ELSEVIER

Polymer 42 (2001) 9921–9927

polymer

www.elsevier.com/locate/polymer

Characterization of chitosan by steric exclusion chromatography

Joel Brugnerotto[a], Jacques Desbrières[a], George Roberts[b], Marguerite Rinaudo[a,*]

[a]*Centre de Recherches sur les Macromolécules Végétales (CNRS), Joseph Fourier University, BP 53, 38041 Grenoble cedex 9, France*
[b]*Design of Materials Group, Department of Fashion and Textiles, Nottingham Trent University, Burton Street, Nottingham NG1 4BU, UK*

Received 2 April 2001; received in revised form 13 July 2001; accepted 6 August 2001

Abstract

In this paper, the gel permeation chromatography of chitosan with different degrees of acetylation ($0 < DA < 60$ mol%) was analyzed using cationic porous supports in acidic conditions. A light scattering detector allowed the determination of the molecular weight distribution and the variation of the radius of gyration as a function of the molecular weight. From these data, the persistence length (L_p) of chitosan as a function of DA is discussed; the role of the method of preparation of the samples is also pointed out. For heterogeneously acetylated chitosan, $L_p = 110$ Å is found to be independent on DA in the range $0 < DA < 25$ mol%. For homogeneously reacetylated samples, L_p increases slightly from 110 to 150 Å when DA increases up to 60%. These conclusions may be related with the acetyl group distribution along the chains as examined by NMR.

Keywords: SEC analysis; Molecular weight distribution; Role of DA

1. Introduction

Chitin and chitosan are important polysaccharides proposed for many applications but mainly for water clarification, biomedical, pharmaceutical and cosmetic uses. It is important to be able to characterize these polymers to control the production and their final properties. Nevertheless, it is not easy due to their heterogeneities.

Chitin is a constituent of the shell of crustaceous organisms or fungi cell walls; it consists of $\beta(1 \rightarrow 4)$ *N*-acetyl D-glucosamine chains. It is insoluble in all common solvents such as dilute acid solutions or organic solvents. It is soluble in DMAc (dimetylacetamide)/LiCl mixtures [1].

Chitin is partially deacetylated, usually under heterogeneous conditions in industrial processes, to produce a variety of polymers; when the average degree of acetylation (DA expressed as molar percentage) becomes lower than 50 mol%, the product is called chitosan and becomes soluble in acidic conditions due to the protonation of the $-NH_2$ group in the C-2 position of the glucosamine unit. The chemical structure is given in Fig. 1.

The role of the distribution of the acetyl substituents along the polymeric chain is of great importance in controlling the solution behavior [2]; the distribution can be investigated using NMR as firstly described by Varüm et al. [3,4]. In their work, the authors conclude on partially hydrolyzed samples that homogeneously prepared samples with the same DA give a random distribution in agreement with the Bernouillan distribution. Our results obtained by NMR on the same samples as used for GPC will be discussed in a future paper [5]. The second important characteristic is the molecular weight distribution; our objective in this paper is to analyze the experimental data obtained by gel permeation chromatography (GPC) or steric exclusion chromatography (SEC) of chitosan solubilized in aqueous acidic solutions.

For that purpose, different chitosan samples were chosen having different average DA values and different distributions of the acetyl groups depending on the conditions of preparation.

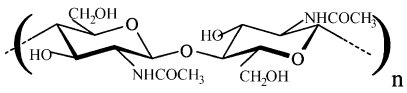
2. Experimental

2.1. Sample characteristics

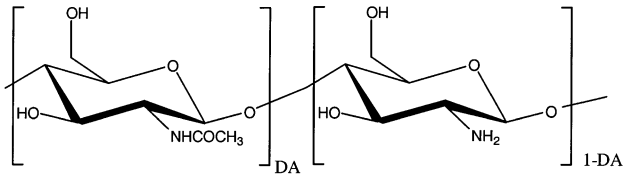
Chitosan samples from different commercial origins were carefully purified as previously described [6,7]. They were characterized by their average value of DA determined by liquid state ^{1}H NMR in acidic D_2O conditions (addition of HCl to pD ~ 4) [8]. The NMR equipment was an AC 300 from Brüker. The spectral measurements were performed at 353 K after freeze drying and redissolution of the chitosan sample three times to exchange labile H atoms. The values

* Corresponding author. Tel.: +33-476-037-627; fax: +33-476-547-203.
E-mail address: marguerite.rinaudo@cermav.cnrs.fr (M. Rinaudo).

0032-3861/01/$ - see front matter
PII: S0032-3861(01)00557-2



CHITIN



CHITOSAN

Fig. 1. Chemical structure of chitin and chitosan (DA is the degree of acetylation).

of DA are listed in Table 1. Some samples are characterized by their intrinsic viscosity $[\eta]$ determined in the same conditions as SEC is performed. The values of the weight average molar mass M_w obtained from SEC are also given.

Some samples were prepared by homogeneous reacetylation of highly deacetylated chitosan in a large range of DA (0–60 mol%) and different average molar masses (Table 2). The conditions used are described below.

2.2. Preparation of chitosan samples

The starting chitosan was a sample supplied by Aber Technologies, France (Batch Number A32E03). Four portions (10 g) were each dissolved in 0.1 M acetic acid (1 l) and the following volumes of $NaNO_2$ solution (0.9 g l^{-1}) added: A — 0 ml; B — 10 ml; C — 20 ml; D — 30 ml allowing to obtain different molar masses [9]. The viscosities of the solutions were measured after standing for 20 h at room temperature, using a Brookfield Rotational Viscometer. The viscosities were: A — 136 cps; B — 67 cps; C — 53 cps; D — 31 cps. Following this a 1% (w/v) NaOH solution was added to the previous solutions up to neutralization (pH = 7.5). The precipitated chitosans were filtered off and washed with distilled water until neutral. They were then dried at 60°C under vacuum [10].

Table 1
Characteristics of the different purified commercial samples obtained by heterogeneous deacetylation

Samples	DA (mol%)	$[\eta]$ (ml g^{-1})	M_w (g mol^{-1})
1	17	830	210000
2	12.5	650	193000
3	6	–	105000
4	3	300	70000
5	6	700	160000
6	Traces	1060	225000
7	22	800	226000
8	23	220	60000
9	12	600	285000
10	3	920	225000
11	25	730	210000
12	13	950	260000
13	8	880	235000

Table 2
Characteristics of the samples obtained by homogeneous reacetylation to cover a wide range of degrees of acetylation and different molar masses (0, 1, 2 are referred to initial chitosan samples with different initial weight average molar masses M_w. A, B, C, D, E refer to samples with different degrees of acetylation obtained from the same initial sample)

Samples	DA (mol%) from ^{1}H NMR	M_w (g mol^{-1}) from GPC
0D	Traces	110000
0A	12	135000
0B	24	127000
0C	40	101000
0E	56	115000
1D	Traces	77000
1A	12	94000
1B	24	95000
1C	41	84000
1E	61	75000
2D	Traces	73000
2A	12	75000
2B	22	85000
2C	42	70000
2E	56	75000

2.3. Preparation of N-acetylchitosans

Portions (2 g) of each product were redissolved in 0.1 M acetic acid (200 ml) and diluted with methanol (250 ml). Then to each solution was added, with vigorous stirring, a further 50 ml of methanol containing the amount of acetic anhydride required to give the target level of *N*-acetylation (calculated assuming a reaction efficiency of 100%) [11]. After standing for 24 h at 25°C the *N*-acetylchitosans were precipitated out by addition of concentrated NH_4OH and isolated by centrifugation. The samples were then washed to neutral with 75% aqueous methanol and dried at 60°C under vacuum. It was demonstrated that no *O*-acetylchitosan is formed from ^{13}C NMR measurements.

2.4. Chromatography conditions

SEC was performed using multi-detector equipment; the chromatograph was a 150C Waters with a differential refractometer to determine polymer concentration; a multi-angle laser light scattering detector from WYATT (DAWN DSP-F) was added on line to get the radius of gyration and the molar mass of the samples during elution.

The solvent adopted was that proposed previously [12]: acetic acid 0.3 M/sodium acetate 0.2 M (pH = 4.5); two columns in series type Eichrom MICRA-Gold CATSEC 100 and 1000 (USA) were used and the temperature adopted was 25°C. The polymer concentration injected was around

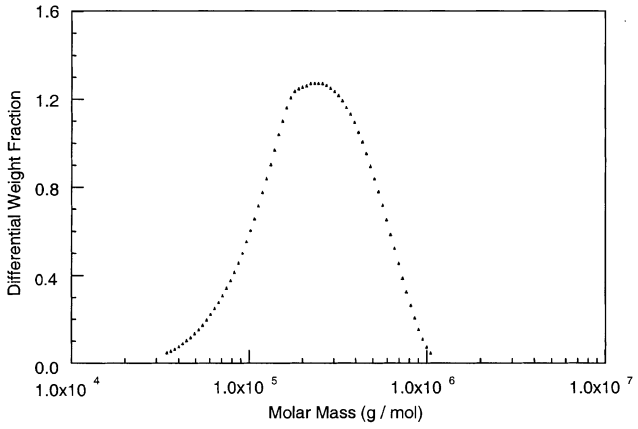


Fig. 2. Examples of differential distribution of molar masses obtained by SEC on a chitosan (sample 12).

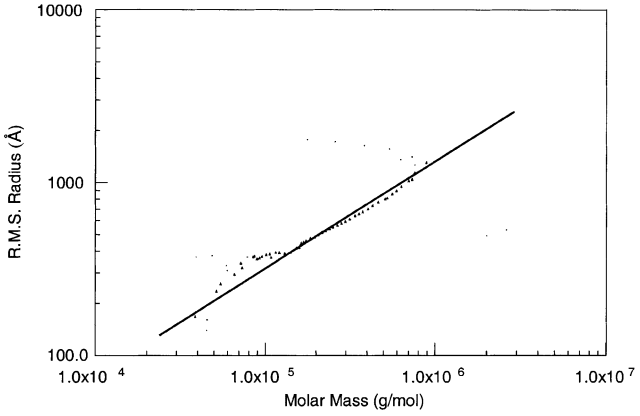


Fig. 3. Radius of gyration (RMS) expressed in Å plotted as a function of the molar mass (g mol^{-1}) (sample 12).

0.5 g l^{-1}. In all these experiments the solvent and solutions were filtered through a 0.1 μm filter (Sartorius).

The d*n*/d*c* was determined for the different DA values in the same solvent using a special home made loop to work strictly in the same experimental conditions (temperature, solvent, wavelength). The values are discussed later.

2.5. Viscosity measurements

The viscosity measurements were performed with an Ubbelohde capillary viscometer (inner diameter 0.58 mm) at 25°C ± 0.01 after having controlled that no shear rate effect exists in the range of molar masses and polymer concentrations covered: hence under these applied experimental conditions Newtonian flow behavior could be assumed and controlled from separated rheological experiments using a Low Shear 30 from Contraves (Germany). The solvent is the same as for SEC experiment.

3. Results and discussion

This paper has the objective to present SEC data and to analyse this data using the theoretical model recently developed in our laboratory [13]. This model assumes that polysaccharides behave as semi-rigid chains and that they are well described as wormlike chains in which the role of the electrostatic contribution in the expansion of the chain is taken into account. The light scattering detector allows direct determination of the molar mass distribution and that of the dependence of the radius of gyration as a function of M in the solvent conditions used. The model adopted is described in the first part, followed by the conditions used for the SEC measurements, and finally the analysis of the experimental data.

3.1. Theoretical approach

This model has been described previously [13–15]; the chains are characterized by an intrinsic persistence length L_p, characterizing the local stiffness of the chain. If L_p is determined for the neutral equivalent chain (screening of the electrostatic repulsions by salt addition) then for a charged molecule, such as chitosan in aqueous acidic solution, the effective persistence length is increased due to electrostatic repulsion between neighboring ionic sites by L_e, the electrostatic persistence length which can be calculated. Then, the persistence length in our experimental conditions is

$$L_t = L_p + L_e \quad (1)$$

For wormlike chains in θ-conditions the relation between the radius of gyration R_g, the contour length L and L_p is that given by Benoit-Doty [16]:

$$R_g^2 = LL_p/3 - L_p^2 + 2L_p^3/L - 2(L_p^4/L^2)[1 - \exp(-L/L_p)] \quad (2)$$

In the model, for given thermodynamic conditions, two electrostatic contributions are added following the Odijk treatment [17]: (1) an electrostatic excluded volume coefficient which can be calculated for each charge parameter of the poyelectrolyte, each M and each salt concentration; (2) the electrostatic persistence length contribution mentioned previously.

Following this treatment, for each set of R_g–M values at a given salt concentration, we are able to calculate from the best fit the intrinsic persistence length. This is a very interesting and important information characterizing the stiffness of the macromolecular chain. Using a home-made software, we are able to determine the characteristic L_p for each experimental $R_g(M)$ curve obtained from the multidetector GPC analysis.

Then for each sample, whatever is the average DA assuming a not too large heterogeneity which may perturb the L_p, one gets a L_p value which can be discussed in relation to the polymer characteristics.

Very recently, molecular modeling was performed on chitin and chitosan molecules [18]. In this modeling the role of the distribution of acetyl groups along the chain was also examined. The main conclusions were that chitin

Table 3
Determination of the increment of refractive index dn/dc fro chitosan samples with different degrees of acetylation in AcOH 0.3 M/AcONa 0.2 M as solvent at 25°C

Samples	DA (mol%)	dn/dc (±0.005) (ml g^{-1})
12	13	0.194
9	12	0.193
1A	12	0.190
1B	24	0.190
1C	41	0.191
1D	Traces	0.189
1E	61	0.188

is a little stiffer than chitosan and that L_p increases moderately as the DA increases from the fully deacetylated chitosan chain. The L_p varies from 90 to 125 Å at 50°C from DA = 0 to 100 mol% respectively. From our experience, the methods adopted for molecular modeling of polysaccharides were validated by other examples, i.e. on hyaluronan and galactmannan [19,20].

3.2. SEC characterization of chitosans

The different samples were passed through the equipment to get the molar mass distribution (Fig. 2) from which the function $R_g(M)$ is obtained directly (Fig. 3). But in a first step, some constants, such as dn/dc, or the second virial coefficient A_2, have been determined to calculate the exact value of M by SEC coupled with a multi-angle laser light scattering detector with the WYATT software (ASTRA 4 for Windows). The value of dn/dc has been studied as a function of DA. The values are given in Table 3. This shows that there is no significant influence of DA on dn/dc and so the average value 0.190 ± 0.005 was adopted (when the concentration is in ml g^{-1}). This value is in good agreement with values given previously in the literature and listed in Table 4.

Light scattering was also used to measure in static conditions the weight average molar mass (M_w) and the second virial coefficient (A_2) of two samples. Every sample measurement was repeated three times. The scattered light intensity of the solution is measured at angles between 36 and 160° at a temperature of 25 ± 0.1°C. The M_w and A_2 were calculated from the Zimm plots processed by the WYATT software. This resulted in an average A_2 value of 1.5×10^{-3} mol ml g^{-2} demonstrating that the adopted solvent is a good solvent for chitosan. There is a good correlation between M_w values determined by static light scattering and by SEC coupled with multi-angle laser light scattering detector.

Table 5
Parameters K',ν (relating R_g and M) and K, a (relating $[\eta]$ and M) for different chitosans obtained by homogeneous reacetylation. Determination of the intrinsic persistence length. Solvent: AcOH 0.3 M/AcONa 0.2 M; T = 25°C. These parameters are given when R_g is expressed in Å, M in g mol^{-1} and $[\eta]$ in ml g^{-1}

Samples	DA (mol%)	L_p (Å)	K'	ν	K	a
0D	2	110	0.63	0.546	0.079	0.796
0A	12	130	0.66	0.546	0.075	0.809
0B	24	130	0.65	0.547	0.070	0.810
0C	40	150	0.66	0.548	0.063	0.823
0E	56	150	0.64	0.549	0.057	0.825
1D	2	110	0.63	0.546	0.079	0.796
1A	12	130	0.62	0.547	0.074	0.798
1B	24	130	0.65	0.547	0.070	0.810
1C	41	150	0.63	0.547	0.062	0.813
1E	61	150	0.63	0.549	0.056	0.821

The influences of the solution concentration, the flow rate and the ionic strength on the molecular characteristics were also examined. It was shown that the initial solution concentration injected between 0.5 and 1.5 g l^{-1} does not have any effect on the results obtained. The flow rate was fixed at 0.3 ml min^{-1} to have good separation from the solvent signal. It is important to note that, generally, more than 95% of the product was eluted from the columns; this implies no adsorption and good solubility. This result permits us to conclude there is no aggregation when the solvent AcOH (0.3 M)/AcONa (0.2 M) is used.

Some experiments were also performed with the solvent AcOH (0.3 M)/AcONa (0.05 M) (pH = 3.95) and no significant influence on the M_w values was obtained compared to

Table 4
List of the dn/dc values obtained from the literature

Solvent	DA (mol%)	Wavelength (nm)	dn/dc (ml g^{-1})	Ref.
AcOH (0.1 M)/NaCl (0.2 M)	15; 42	633	0.191; 0.180	[21]
HCl (0.01N)/NaCl (0.19N)	17	633	0.189	[22,23]
0.2 M NaCl on the chlorhydrate form of chitosan	0–23	632.8	0.170	[24]
AcOH (1%)/AcONa (0.2 M)	13–22	546	0.160	[25]
AcOH (0.2 M)/AcONa (0.1 M)	0–31	632.8	0.208–0.175	[26]
Acetate buffer (0.02 M)/NaCl (0.1 M)	7–25	436	0.203	[27]
AcOH (0.333 M)/AcONa (0.1 M)	0–24	632.8	0.181	[28]
Acetate ammonium (0.2 M)	0–60	632.8	0.162	[29]
AcOH (0.1N)/NaCl (0.2 M)	5; 28; 42;	632.8	0.201; 0.183; 0.180	[30]
AcOH (0.3 M)/AcONa (0.2 M)	2–21	632.8	0.163	[31]

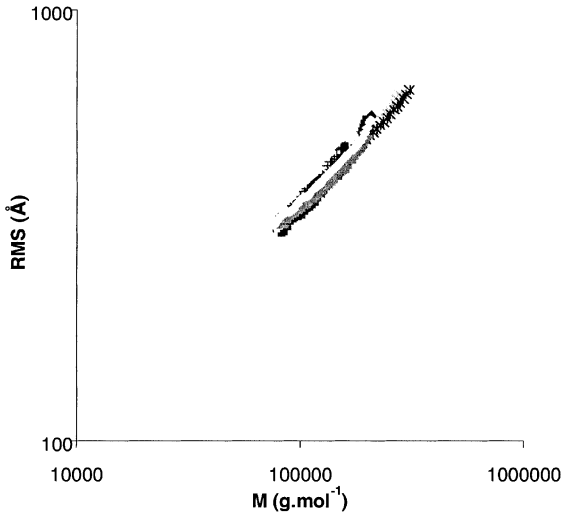


Fig. 4. Radius of gyration (RMS) expressed in Å plotted as a function of the molar mass (g mol^{-1}) for the homogeneous samples with degrees of acetylation from 0 to 61 mol% (series 0 and 1, see Table 2).

the results obtained in AcOH (0.3 M)/AcONa (0.2 M) (pH = 4.5).

3.3. SEC analysis and $R_g(M)$ dependence

It must be mentioned that no evidence of aggregation appears on the chromatograms obtained for samples injected at a concentration lower than the overlap concentration. Then, the SEC analysis is performed with no difficulties.

A series of $R_g(M)$ curves for samples in the same average molar mass range but with different DA values, obtained by homogeneous *N*-reacetylation of chitosan was analyzed (Table 5). Over the range of average DA values from 0 to 60 mol%, the samples are perfectly soluble and show only a very slight deviation in the curves as shown in Fig. 4. The fact that the different curves can be nearly superimposed indicates that the DA has no significant role on the hydrodynamic volume and confirms our previous results [12]. The intrinsic persistence length and the parameters (K', ν) of the relationship:

$$R_g = K'M^{\nu} \quad (3)$$

were determined for each curve $R_g(M)$ and the values obtained are given in Table 5. It can be concluded that the exponent ν and the constant K' have average values equal 0.548 ± 0.002 and 0.64 ± 0.002 respectively (when R_g is in Å and M in g mol^{-1}) over the range of DA values studied. From the theoretical treatment, the L_p values are calculated for each $R_g(M)$ curve separately; it is shown that L_p value varies slightly when DA increases from $L_p = 110$ Å for DA = 2 mol% up to 150 Å for DA ~ 60 mol%. These L_p values are larger than the values calculated from molecular modeling; at 25°C, the predicted values are $L_p = 95$ Å and $L_p = 125$ Å for DA = 0 and 50 mol% respectively, assuming a random distribution of the acetyl groups. The values of L_p obtained for homogeneous *N*-reacetylated chitosan are higher than those predicted for the two limits given previously. The reason for these higher values could be related to an alternated *N*-acetyl group distribution along the chain; indeed, the molecular modeling also predicted that for an alternating distribution of acetylated and non-acetylated glucosamine units, the chain is stiffer [18]. More investigations on the relation between the chemical modification of chitin and the distribution of substituents along the chain are needed.

The $R_g(M)$ data obtained for the series of samples prepared by heterogeneous deacetylation from different sources (Table 1) are nearly superimposable, with approximately the same position than the first series obtained with homogeneous samples. The characteristic values are given in Table 6. From all these data it is confirmed that heterogeneous chitosans with different DA values over the range 0–25 mol% have the same behavior i.e. the stiffness does not change to any significant extent. In this case, the relation between R_g and M (Relation (3)) gives a value for the constant K' ($K' = 0.61 \pm 0.02$) slightly lower than the average values given for the series of homogeneous samples; and the value for the exponent ν ($\nu = 0.546$) is very close to the value of the previous series. Comparison of Tables 5 and 6 shows that for different samples with DA ~ 0 mol% the parameters K' and ν are the same. From the analysis of the data of Table 6, L_p is found to be independent on DA and equal to 110 Å over the range of $0 < DA < 25$ mol%, in good agreement with the value calculated by molecular modeling. This conclusion confirms our previous results [12].

The role of the imposed intrinsic persistence length on the K' and ν parameters were tested independently; few values calculated for the same range of molar masses are given in

Table 6
Parameters K', ν (relating R_g and M) and K, a (relating $[\eta]$ and M) for different chitosans obtained by heterogeneous deacetylation. The intrinsic persistence length determined is equal to 110 Å for all the samples. Solvent: AcOH 0.3 M/AcONa 0.2 M; $T = 25$°C. These parameters are given when R_g is expressed in Å, M in g mol^{-1} and $[\eta]$ in ml g^{-1}

Samples	DA (mol%)	K'	ν	K	a
1	17	0.61	0.547	0.072	0.799
2	12.5	0.62	0.547	0.074	0.798
3	6	0.62	0.546	0.077	0.797
4	3	0.63	0.546	0.078	0.797
5	6	0.62	0.546	0.077	0.797
6	0.5	0.63	0.546	0.080	0.796
7	22	0.61	0.547	0.069	0.799
8	23	0.61	0.547	0.069	0.799
9	12	0.62	0.547	0.074	0.798
10	3	0.63	0.546	0.078	0.797
11	25	0.60	0.547	0.068	0.800
12	13	0.62	0.547	0.073	0.798
13	8	0.62	0.546	0.076	0.797

Table 7
Calculated values of the parameters K, K', ν, a for a chitosan with DA = 0 mol% and different intrinsic persistence lengths imposed. Solvent: AcOH 0.3 M/AcONa 0.2 M; $T = 25$°C. These parameters are given when R_g is expressed in Å, M in g mol^{-1} and $[\eta]$ in ml g^{-1}

L_p (Å)	K'	ν	K	a
50	0.41	0.557	0.053	0.774
100	0.60	0.547	0.080	0.789
150	0.72	0.546	0.082	0.817

Table 7. It is demonstrated that $L_p = 50$ Å gives very different parameters compared with $L_p = 100$ Å when, for L_p in the range 100–150 Å, only a very small influence is observed, especially on ν values. This conclusion allows to justify that the values of ν in Table 5 are nearly constant for L_p values given in the range of 110–150 Å.

3.4. Intrinsic viscosity–M relationship

From L_p it is also possible to predict the intrinsic viscosity in the experimental conditions adopted for a given molar mass as well as the Mark-Houwink parameters for the different DA values in the relation

$$[\eta] = KM^a \quad (4)$$

Due to the $R_g(M)$ dependence obtained from SEC analysis (Relation (3)), we must predict that K and a would be only slightly dependent on DA. The calculated values are given in Tables 5 and 6 (when $[\eta]$ is given in ml g^{-1} and M in g mol^{-1}). The prediction is that K decreases and a increases when DA increases. The evolution of K and a with the degree of acetylation, whatever the origin of the chitosan, is given in Table 8.

The values of $[\eta]$ calculated using the model developed using these characteristics are usually larger than the values obtained by experimental determination (Table 1). A very large discrepancy between the $[\eta]$ values was previously observed on different systems; it seems that it may be attributed to the indirect approach involved in the use of the Yamakawa-Fujii treatment. This point must be examined more precisely to predict the intrinsic viscosity of a polymer with a given persistence length.

Table 8
Mark-Houwink parameters predicted from the wormlike chain model from GPC experiments for homogeneous and heterogeneous samples. These parameters are given when M is expressed in g mol^{-1} and $[\eta]$ in ml g^{-1}

DA (mol%)	K	a
0–3	0.079	0.796
12	0.074	0.8
22–24	0.070	0.810
40	0.063	0.83
56–61	0.057	0.825

4. Conclusion

This paper describes the gel permeation chromatography of chitosan samples covering a large range of degrees of acetylation. The use of a laser multi-angle light scattering detector allows the molar mass distribution to be obtained without any calibration, together with the curves relating the radius of gyration with the molar mass.

Considering the wormlike chain behavior of this polymer, one gets the intrinsic persistence length as a function of DA; it remains constant with $L_p = 110$ Å for heterogeneous chitosans with low degrees of acetylation (DA < 25 mol%); for homogeneous chitosans, L_p increases moderately when DA increases. At the same degree of acetylation DA the stiffness of chitosan appears greater for homogeneously *N*-reacetylated chitosan, in agreement with the prediction from molecular modeling on the effect of an alternating distribution of the acetyl groups.

The prediction of the intrinsic viscosity from the model proposed using the L_p determined from the radius of gyration gives values larger than the experimental values. This conclusion was also previously obtained on other systems such as hyaluronan [32] and poses the problem of the application of the theoretical approach proposed for viscosity.

Acknowledgements

The authors thank the valorization company of the University of Liège (Belgium), the research group on chitin-chitosan headed by Doctor M-.F. Versali for their financial support.

References

[1] Hasegawa M, Isogai A, Onabe F. Carbohydr Res 1994;262:161.
[2] Aiba SI. Int J Biol Macromol 1991;13:40.
[3] Varüm KM, Anthonsen MW, Grasdalen H, Smidrod O. Carbohydr Res 1991;211:17.
[4] Varüm KM, Anthonsen MW, Grasdalen H, Smidrod O. Carbohydr Res 1991;217:19.
[5] Brugnerotto J, Mazeau K, Auzely R, Desbrières J, Rinaudo M. Submitted for publication.
[6] Rinaudo M, Milas M, Desbrières J. In: Goosen MFA, editor. Applications of chitin and chitosan. Lancaster: Technomic Pub Co Inc, 1997. p. 89–102.
[7] Brugnerotto J, Lazardi J, Goycoolea FM, Argüelles-Monal W, Desbrières J, Rinaudo M. Polymer 2001:3569.
[8] Desbrières J, Milas M, Rinaudo M. In: Karnicki ZS, Brzeski MM, Bykowski PJ, Wojtasz-Pajak A, editors. Chitin world, 1994. p. 73–90.
[9] Allan GG, Peyron M. In: Skjak-Braek G, Anthonsen T, Sanford P, editors. Chitin and chitosan. Barking, England: Elsevier Applied Science, 1989. p. 443–66.
[10] Roberts GAF, Wood FA. J Biotechnol 2001;89:297–304.
[11] Maghami GG, Roberts GAF. Makromol Chem 1988;189:195.
[12] Rinaudo M, Milas M, Le Dung P. Int J Biol Macromol 1993;15:281.
[13] Chazeau L, Milas M, Rinaudo M. Int J Polym Anal Character 1995;2:21.

[14] Reed W. In: Schmitz K, editor. Macroion characterization from dilute solutions to complex fluids. ACS Symp Ser 548. 1994, p. 297–314.
[15] Rinaudo M, Roure I, Milas M. Int J Polym Anal Character 1999;5:277.
[16] Benoit H, Doty P. J Phys Chem 1953;57:958.
[17] Odijk T. Biopolymers 1979;18:3111.
[18] Mazeau K, Perez S, Rinaudo M. J Carbohydr Chem 2000;19:1269.
[19] Haxaire K, Braccini I, Milas M, Rinaudo M, Perez S. Glycobiology 2000;10:587.
[20] Petkowicz CLO, Rinaudo M, Milas M, Mazeau K, Bresolin T, Reicher F, Ganter J. Food Hydrocoll 1999;13:263.
[21] Terbojevich M, Cosani A, Conio G, Marsano E, Bianchi E. Carbohydr Res 1991;209:251.
[22] Tsaih ML, Chen RH. Int J Biol Macromol 1997;20:233.
[23] Chen RH, Tsaih ML. Int J Biol Macromol 1998;23:135.
[24] Domard A, Rinaudo M. Int J Biol Macromol 1983;5:49.
[25] Muzzarelli RAA, Lough C, Emmanuelli M. Carbohydr Res 1987;164: 433.
[26] Wang W, Bo S, Li S, Quin W. Int J Biol Macromol 1991;13:281.
[27] Berth G, Dautzenberg H, Peter MG. Carbohydr Polym 1998;36:205.
[28] Beri RG, Walker J, Reese ET, Rollings JE. Carbohydr Res 1993;238:11.
[29] Ottoy MH, Varum KM, Christensen BE, Anthonsen MW, Smidsrod O. Carbohydr Polym 1996;31:253.
[30] Terbojevich M, Cosani A, Focher B, Naggi A, Torri G. Carbohydr Polym 1992;18:35.
[31] Rinaudo M, Milas M, Le Dung P. Int J Biol Macromol 1993;15: 281.
[32] Fouissac E, Milas M, Rinaudo M, Borsali R. Macromolecules 1992;25:5613.