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High-performance gel-permeation chromatography of chitosan samples

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ABSTRACT

The conformational properties of chitosan, a copolymer of 2-acetamido-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose, have been examined both in solution and in the solid state. Little has been reported previously on the determination of molecular weight using high-performance gel-permeation chromatography (HPGPC) and no attempt has been devoted to an examination of molecular weight distribution. An HPGPC method for evaluating the above-mentioned parameters for chitosan samples having different molecular weights and different degrees of acetylation was therefore developed. Calibration using sodium polystyrene sulfonate commercial standards of narrow molecular weight distribution could not be carried out in the solvent system used for chitosan. Calibration was therefore performed by means of chitosan samples obtained by depolymerization.

INTRODUCTION

Chitosan is the *N*-deacetylated product of chitin; the name does not identify a single product but rather a product family, each product differing in the degree of acetylation (Ac%), type of sequence arrangement, chain length (M), and molecular weight distribution (MD).

The properties of chitosan have been examined both in solution[1–3] and in the solid state[4,5]. The average molecular weight was determined by membrane osmometry and static light-scattering, but little has been reported using HPGPC. Until now, the polysaccharide has been characterized with Glycophase G/CPG and aqueous 2% acetic acid as the eluent[6], and cationic porous silica gels with an eluent containing 0.05 M ammonium acetate[7]. We now report an HPGPC method for evaluating the MD for chitosan samples having different molecular weights and

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different degrees of acetylation. Indeed, information on this property is highly desirable in view of relevant theoretical and practical implications, as it is in general for polymer and biopolymer materials.

EXPERIMENTAL

Materials.—Sodium polystyrene sulfonate (PSSNa) with molecular weights (M_p) in the range 4.6×10^3–1.2×10^6 were obtained from Polymer Laboratories Ltd. (UK). M_p corresponds to the peak molecular weight as determined by HPGPC. The M_w/M_n values were < 1.1.

Samples of commercial chitosan of 42 and 28 Ac% (Chit-42 and Chit-28) from Rybex Krill (*Euphausia superba*; Fisheries Central Board, Szczecin, Poland), and 15 Ac% (Chit-15) from *Chionocetes japonicus* (Katakura Chikkarin, Tokyo, Japan) were used. The Ac% values specified by the producers were confirmed by ^{1}H NMR and UV spectroscopy[8].

In order to obtain samples with a lower intrinsic viscosity [η], commercial samples Chit-42 and Chit-15 were hydrolyzed in acid media under different conditions of pH and temperature. As previously reported[3], acid hydrolysis of glycosidic linkages occurs without involving deacetylation. The products were recovered by precipitation with M NaOH. Their characteristics are reported in Table I. Intrinsic viscosity measurements were performed in 0.5 M AcOH–0.2 M NaOAc. Hydrolyzed samples sometimes exhibit ageing phenomena, which render them partially or totally insoluble.

Preparation of solutions.—To prepare chitosan solutions, the material was added, with stirring, to 0.5 M AcOH. After solubilization of the sample, NaOAc was added to reach the desired ionic strength and the solution was centrifuged at 25 000*g* for 1 h.

TABLE I

Characteristics of chitosan samples

Sample	Hydrolysis conditions	$[\eta]_{dL/g}$
Chit-42 [a]		13.7
Chit-42 D_3	0.6 M HCl, 25°C, 367 h	5.2
Chit-42 D_4	0.6 M HCl, 25°C, 818 h	3.4
Chit-42 C_1	0.6 M HCl, 50°C, 12 h	2.6
Chit-42 H	Aq 1% AcOH, reflux, 43 h	2.4
Chit-28		14.6
Chit-15		16.4
Chit-15 H_1	0.6 M HCl, 50°C, 30 min	10.8
Chit-15 H_4	0.6 M HCl, 50°C, 12 h	4.0
Chit-15 H_5	0.6 M HCl, 50°C, 33 h	2.2
Chit-15 H	Aq 1% AcOH, reflux, 64 h	2.1

[a] Pretreated by dissolution in 1% AcOH and reprecipitation[3].

We have followed the depolymerization of chitosan samples in this solvent system, at room temperature, both by viscometric and chromatographic methods: we conclude that the solution of chitosan must be analyzed as soon as possible after preparation, because $[\eta]$ decreases with time and the elution patterns indicate a variation in peak position, modifications in curve shape, and increase of low molecular weight material.

Light scattering (LS).—These measurements were performed at 20°C using a Sofica Model 42 000 photometer with cylindrical cells immersed in toluene. Non-polarized laser light (633 nm) was used, covering scattering angles (ϑ) between 30 and 150°. A Rayleigh ratio $R_{90°} = 8.96 \times 10^{-6}$ cm^{-1} was used for calibration of the instrument with benzene[9]. Solutions and solvents were clarified by additional centrifugation at 25 000g for 3 h. The data were treated as previously reported[3].

Intrinsic viscosity.—Viscosities were determined using a multigradient suspended-level Ubbelohde viscometer at 25.0 ± 0.1°C with solvent flow times of ~ 150 s. Relative viscosities ranged between 1.1 and 1.6. Dilutions were made directly in the viscometer. No shear effect was observed up to $[\eta] = 8$–10 dL/g. Higher viscosities were extrapolated to zero flow-velocity gradient[10]. Intrinsic viscosity data were calculated by the Huggins equation[11].

Liquid chromatography.—The HPGPC measurements were performed in 0.5 M AcOH–0.2 M NaOAc as the mobile phase. Eluent and polymer solutions were filtered using 0.45-μm Millipore filters (HAWP01300). The injected volume was always 100 μL. The polymer concentration did not exceed the critical concentration $c^* \sim 1/[\eta]$, according to literature suggestions[12].

Commercially available Bio-Gel TSK columns (ToyoSoda, Tokyo) were used. The 50 XL column (300 × 7.5 mm) worked in the retention volumes (V_r) ranging from 5 mL (V_0) and 11.3 mL (V_m): the total V_m for the system with two columns (one 50XL, one DNAXL) was 22 mL, while the excluded volume could not be determined experimentally, due to unavailability of well-characterized water-soluble high molecular weight standards. According to the manufacturer, the exclusion limit of the DNAXL column corresponds to a mol wt of 8×10^6 for poly(ethylene glycol). It can be assumed from the data obtained using chitosan samples that V_0 does not exceed 10 mL.

A Knauer HPLC pump Type 64.00 and Rheodyne Injector were used, with a refractive index detector Model ERC 7512 (Erma CR. Inc., Japan) and a flow rate of 0.6 mL/min.

Elution volumes were calculated from flow rate time. Ethanol was used as external standard and "Donnan salt peak" as internal standard.

RESULTS

HPGPC measurements.—Chitosans having *N*-acetyl contents of the order of 40–50% are soluble in dilute aqueous AcOH, in which the polymer behaves as a cationic polyelectrolyte. In contrast to neutral hydrophilic polymers, the characterization of polyelectrolytes by aqueous GPC is more complex.

Firstly, the size of the polyelectrolyte molecule is highly dependent upon ionic strength and hence on its own concentration in solution and in the presence of other ionic species.

Secondly, ion-inclusion effects are present. Whereas ion-inclusion cannot be easily eliminated, ion-exclusion may be overcome by the addition of an appropriate low molecular weight electrolyte to the mobile phase.

On the other hand, the charge density on the polyelectrolyte and that which may be present on the support can cause ionic interactions. This phenomenon is known for cationic substances, because negatively charged groups are present in many gel matrices.

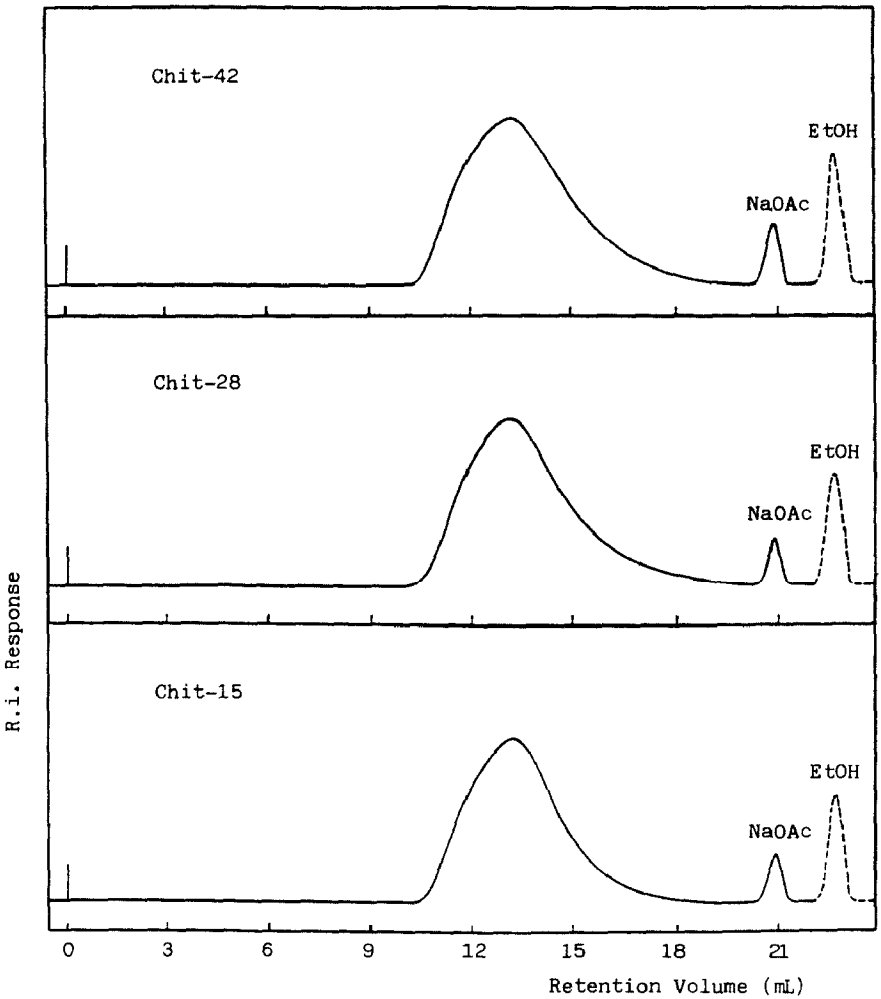

Fig. 1. Elution patterns of chitosan samples in 0.5 M AcOH–0.2 M NaOAc. Columns: two (300 × 7.5 mm) Bio-Gel TSK (one 50XL, one DNAXL).

The pH of the solution is also important in modulating the degree of ionization both of the polymer species and of the functional groups on the surface of the support.

All of these solute–gel matrix interactions are undesirable in the determination of the MD of polycations.

Keeping these problems in mind, HPGPC measurements of chitosan solutions were performed using commercially available Bio-Gel TSK columns and aqueous AcOH (0.5 M) as the mobile phase: this eluent is expected to eliminate ionization of carboxyl groups present on the support surface and to reduce adsorption of chitosan.

The addition of NaOAc (0.2 M) generates enough ionic strength to overcome ion-exclusion effects; too high a concentration of salt should be avoided, since this causes an increase in the pH. In addition, hydrophobic interactions between polyelectrolyte molecules and the stationary phase may lead to absorption. It is not possible to use 0.3 M sodium sulphate[13], which causes little corrosion of stainlees steel at low pH, because chitosan sulphate is insoluble in water.

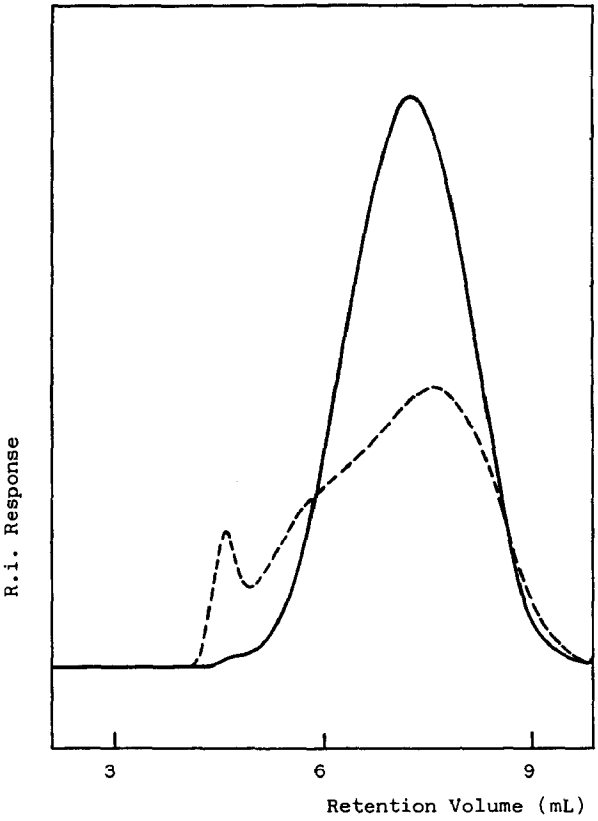

Fig. 2. Elution patterns of Chit-42 C_1 (———) and Chit-42 H (- - - - -) in 0.5 M AcOH–0.2 M NaOAc. Column: Bio-Gel TSK-50XL (300×7.5 mm).

The elution patterns for commercial chitosan samples, using the foregoing solvent system, are shown in Fig. 1. Exclusion peaks for salt and ethanol were observed and their elution is in agreement with the GPC theory of polyelectrolytes[12].

All of the polymeric material was eluted within the separation range of the chromatographic system and the resolution was good both at high and low retention volumes.

The results were very different if the experimental conditions described above were changed. In particular at concentrations of NaOAc less than 0.2 M, chitosans were eluted in a nonreproducible fashion or not eluted at all, because electrostatic interactions between polycation and gel matrix were present.

Calibration with narrow-MD standards.—The calibration curve (log M_p vs. V_r) for chitosan samples can be obtained from the calibration curve of standards having narrow MD by applying the universal calibration technique: once the a and k viscometric parameters are known, the molecular weights of different polymers can be related directly to the corresponding retention volumes in GPC[14].

At first, we used commercial samples of PSSNa as the standards. Their a and k values are available at different ionic strengths[15]. These parameters have also been reported in the literature for chitosan samples and are not dependent on Ac%[16].

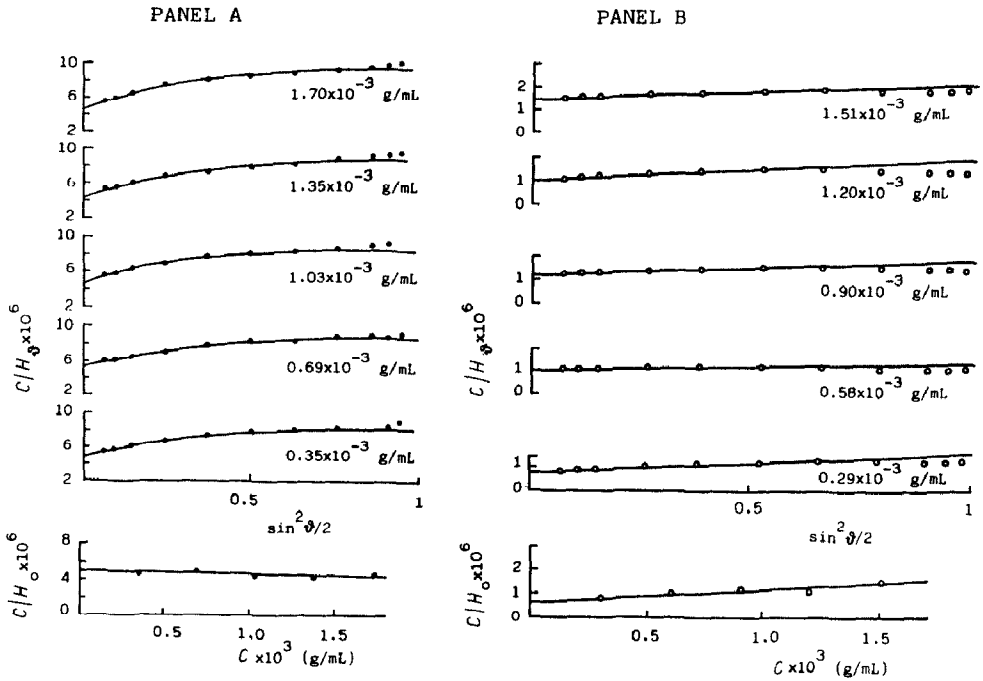

Fig. 3. Plots of c/H_ϑ *versus* $\sin^2\vartheta/2$ at different concentrations and plot of c/H_0 vs. c, for Chit-15 H (Panel A) and Chit-15 H_5 (Panel B)

HPGPC measurements indicated that PSSNa samples of low M_n are eluted in 0.5 M AcOH–0.2 M NaOAc beyond the V_m of the column. Probably, the high ionic strength causes hydrophobic interactions between polyelectrolyte molecules and the stationary phase. In fact, as ionic strength decreases (0.1, 0.05, 0.02 M), all peaks fall in the column separation range. However, only at 0.02 M NaOAc could $[\eta] \times M$, proportional to the hydrodynamic volume of the polymer molecule, be used as a universal parameter. Unfortunaly, chitosan samples are not eluted at ionic strengths lower than 0.2 M, as previously reported.

It is evident that the peculiar conditions at which chitosan samples are eluted are not appropriate for polymer chains having different chemical structure. Therefore, the simplest approach for a satisfactory calibration requires the availability of narrow fractions of the test polymer.

Attempts to perform molecular weight fractionation of commercial chitosan samples were only partially successful, both according to literature procedures[17] and by performing the separation techniques reported for polyanions (selective precipitation with ethanol from aqueous salt solutions[18] or ion-exchange absorption[19]). The reasons for this possibly arise from depolymerization of chitosan chains during the experiments and only partial recovery of the polymeric material from the chromatography column.

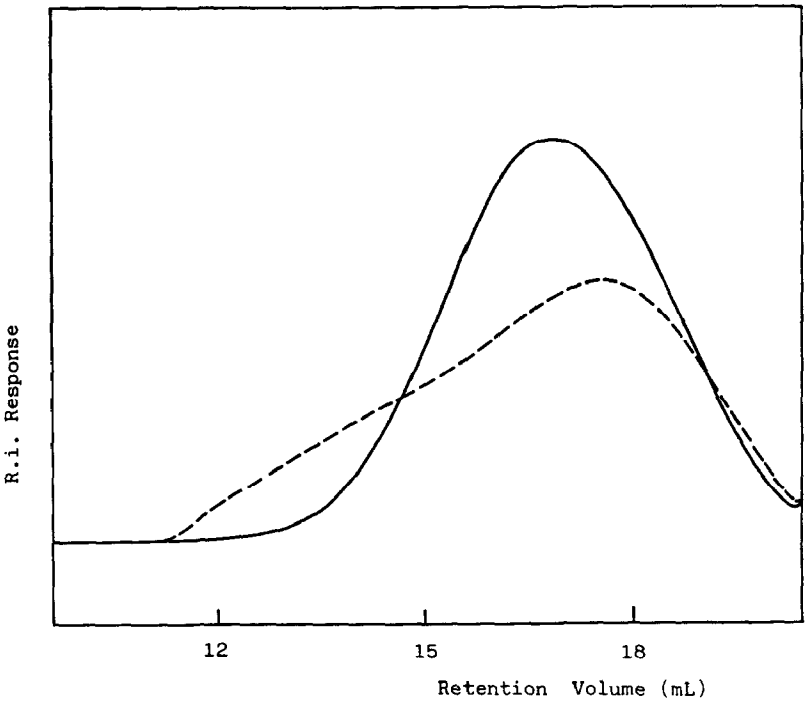

Fig. 4. Elution patterns of Chit-15 H_5 (———) and Chit-15 H (- - - - -) in 0.5 M AcOH–0.2 M NaOAc. Columns: two (300×7.5 mm) Bio-Gel TSK (one 50XL, one DNAXL).

Calibration with broad-MD standards.—Since narrow-MD samples are not available, a practical approach[14] makes use of theoretical MD and average M values of standards to provide a sufficiently reliable calibration curve (integral MD method).

We suppose that depolymerisation of chitosan samples in aqueous acid solutions gives products with a Flory "most probable distribution" represented by eq *1* where W_X is the weight fraction of polymer with X repeat units of molecular weight M_0, and p is equal to $(M_n - M_0)/M_n$ (M_n is the number-average molecu-

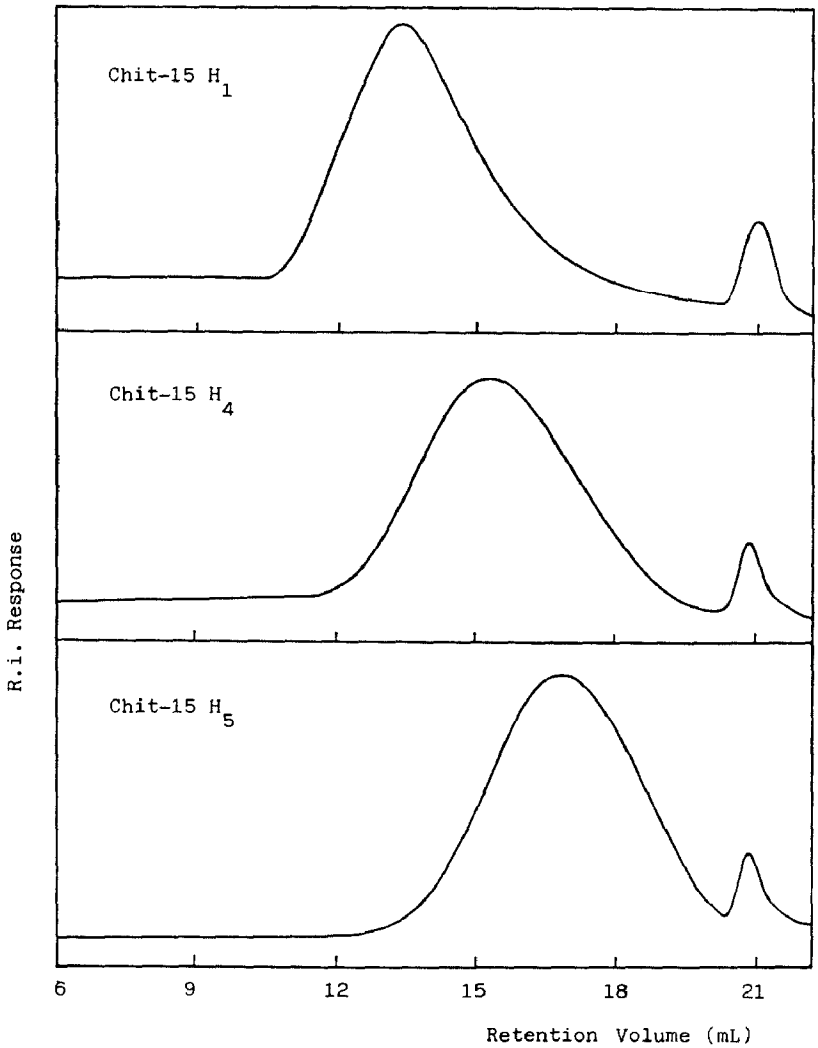

Fig. 5. Elution patterns of depolymerized chitosan samples. Eluent: 0.5 M AcOH–0.2 M NaOAc. Columns: two (300×7.5 mm) Bio-Gel TSK (one 50XL, one DNAXL).

lar weight). As the value for p approaches unity, the value for polydispersity (M_w/M_n) approaches 2.

$$W_X = (1-p)^2(X)(p^{X-1}) \quad (1)$$

After depolymerization, the chitosan samples of different intrinsic viscosity can be characterised by LS techniques, in order to obtain the weight-average molecular weight (M_w).

A most probable MD requires that all glycosidic bonds have the same probability of being hydrolyzed. It is known[21] that the reactivity of a glycosidic bond close to the acetamido group is greater than the reactivity of a glycosidic bond close to a

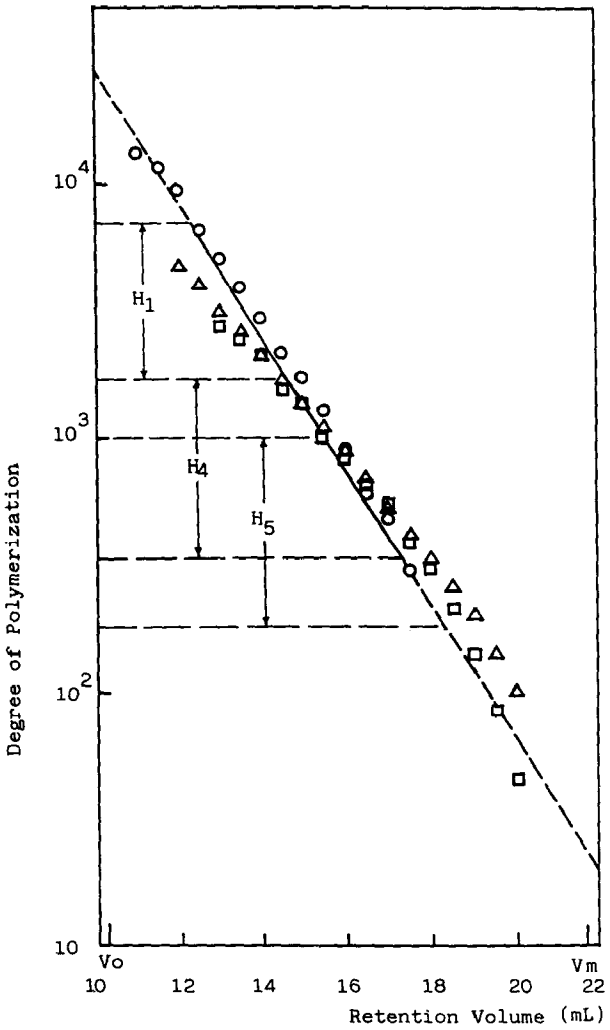

Fig. 6. Calibration curve obtained by using Chit-15 H_1 (○), Chit-15 H_4 (△), and Chit-15 H_5 (□).

protonated amino group. Therefore, we chose the chitosan sample of lowest Ac% (chit-15) to prepare our standard material.

As for the hydrolysis conditions, it was recently reported[3] that the properties of hydrolyzed products are related to the ability of the hydrolytic system to break the crystalline regions of the polymer during the degradative process. The authors suggested that only in 0.6 M HCl does a total dissolution of the crystalline regions occur, while AcOH leaves crystalline regions unaffected: as hydrolysis proceeds, crystallites promote association and originate species of high molecular weight. These findings observed during the hydrolysis of chitosans of 42 and 28 Ac% were confirmed by chromatographic analysis, using the system described above. The elution patterns of chitosan samples Chit-42 H and Chit-42 C_1, having almost identical low $[\eta]$, but obtained using different hydrolysis conditions (Table I), showed important differences (Fig. 2): depolymerization performed under mild conditions causes multimerization phenomena. The low degree of acetylation present in the chitosan of 15 Ac% should not exibit this behaviour, because of its small percentage of crystalline structure[21]. On the contrary, both the light-scattering and chromatographic results (Figs. 3 and 4) are in agreement with a strong difference between samples Chit-15 H_5 and Chit-15 H (Table I): once more, depolymerization in 1% AcOH, under reflux, gave materials which were difficult or impossible to characterize in solution.

Because of these results, we used the samples Chit-15H_1, Chit-15H_4 and Chit-15H_5, prepared by hydrolysis in 0.6 M HCl at 50°C, as broad-MD standards. They have been characterized by LS techniques, in the same solvent system used in HPGPC: the data gave linear angular plots and there was a high positive dependence on concentration, which ruled out multimerization phenomena of chitosan chains.

Table II contains the M_w values evaluated by this method, and the elution patterns in 0.5 M AcOH–0.2 M NaOAc are reported in Fig. 5. Following the method suggested by Swartz[22], integral MD obtained by GPC (cumulative weight

TABLE II

Comparison of molecular weights obtained for various samples of chitosans by LS and GPC techniques

Sample	$(M_w)_{LS} \times 10^{-5}$	$(M_w)_{GPC} \times 10^{-5}$	
		Fig. 6 (15 Ac%)	Fig. 8 (42 Ac%)
Chit-15 H_1	7.2	6.5	6.1
Chit-15 H_4	2.0	2.2	2.5
Chit-15 H_5	1.1	1.1	1.4
Chit-15	10.0	8.5	
Chit-28	9.0	8.0	
Chit-42	13.6	8.5	7.5
Chit-42 C_1	2.0	1.3	
Chit-42 D_3	3.4	2.9	
Chit-42 D_4	2.6	2.0	

fractions W_x vs retention volumes) were compared to the integral theoretical MD (cumulative weight fractions W_x vs degree of polymerization), in order to obtain the values X–V_r for the calibration curves reported in Fig. 6. All three samples were used, because none of these spans above the whole range V_m–V_o and the method does not permit an extrapolation outside experimental GPC values. Superimposition of the straight lines corresponding to each sample is good only if one takes the values of cumulative W_x ranging for 0.2 to 0.8, where the error is very small. A calibration curve can be thus obtained, which ranges from $X = 7000$ ($M = 1.18 \times 10^6$) at cumulative $W_x = 0.8$ for Chit-15 H_1 to $X = 190$ ($M = 3.2 \times 10^4$) at cumulative $W_x = 0.2$ for Chit-15 H_5. The extrapolated data were evaluated by a best-fitting approach, by using $(M_w)_{LS}$ values of Table II; the whole calibration curve gives $(M_w)_{GPC}$ values with an error of $\pm 10\%$ (see Table II).

DISCUSSION AND CONCLUSION

In order to test the calibration curve of Fig. 6, evaluated by means of the Integral MD Method with slight modifications, $(M_w)_{GPC}$ values for commercial

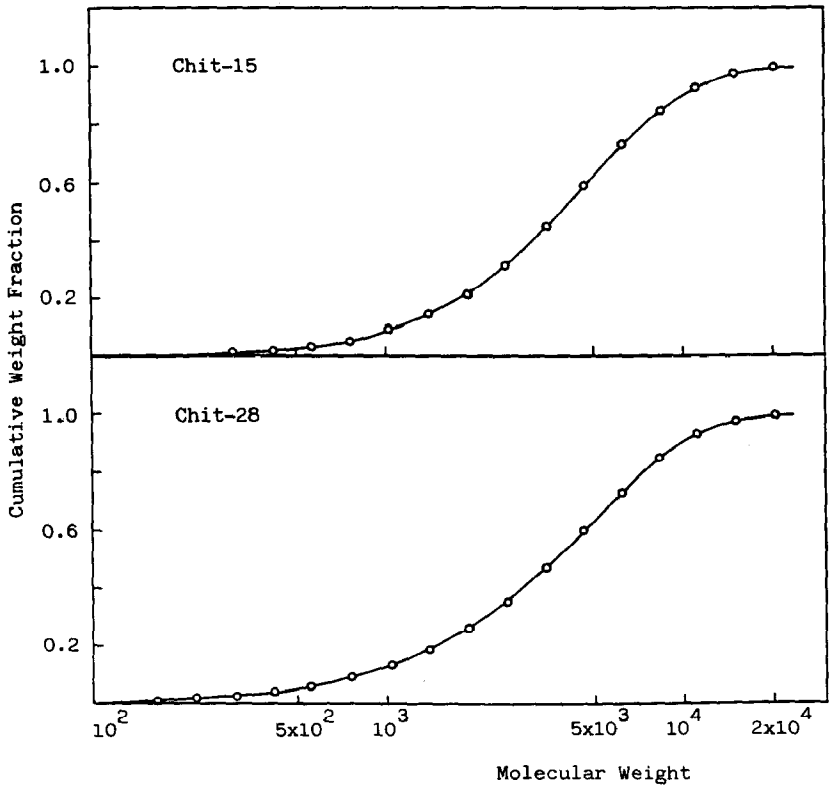

Fig. 7. Integral distribution curves of chitosan samples.

chitosans Chit-15, Chit-28, and Chit-42 were calculated and compared to $(M_w)_{LS}$ (Table II). The agreement is remarkably good for samples of 15 and 28 Ac%, and the small difference may be due to the extrapolated values at high degrees of polymerization, where linearity is often not observed. Therefore, it is reliable to evaluate the M_n values, which are 3.8×10^5 for Chit-15 and 3.1×10^5 for Chit-28, and the corresponding integral distribution curves are reported in Fig. 7. Correction for curve broadening caused by the chromatographic equipment is unnecessary[22]. By contrast, the agreement between $(M_w)_{GPC}$ and $(M_w)_{LS}$ is only fair for samples of 42 Ac%, both at high and low $[\eta]$ (Table II): probably the hydrodynamic volumes of these chitosan chains, proportional to $[\eta] \times M$ (ref 14), are changed with reference to less acetylated samples. This reflects a change in the

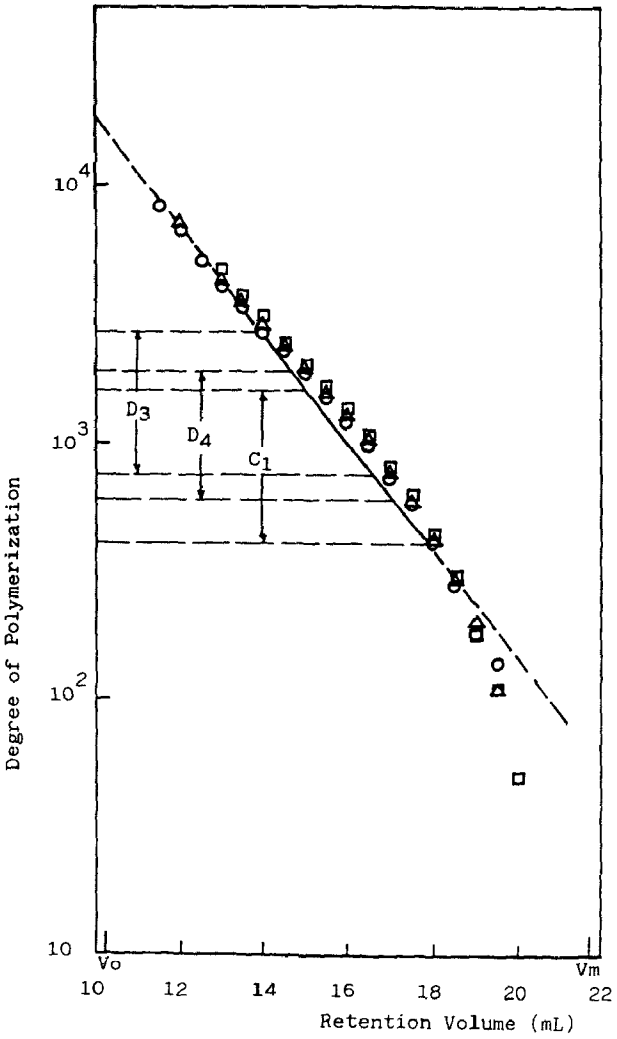

Fig. 8. Calibration curve obtained by using Chit-42 D_3 (○), Chit-42 D_4 (△), and Chit-42 C_1 (□).

viscometer parameters a and k. A plot log $[\eta]$ vs log M_w, using all of the samples in Table II, does not show a large difference among the viscometric parameters: apparently, they are not dependent on the degree of acetylation. On the other hand, this result may not be meaningful, because the chitosan samples used in the $[\eta]$–M relationship are not fractionated samples and have different MD.

Tentatively, for chitosans of 42 Ac%, we also used the integral MD method in order to obtain a new calibration curve. Plotting the theoretical MD (Flory's distribution) versus the MD obtained by GPC gave the curve reported in Fig. 8. $(M_w)_{GPC}$ values evaluated by means of this calibration curve are in great disagreement with the corresponding $(M_w)_{LS}$ values, for samples having both high and low degrees of acetylation. As expected, the MD of chitosans of 42 Ac%, used for calibration, is not Flory's distribution represented by eq *1*.

In conclusion, the method discussed above permits evaluation of MD for chitosan samples having different molecular weights and different degrees of acetylation. The results are only indicative when the degree of acetylation exceeds 30–35%; fortunately, these samples are not frequently used in the theoretical and applied fields.

The test sample is eluted by using the conditions of mobile phase and columns described above. If its degree of acetylation, evaluated by UV or ^{1}H NMR spectroscopy, is ca. 15–20%, this sample can also be hydrolyzed in 0.6 M HCl at 50°C, to give a few broad MD standards. Experimental determinations of M_n or M_w allow the construction of a calibration curve as reported in Fig. 6.

If absolute molecular weight data are not available, M_w can be calculated from viscosity measurements by using the $[\eta]$ and M_w values of Tables I and II of this work.

REFERENCES

1 M. Rinaudo and A. Domard, in G. Skjäk-Break, T. Anthonsen, and P. Sandford (Eds.), *Chitin and Chitosan*, Elsevier Applied Science, London, 1989, pp 71–86.
2 M. Terbojevich, A. Cosani, G. Conio, E. Marsano, and E. Bianchi, *Carbohydr. Res.*, 209 (1991) 251–260.
3 M. Terbojevich, A. Cosani, B. Focher, A. Naggi, and G. Torri, *Carbohydr. Polym.*, 18 (1992) 35–42.
4 H. Struszczyk, *J. Appl. Polym. Sci.*, 33 (1987) 177–189.
5 B. Focher, A. Naggi, G. Torri, A. Cosani, and M. Terbojevich, *Carbohydr. Polym.*, 18 (1992) 43–50.
6 A.C.M. Wu, W.A. Bough, E.C. Conrad, and K.E.E. Alden, *J. Chromatogr.*, 128 (1976) 87–99.
7 A. Domard and M. Rinaudo, *Polym. Commun.*, 25 (1984) 55–58.
8 R.A.A. Muzzarelli and R. Rocchetti, *Carbohydr. Polym.*, 5 (1985) 461–472.
9 B. Millaud and C. Strazielle, *Makromol. Chem.*, 180 (1979) 441–453.
10 M. Terbojevich, E. Peggion, A. Cosani, G. D'Este, and E. Scoffone, *Eur. Polym. J.*, 3 (1967) 681–689.
11 M.L. Huggins, *J. Am. Chem. Soc.*, 64 (1942) 2716–2718.
12 J. Desbrieres, J. Mazet, and M. Rinaudo, *Eur. Polym. J.*, 18 (1982) 269–272.
13 Y. Kato and T. Hashimoto, *J. Chromatrogr.*, 235 (1982) 539–543.
14 W.W. Yau, J.J.K. Kirkland, and D.D. Bly, *Modern Size-Exclusion Liquid Chromatography*, Wiley, New York, 1979, p 289.
15 A. Takahashi, T. Kato, and M. Nagasawa, *J. Phys. Chem.*, 71 (1967) 2001–2010.

16 G.G. Maghani and G.A.F. Roberts, *Makromol. Chem.*, 189 (1988) 195–200.
17 L.A. Nudiga, E.A. Plisko, and S.N. Danilov, *Zh. Obshch. Khim.*, 41 (1970) 2555–2558.
18 V.F. Felicetta, A. Ahola, and J.L. McCarthy, *J. Am. Chem. Soc.*, 78 (1956) 1899–1904.
19 R.L. Cleland, *Biopolymers*, 23 (1984) 647–666.
20 R.C.G. Moggridge and A. Neuberger, *J. Chem. Soc., C*, (1938) 745–750.
21 K. Kurita, T. Sannan, and Y. Iwakura, *Makromol. Chem.*, 178 (1977) 3197–3202.
22 T.D. Swartz, D.D. Bly, and A.S. Edwards, *J. Appl. Polym. Sci.*, 16 (1972) 3353–3360.