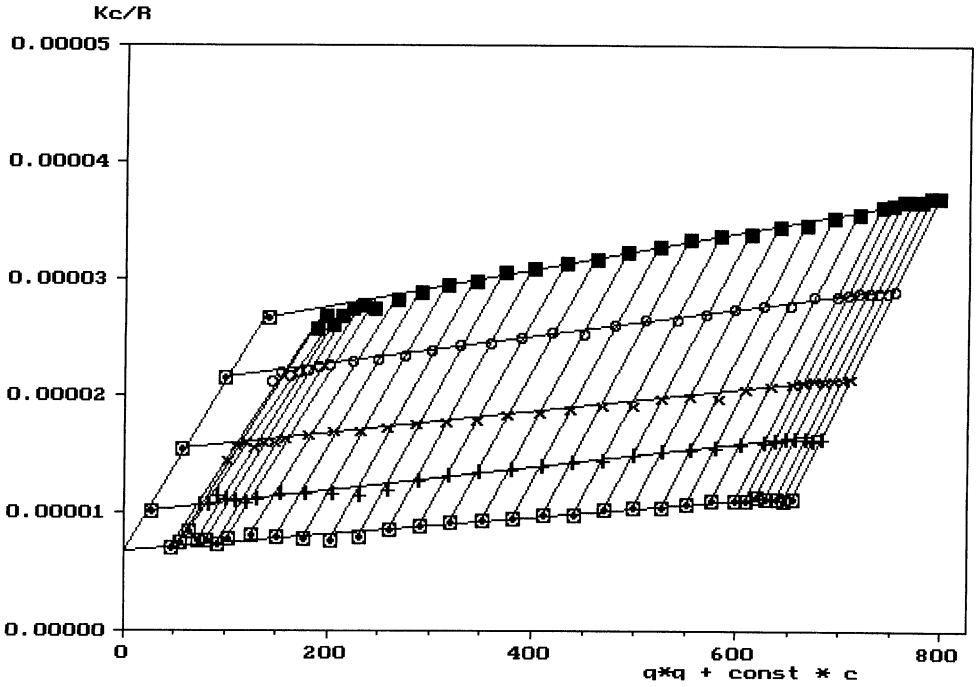

Fig. 9. Zimm plot for Chit 30: $M_w = 144,000$ g/mol; $R_{G,z} = 55$ nm; $B = 5.22 \times 10^{-3}$ ml mol/g^2.

Polydispersity is one of the major problems we have been faced with during polysaccharide characterisation (Berth & Dautzenberg, 1998). In contrast to the strongly varying polydispersities in a series of fractions from GPC, the parent chitosan samples in Berth et al. (1998) have revealed M_w/M_n values around 2. Assuming a similar situation for all types of chitosans, it would make sense to analyse the parent samples and compare them with chitosans from various sources. In the case of *unique* polydispersities, the scaling exponent a_R of the relationship $R_{G,z} = \text{const}^* M_w^{a_R}$ is characteristic of the chain conformation (same excluded volume effect provided).

3.2.2. Physico-chemical characterisation of the parent samples

The parent samples were analysed by SLS and capillary viscometry in order to complement the set of data. Results are listed in Table 1.

The Zimm plot in Fig. 9 may illustrate the quality of SLS measurements achieved if clarification succeeded. It is representative of the whole sample set and shows a slightly distorted shape as found previously (Berth et al., 1998; Cölfen et al., 2001). The steadily increasing downward curvature at increasing concentrations particularly in the lower angular range may arise from slightly increasing amounts of residual particulate matter (see Berth et al., 1998) as well as a concentration-dependent association. In the light of the extreme difficulties with clarification we have described previously, we tend to ascribe the effect to particles. (Zimm plots for comparatively high-molecular weight fractions from GPC were *not* distorted in this way suggesting that GPC removed these particles.) The interpretation of Zimm plots such as in Fig. 9 leaves a little room for uncertainty. It seems, however, fair to mention that the small arbitrary constants in our Zimm plots allow such detailed insights whereas the often used big arbitrary constants make the angular dependencies extremely steep giving the Zimm plots a deceptive beauty.

The M_w data in Table 1 (column 4) decreases with increasing DA. This suggests some degradation or fractionation during acetylation or precipitation. Columns 5–8 contain the corresponding radii of gyration, virial coefficients B, intrinsic viscosities, and Huggins constants. The latter seem extraordinarily high. There is a tendency for B to decrease with increasing DA. This is consistent with the previous discussion on the dominating electrostatic contribution to the excluded volume (Berth et al., 1998) and was also found elsewhere (Anthonsen, 1993). However, the decrease does not exceed the deviations measured on several chitosans having nearly the same DA. It is in agreement with the general experience that B values are difficult to reproduce (see also Cölfen et al., 2001).

Fig. 10 presents the molecular masses, radii of gyration and intrinsic viscosities from Table 1 plotted together with numerous data reported in the literature. Although having mixed data for samples of widely varying DAs (covering the range between 5 and 55%) and different origin (chitin sources, way of preparation, commercial de-acetylated products as well as in the laboratory re-acetylated samples) in solvents of various ionic strengths (~0.1–~0.3 M) the

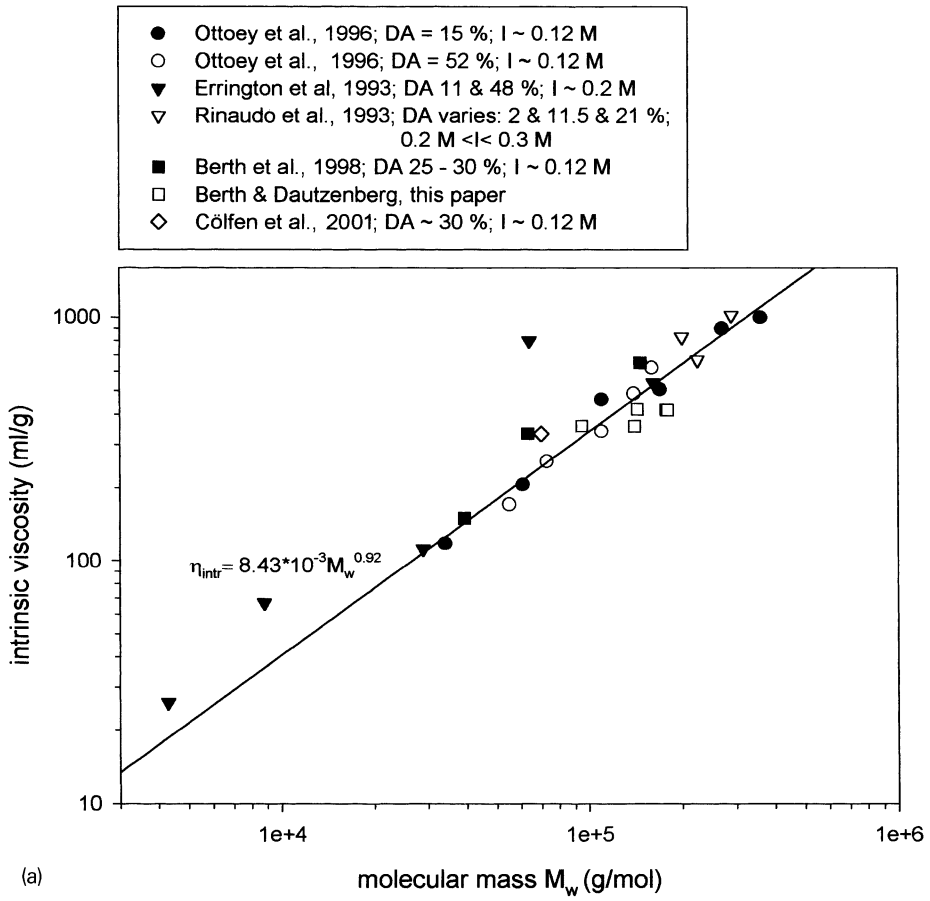

Fig. 10. Plots of (a) intrinsic viscosity $[\eta]$; and (b) radius of gyration $R_{G,z}$ versus the molecular mass M_w measured on the parent samples together with data from the literature (I = ionic strength).

points straddle straight lines that obey the following equations:

$$[\eta] = 8.43 \times 10^{-2} M_w^{0.92}$$

$$R_{G,z} = 7.5 \times 10^{-2} M_w^{0.55}$$

Taking our eight data pairs by themselves, the figures would not be distinctly different.

The data sets published by Tsaih and Chen (1999) and Yomota, Miyazaki and Okada (1993) were omitted. They did not fit the above relationship because of the significantly higher molecular masses. The $[\eta]$–M_w data reported by Rinaudo et al. (1993) fit the other data well. So does their scaling exponent of 0.54 in Fig. 10b but not the position of the line in this plot (taken from Fig. 2 in Rinaudo et al. (1993)).

The data in Fig. 10 were derived from several techniques. For the average molecular mass, this is analytical ultracentrifugation (Cölfen et al., 2001; Errington, Harding, Vårum & Illum, 1993), SLS standing alone (Berth et al., 1998; Terbojevich, Cosani, Conio, Marsano & Bianchi, 1991; Wu, Zhou & Wang, 1995; Berth and Dautzenberg, this paper) and high-performance size exclusion chromatography with on-line low-angle laser light scattering (Ottøy et al., 1996) or multi-angle laser light scattering detection (Beri, Walker, Reese & Rollings, 1993; Rinaudo et al., 1993). The majority of intrinsic viscosities was determined in an Ubbelohde-type viscometer (Berth et al., 1998; Errington et al., 1993; Ottøy et al., 1996; Berth & Dautzenberg, this paper). Rinaudo et al. (1993) used on-line viscosity detection claiming they could not succeed with stand-alone viscosity or light scattering measurements on the parent samples. In our experience, stand-alone measurements on concentration series by light scattering and analytical ultracentrifugation are often more difficult to manage than chromatographic procedures with serially connected detectors.

Considering the experimental error of each of the methods one can hardly derive any systematic effect of the DA on both the $[\eta]$–M_w (Fig. 10a) or R_G–M_w relationship (Fig. 10b) and hence the chain conformation. The overall scaling exponents are consistent with the model of a nearly free-draining wormlike chain presented in Berth et al. (1998) and Cölfen et al. (2001) and the effects of salinity, chitin source, sample preparation, etc., on the chain conformation seem

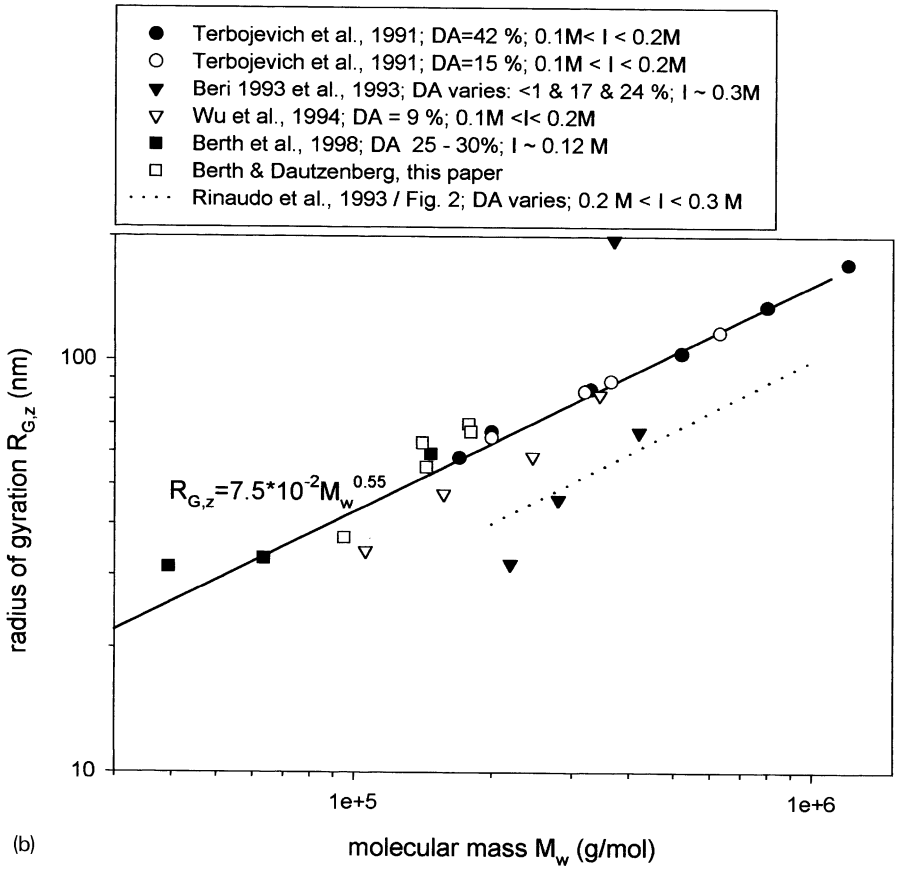

Fig. 10. (*continued*)

lower than often claimed. Comparing our own data in Figs. 8a and 10a, one will see the slopes are practically identical but the line in Fig. 10a is shifted to somewhat higher molecular masses. The reason is likely to be that the figures in Fig. 10a are overall quantities involving the levelling-off region in Fig. 8a where the molecular masses increase but the viscosities do not.

4. Conclusions

Whereas the chemical characterisation of chitosans in terms of DA is straightforward, the macromolecular characterisation remained a challenge even on samples that had been carefully prepared in the laboratory. The chemical characterisation in this paper could not provide a clue to explain the difficulties of the physico-chemical characterisation. Despite some progress achieved during the last decade by several workers, not everything about the behaviour of chitosan is yet understood. Chitosans from different sources seem to have much more in common than has often been claimed. Parts of the mystery about chitosans in solution might have come from artefacts. So we agree with Smidsrød, Ottøy, Anthonsen and Vårum (1997) that '…determination of a correct molecular weight of any polysaccharide is still … a research project of a certain difficulty…'. Further efforts to develop methodology seem desirable.

Acknowledgements

G.B. thanks the Deutsche Forschungsgemeinschaft (DFG) as well as the Wella AG, Germany, for financial support. Professor Markus Antonietti is thanked for the opportunity to study chitosans in his department in a pleasant scientific atmosphere.

References

Aiba, S. (1986). Studies on chitosan: 1. Determination of the degree of *N*-acetylation of chitosan by ultraviolet spectrophotometry and gel permeation chromatography. *International Journal of Biological Macromolecules*, *8*, 173–176.

Aiba, S. (1991). Studies on chitosan: evidence for the presence of random and block copolymer structures in partially *N*-acetylated chitosans. *International Journal of Biological Macromolecules*, *13*, 40–44.

Anthonsen, M. A. (1993). *Chitosan, chemical structure and physical properties*. PhD thesis, University of Trondheim (NTH), Norway.

Beri, R. G., Walker, J., Reese, E. T., & Rollings, J. E. (1993). Characterisation of chitosans via coupled size-exclusion chromatography and multi-angle laser light scattering technique. *Carbohydrate Research*, 11–26.

Berth, G., & Dautzenberg, H. (1998). Solution behaviour of some selected polysaccharides studied preferentially by static light scattering. *Recent Research Development in Macromolecular Research*, *3*, 225–248.

Berth, G., Dautzenberg, H., & Peter, M. G. (1998). Physico-chemical characterization of chitosans varying in degree of acetylation. *Carbohydrate Polymers*, *36*, 205–216.

Cölfen, H., Berth, G., & Dautzenberg, H. (2001). Hydrodynamic characterisation of chitosans in aqueous solution. *Carbohydrate Polymers*, (in press).

Dautzenberg, H. (2001). In T. Radeva, *Physical chemistry of polyelectrolytes*. New York: Marcel Dekker (in press).

Dautzenberg, H., Hartmann, J., Grunewald, S., & Brand, F. (1996). Stoichiometry and structure of polyelectrolyte complex particles in dilute solutions. *Berichte der Bunsen-Gesellschaft*, *100*, 1024–1032.

Errington, N., Harding, S. E., Vårum, K. M., & Illum, L. (1993). Hydrodynamic characterization of chitosans varying in degree of acetylation. *International Journal of Biological Macromolecules*, *15*, 113–117.

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

Hattori, T., Katai, K., Kato, M., Izume, M., & Mizuta, Y. (1999). Colloidal titration of chitosan and critical unit of chitosan to the potentiometric colloidal titration with poly(vinyl sulfate) using Toluidine Blue as indicator. *Bulletin of the Chemical Society of Japan*, *72*, 37–41.

Hugerth, A., Caram-Lelham, N., & Sundelöf, L. O. (1997). The effect of charge density and conformation on the polyelectrolyte complex formation between carrageenan and chitosan. *Carbohydrate Polymers*, *34*, 149–156.

Maghami, G. G., & Roberts, G. A. F. (1988). Studies on the adsorption of anionic dyes on chitosan. *Makromolekulare Chemie*, *189*, 2239–2243.

Ottøy, M. H., Vårum, K. M., Christensen, B. E., Anthonsen, M. W., & Smidsrød, O. (1996). Preparative and analytical size-exclusion chromatography of chitosans. *Carbohydrate Polymers*, *31*, 253–261.

Rinaudo, M., Milas, M., & Dung, P. L. (1993). Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. *International Journal of Biological Macromolecules*, *15*, 281–285.

Roberts, G. A. F. (1992). *Analysis of chitin and chitosans in chitin chemistry*, Houndmills/Basingstoke/Hampshire/London, UK: Macmillan Press.

Roberts, G. A. F. (1997a). Chitosan production routes and their role in determining the structure and properties of the product. *Advances in Chitin Science*, *2*, 22–31.

Roberts, G. A. F. (1997b). Determination of the degree of *N*-acetylation of chitin and chitosan. In R. A. A. Muzzarelli & M. G. Peter, *Chitin handbook* (3rd ed.), *Physical Chemistry of Macromolecules*. New York: Wiley (European Tanford, Ch. (1965)).

Smidsrød, O., Ottøy, M. H., Anthonsen, M. W., & Vårum, K. M. (1997). Solution properties of chitosan. *Advances in Chitin Science*, *2*, 402–409.

Tanford, Ch. (1965). *Physical chemistry of macromolecules* (3rd ed.), New York: Wiley.

Terbojevich, M., Cosani, A., Conio, G., Marsano, E., & Bianchi, E. (1991). Chitosan: chain rigidity and mesophase formation. *Carbohydrate Research*, *209*, 251–260.

Tsaih, M. L., & Chen, R. H. (1999). Molecular weight determination of 83% degree of deacetylation chitosan with non-Gaussian and wide range distribution by high-performance size exclusion chromatography and capillary viscometry. *Journal of Applied Polymer Science*, *71*, 1905–1913.

Wassmer, K. -H., Schroeder, U., & Horn, D. (1991). Characterization and detection of polyanions by direct polyelectrolyte titration. *Makromolekulare Chemie*, *192*, 553–565.

Wu, C., Zhou, S. Q., & Wang, W. (1995). A dynamic laser light scattering study of chitosan in aqueous solution. *Biopolymers*, *35*, 385–392.

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