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PII: S0144-8617(97)00244-0

Carbohydrate Polymers **35** (1998) 223–231

0144–8617/98/$19.00+0.00

Low-molecular-weight chitosans derived from β-chitin: preparation, molecular characteristics and aggregation activity

Manabu Shimojoh[a],*, Kazuo Fukushima[b] and Keisuke Kurita[c]

[a]*Research and Development Department, 3rd Section, Toyo Suisan Kaisha, Ltd, 2-13-40, Konan, Minato-ku, Tokyo 108, Japan*
[b]*Department of Microbiology, School of Dentistry at Matsudo, Nihon University, Matsudo, Chiba 271, Japan*
[c]*Department of Industrial Chemistry, Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180, Japan*

(Received 17 June 1997; revised 10 November 1997; accepted 11 November 1997)

Preparation, molecular characteristics, and aggregation activity of low-molecular-weight chitosans derived from β-chitin have been studied in comparison with those of chitosans from α-chitin. Chitosan derived from β-chitin was partially degraded with alkali and acid to prepare chitosans with reduced molecular weights. The reaction was also conducted with chitosan from α-chitin, but it was less susceptible to the degradation than chitosan from β-chitin. The resulting two series of chitosans had molecular weights ranging from 11 to 436 kDa. GPC analysis showed similar changes in the molecular weight distribution in the progress of main chain cleavage of the two kinds of chitosans. The polydispersity values were 2.01–4.16, indicating relatively narrow molecular weight distributions. These chitosans aggregated bovine serum albumin efficiently, and the aggregation behavior was dependent on the molecular weight and concentration of chitosan in addition to the pH of the media and concentration of sodium chloride. The aggregation activity of chitosans from β-chitin was found to be somewhat higher than that of chitosans from α-chitin.

Key words: β-chitin, chitosan, molecular weight, bovine serum albumin, aggregation.

INTRODUCTION

Chitin is one of the most important biomass resources, but the physicochemical and biological characteristics have not been fully disclosed yet owing to the intractable nature. In view of potential applications, chitosan, a deacetylated form of chitin, is particularly interesting for its marked ability of adsorption or aggregation. Besides the high coagulating ability for activated sludge and various other suspensions, chitosan aggregates L1210 leukemia cells (Sirica & Woodman, 1971), yeast cells (Weir, Ramaden & Hughes, 1993), and anionic polymers (Terayama, 1952). It also adsorbs various substances including iodine (Gaillard & Bailey, 1966), dyes (Maeda & Ishida, 1967; Knorr, 1983; Yamamoto, 1984), solvents (Castle, Deschamps & Tice, 1984), and lipids (Nauss, Thompson & Nagyvary, 1983). Although chitin does not bind to bovine serum albumin (BSA) (Montgomery & Kirchman, 1993), carboxymethylchitin (Nishimura, Ikeuchi & Tokura, 1984) shows some affinity toward BSA.

Of three crystalline forms of chitins, α-, β-, and γ-chitins, α-chitin has been studied most extensively because of the abundance and easy accessibility. Chitosan is thus prepared almost exclusively from α-chitin. Although only little attention has been paid to β-chitin, it may be a promising alternative source of chitin with distinctive features. β-Chitin is characterized by weak intermolecular forces (Rudall, 1963; Gardner & Blackwell, 1975) and has been confirmed to exhibit higher reactivity in various modification reactions as well as higher affinity for solvents than α-chitin (Kurita, Tomita, Tada, Ishii, Nishimura & Shimoda, 1993a; Kurita, Tomita, Ishii, Nishimura & Shimoda, 1993b;

*Corresponding author.

Table 1. Preparation and molecular charactersitics of chitosans

Sample code	Starting material	Preparation conditions	Molecular weight ($\times 10^3$)			Mw/Mn[d]
			Mv[a]	Mn[b]	Mw[c]	
Sp-chitosan	SP-chitin	(40% NaOH, 100°C, 2 h) × 4	600	–	–	–
SP-1	SP-chitosan	(40% NaOH, 100°C, 2 h)	426	162	326	2.01
SP-2	SP-chitosan	(40% NaOH, 100°C, 2 h) × 3	220	118	300	2.54
SP-3	SP-2	(conc. HCL, 30°C, 2 h)	70	50	184	3.68
SP-4	SP-2	(conc. HCL, 68°C, 2 h)	13	12	28	2.33
CC-chitosan			670	–	–	–
CC-1	CC-chitosan	(40% NaOH, 100°C, 2 h) × 4	436	137	300	2.19
CC-2	CC-chitosan	(40% NaOH, 100°C, 3 h) → (6N HCL, 30°C, 3 h)	220	97	270	2.78
CC-3	CC-chitosan	(40% NaOH, 100°C, 3 h) → (conc. HCL, 30°C, 2 h)	110	61	254	4.16
CC-4	CC-chitosan	(40% NaOH, 100°C, 3 h) → (conc. HCL, 70°C, 2 h)	11	15	32	2.13

[a] Molecular weight determined by viscometry.
[b] Number average molecular weight by GPC.
[c] Weight average molecular weight by GPC.
[d] Polydispersity.

Kurita, Ishii, Tomita, Nishimura & Shimoda, 1994). It is noteworthy that chitosan prepared from β-chitin also exhibited high reactivity compared to that from α-chitin (Kurita, Tomita, Tada, Nishimura & Shimoda, 1993c) and significant bactericidal activity (Shimojoh, Masaki, Kurita & Fukushima, 1996). These results suggest the high potential of chitosan derived from β-chitin as a novel functional biopolymer.

It is therefore worthwhile to establish an efficient preparative procedure for chitosans with desired molecular weights from β-chitin to explore optimum functions including flocculation, adsorption, and antimicrobial activities. This would also make possible discussion of the properties in relation with molecular weight and/or molecular weight distribution. Here we report the formation of chitosans with reduced molecular weights from β-chitin, the molecular characteristics, and the aggregation behavior for BSA in comparison with those of the corresponding chitosans prepared from α-chitin.

MATERIALS AND METHODS

General

The degree of deacetylation (dd) was determined by conductometric titration with a TOA CM-40S. Gel permeation chromatography (GPC) was carried out with a Yokogawa LC100 System connected to a Shodex SE-61 RI detector (column, Shodex OH Pack SB-804 HQ; mobile phase, lactic acid buffer solution (pH 3.4, prepared from 0.45% L-lactic acid and 0.5 mol/l sodium hydroxide); flow rate, 0.5 ml/min). Standard pullulans were used for calibration. Absorbance was determined with a JASCO Ubest-30 as a measure of turbidity. BSA was purchased from Sigma. Water was purified by distillation followed by deionization.

Viscometry

The solution viscosity was measured with an Ubbelohde-type viscometer. Molecular weights of three samples were first determined by viscometry in 0.2 mol/l acetic acid–0.1 mol/l sodium chloride–4 mol/l urea solution with Mark-Houwink's relation for chitosan proposed by Lee (1974) where K and a are 8.93×10^{-4} and 0.71, respectively. The viscosities of the same samples were then measured in a lactic acid buffer solution (0.45% L-lactic acid solution adjusted to pH 5.90 with 0.5 mol/l sodium hydroxide), and K and a values were calculated to be 8.09×10^{-5} and 0.91. The viscosities of the chitosan samples in the lactic acid buffer solution were thus calibrated to the molecular weights with these parameters.

Chitin and chitosans

β-Chitin was isolated from squid (*Dasidicus gigas*) pens by the previously reported method (Kurita et al., 1993a) and pulverized to 0.5 mm mesh with an ultracentrifugal mill Retsch ZM-1 (Germany). The degree of deacetylation was 0.08.

The β-chitin was treated with 40% sodium hydroxide at 100°C for 2 h, filtered, and washed with water. The deacetylation procedure was repeated three more times to give chitosan (SP-chitosan) with a dd of 0.98 and molecular weight of 600 kDa as determined by viscometry.

Chitosan (CC-chitosan), prepared from crab (*Chionoecetes opilio* and *Chionoecetes japonicus*) crusts by the ordinary procedure with hydrochloric acid and aqueous sodium hydroxide, was purchased from Kimitsu Chemical Industries (dd, 0.88; molecular weight, 670 kDa by viscometry; ash, less than 1%). It was further treated with 40% sodium hydroxide at 100°C for 2 h. The alkaline treatment was repeated two more times to ensure complete removal of

the remaining acetyl groups and some possible contaminants. The resulting chitosan had a dd of 1.0 (CC-1).

Partial degradation of chitosan

Two kinds of chitosans, SP-chitosan and CC-chitosan, were treated with aqueous sodium hydroxide and then with hydrochloric acid to prepare chitosans with reduced molecular weights. A typical example is as follows.

A suspension of 10 g of SP-chitosan in 210 ml of 40% sodium hydroxide was heated at 100°C for 2 h. The chitosan was filtered, washed with water, and treated with 40% sodium hydroxide again. After repeating the deacetylation procedure one more time, the solid was washed thoroughly with water and dried to give 7.2 g of chitosan. The dd and molecular weight were 1.0 and 220 kDa.

The chitosan obtained above (1.0 g) was then treated with 25 ml of concentrated hydrochloric acid at 30°C for 2 h, and the mixture was poured into about 100 ml of water. The pH of the resulting solution was raised to 10 with aqueous sodium hydroxide, and the precipitate was collected by filtration. It was washed thoroughly with water to give 0.79 g of the product with a dd of 1.0 and a molecular weight of 70 kDa.

Aggregation assay

Chitosan (50 mg) was dissolved in a lactic acid solution prepared by diluting 0.25 ml of 89% aqueous L-lactic acid with 30 ml of water, and the pH was adjusted to 5.90 with 0.5 mol/l sodium hydroxide. The solution was autoclaved at 121°C for 15 min, and the final pH and volume were adjusted to 5.90 and 50 ml to prepare a 0.45% L-lactic acid solution containing 0.1% chitosan. Chitosan solutions of various concentrations were prepared by serial two-fold dilutions with 0.45% L-lactic acid buffer solution of pH 5.90. In a similar manner, BSA (125 mg) was dissolved in the aqueous lactic acid solution, and the final pH and volume were adjusted to 5.90 and 50 ml.

A mixture of 1 ml of the BSA solution and 1 ml of the chitosan solution of a certain concentration was incubated at room temperature for 10 min, and the turbidity due to aggregation was measured at 660 nm. To confirm the reproducibility, the experiments were repeated four times in the aggregation study for elucidating the influence of the molecular weight, and three data were used to calculate the average and standard deviation vFig. 7 Fig. 8).

The influence of a salt was examined with a chitosan solution and a BSA solution both containing sodium chloride of the same concentrations.

RESULTS AND DISCUSSION

Determination of molecular weight

In order to follow the progress of partial degradation of chitosan, molecular weights of the products were measured by viscometry. The viscosities were determined in aqueous L-lactic acid instead of aqueous acetic acid, since lactic acid is more acceptable in terms of low toxicity and easy handling especially in the fields of food, cosmetics, and medicine. Molecular weight can be calculated from the intrinsic viscosity with Mark-Houwink's relation.

$$[\eta] = K \cdot M\mathrm{v}^{a}$$

Since the values for K and a were proposed for chitosan in an aqueous acetic acid/sodium chloride/urea solution (Lee, 1974), they were modified for aqueous lactic acid. Viscosities of chitosan samples were thus determined in both the aqueous solutions, and K and a values for aqueous lactic acid were determined to be 8.09×10^{-5} and 0.91, respectively.

Molecular weights were also estimated by GPC in aqueous lactic acid solutions to discuss the molecular weight distribution of the resulting chitosans.

Preparation of SP-chitosans and CC-chitosans

Controlled cleavage of the main chain of SP-chitosan prepared from squid pen chitin (β-chitin) was conducted under various conditions to establish an efficient preparative procedure for squid pen chitosans with reduced molecular weights. It was partially degraded with aqueous sodium hydroxide, and subsequent treatment with hydrochloric acid further lowered the molecular weight effectively. The reactions were carried out under mild conditions so as to avoid side reactions. Four kinds of chitosans with reduced molecular weights were thus obtained as summarized in Table 1.

Chitosan prepared from crab crust chitin (α-chitin), CC-chitosan, was also treated with alkali similarly, but more resistive to degradation than SP-chitosan as evident by the molecular weights of SP-2 ($M\mathrm{v} = 220 \times 10^3$) and CC-1 ($M\mathrm{v} = 436 \times 10^3$) prepared under similar conditions. This is consistent with the lower reactivity of CC-chitosan than SP-chitosan in N-phthaloylation (Kurita et al., 1993c). CC-Chitosan was thus degraded under more rigorous conditions to prepare chitosans with similar molecular weights to make possible comparison of the characteristics of the two series of chitosans.

The partially degraded chitosan samples were obtained as white to off-white powdery materials and had molecular weights ranging from 11 to 436 kDa, as listed in Table 1. Deacetylation was complete as confirmed by the dd values of 1.0 in all the cases.

Molecular weight characteristics of chitosans

The properties of chitosans may possibly be influenced not only by molecular weight but also by molecular weight distribution, since the ordinary chitosan samples were shown to have fairly wide distributions of molecular weight (Wu & Bough, 1978). Molecular weight distribution of

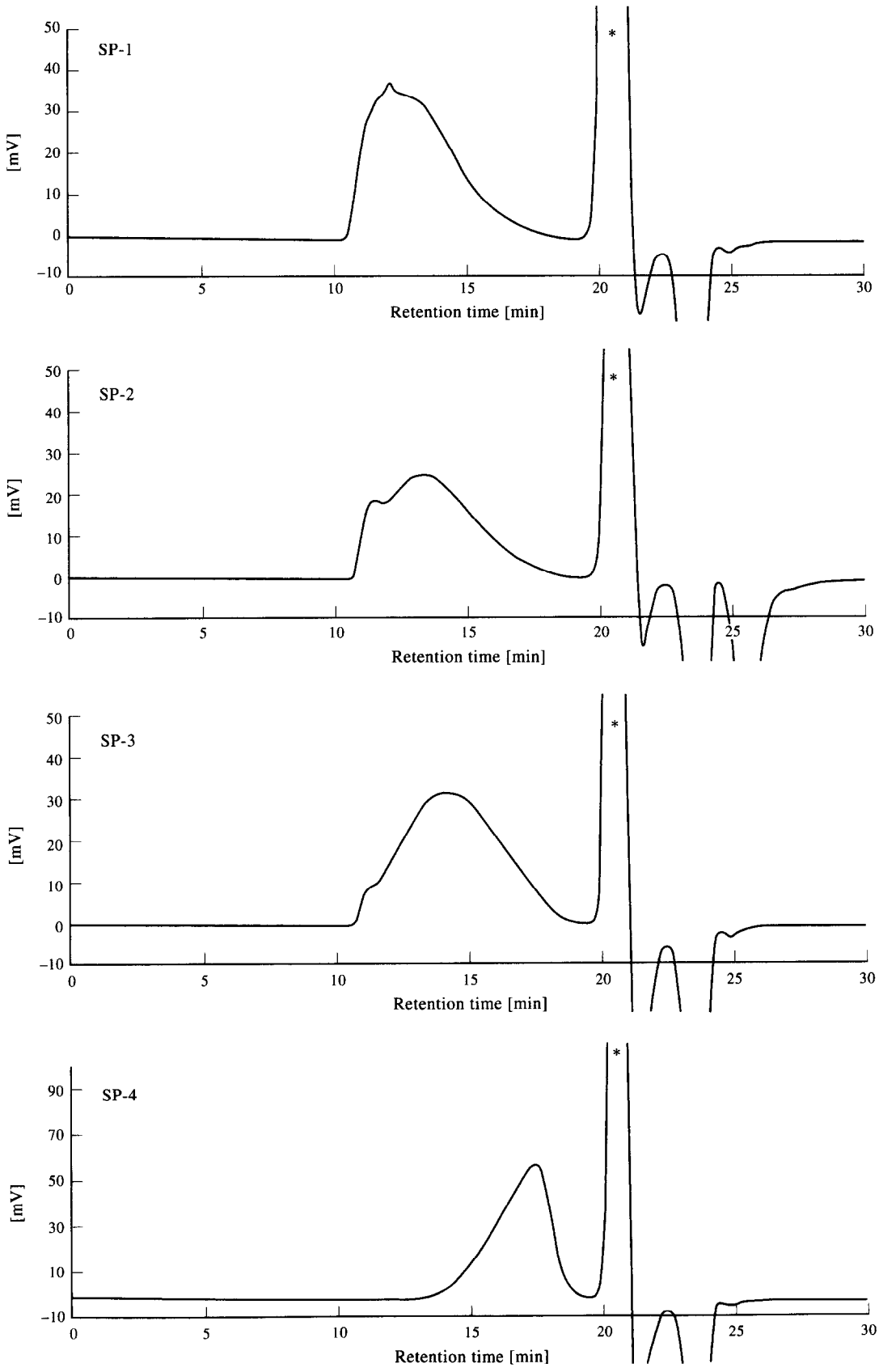

Fig. 1. GPC curves for SP-chitosans (*denotes the peak due to L-lactic acid).

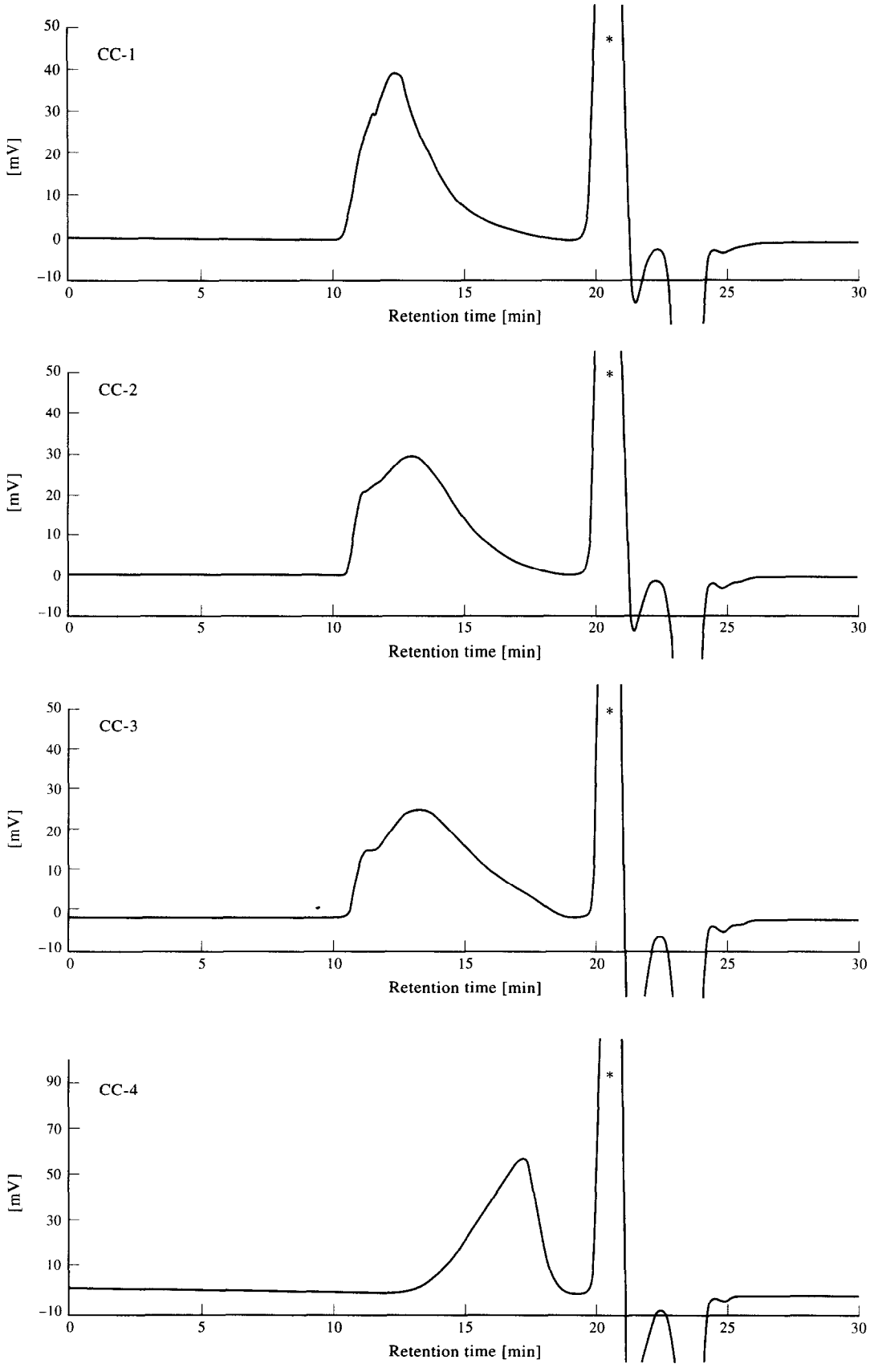

Fig. 2. GPC curves for CC-chitosans (*denotes the peak due to L-lactic acid).

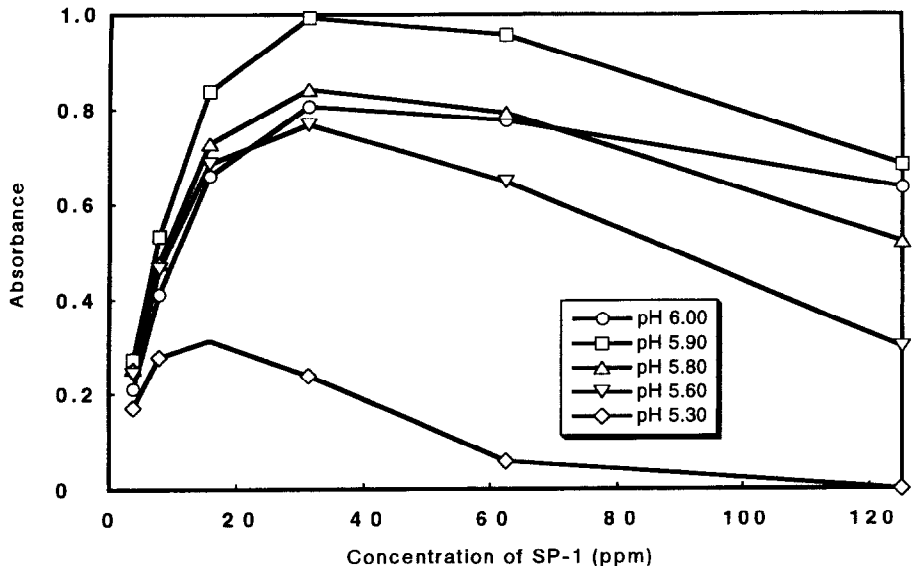

Fig. 3. Effects of pH and concentration of chitosan (SP-1) on the aggregation of BSA.

chitosans prepared here was therefore elucidated by GPC in aqueous lactic acid solution, and the change in the distribution of SP-chitosans is illustrated in Fig. 1. The distribution became wide as the partial degradation proceeded, but then became narrow again when the molecular weight was considerably lowered to about 10 kDa.

The number average molecular weights (Mn) and weight average molecular weights (Mw) were calculated with pullulan standards and are included in Table 1. When the molecular weight is high, the Mn and Mw values are lower than the Mv value determined with Mark-Houwink's relation used in this study, and this was again observed with SP-1 and CC-1 in Table 1. The change in the molecular weight observed by GPC is roughly parallel to that by viscometry. Interestingly, CC-chitosan underwent partial degradation in a substantially similar manner as evident from the change in the GPC patterns in Fig. 2.

Polydispersity (Mw/Mn) is a measure of distribution of molecular weight, and the values in Table 1 clearly support the time courses of the molecular weight distribution implied from the GPC profiles in Fig. 1 and Fig. 2. It is worthy of remark that the polydispersity values reported for chitosans prepared by treating α-chitin with 50% sodium hydroxide at 100°C were almost constant (4.63–4.98) independent of the molecular weight (Wu & Bough, 1978). Moreover, the polydispersity data indicate that the molecular weight distributions of chitosans prepared here were much narrower than those by the conventional method with sodium hydroxide alone.

Aggregation of BSA with chitosans

Influence of pH and concentration of chitosans

Aggregation of BSA with chitosan would be influenced by

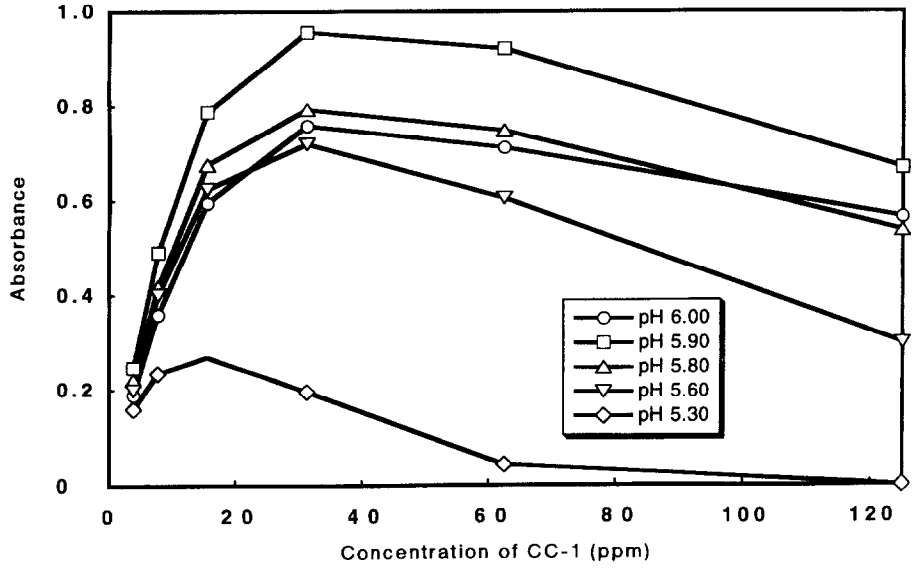

Fig. 4. Effects of pH and concentration of chitosan (CC-1) on the aggregation of BSA.

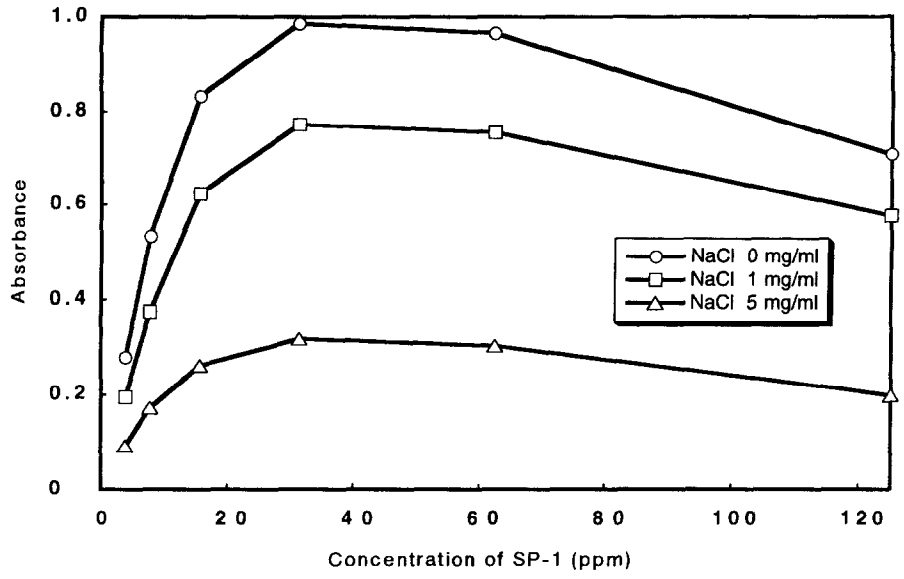

Fig. 5. Aggregation of BSA with chitosan depending on the concentrations of sodium chloride and chitosan (SP-1) at pH 5.90.

the change in the ionic state, and thus the effects of pH were first examined. The aggregation with SP-1 and CC-1 was evaluated in terms of turbidity of the mixture. The pH value was varied from 5.0, where no aggregation took place, to 6.3, where chitosan precipitated, and the aggregation activities of the two kinds of chitosans turned out to be quite similar to each other, as shown in Fig. 3 and Fig. 4. The aggregation was low at a low pH, but increased sharply with an increase in the pH. Both chitosans exhibited efficient aggregation as the solution became close to neutral, suggesting that ionic interaction plays an important role in the aggregation. In either case, optimum pH was found to be 5.90 (Fig. 3 and Fig. 4).

Chitosan concentration also influenced the aggregation behavior, and aggregations reached maxima at certain concentrations. For instance, at pH 5.90, a maximum was found at 31.2 ppm of chitosan, where concentrations of chitosan and BSA were 7.2×10^{-8} and 1.9×10^{-5} mol/l (Fig. 3). This indicates that the amount of chitosan was about 1/260 equivalent to BSA at this maximum. With further increasing chitosan concentration, the extent of aggregation decreased, and this may probably be attributable to the resulting high concentration of chitosan around the BSA molecules. However, the aggregation became less sensitive to chitosan concentration in the high concentration region at a higher pH where aggregation was quite efficient. High aggregation was observed even at 125 ppm at pH above 5.80 (Fig. 3 and Fig. 4).

Influence of sodium chloride and concentration of chitosans

To assess the ionic interaction in aggregation, the influence of sodium chloride on the aggregate formation was

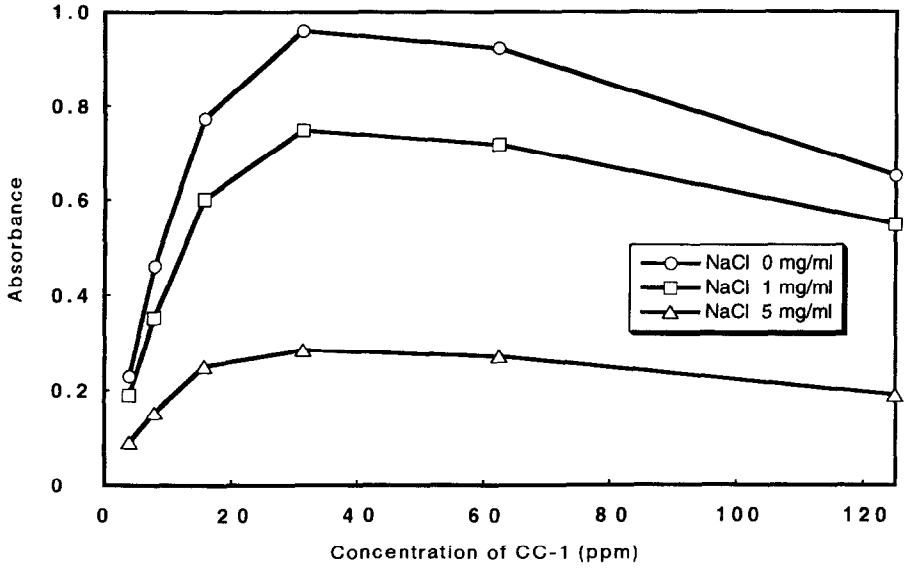

Fig. 6. Aggregation of BSA with chitosan depending on the concentrations of sodium chloride and chitosan (CC-1) at pH 5.90.

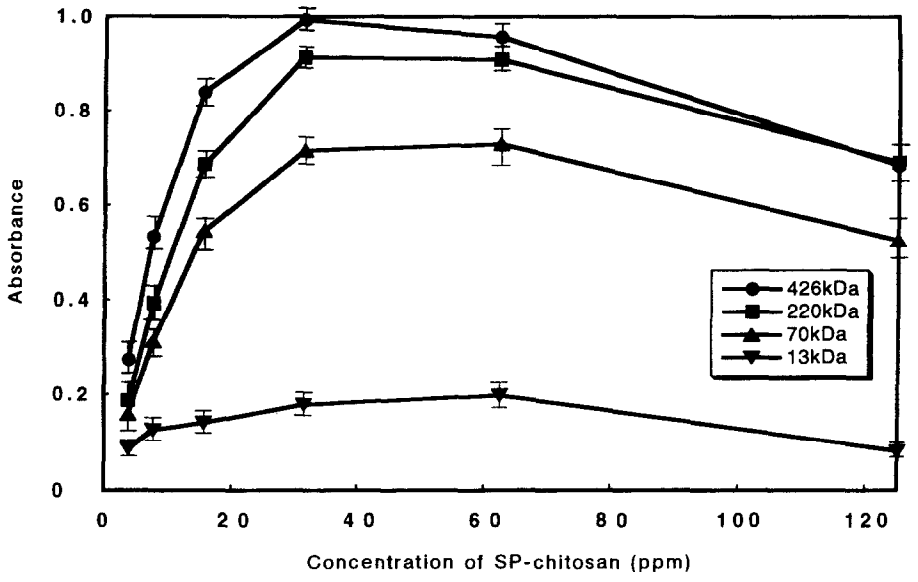

Fig. 7. Dependence of the aggregation of BSA with SP-chitosans on the molecular weight and concentration of chitosan at pH 5.90.

examined at pH 5.90. As illustrated in Fig. 5 and Fig. 6, the addition of the salt interfered with the aggregation drastically for both SP-1 and CC-1, and the effect was significant even at a concentration as low as 1 mg/ml. The aggregation was disturbed further with an increase in the sodium chloride concentration, and only poor aggregation was observed at 5 mg/ml, supporting the importance of the ionic interaction.

Influence of molecular weight and concentration of chitosans

As another factor possibly influencing the aggregation, molecular weight could be important, and the effects were examined with SP- and CC-chitosans of various molecular weights at pH 5.90.

Fig. 7 shows the dependence of aggregation on the molecular weight and concentration of SP-chitosans. The experiments were confirmed reproducible, and the aggregation ability increased with an increase in the molecular weight as expected. Furthermore, maximum aggregations were induced with about 30–60 ppm chitosans independent of the molecular weight. Aggregation then decreased with the chitosan concentration.

Aggregation behavior with CC-chitosans was similar to that with SP-chitosans. Dependence on the molecular weight, however, appeared to be low to some extent compared to that with SP-chitosans as shown in Fig. 8.

CONCLUSIONS

A procedure for the preparation of chitosans with reduced

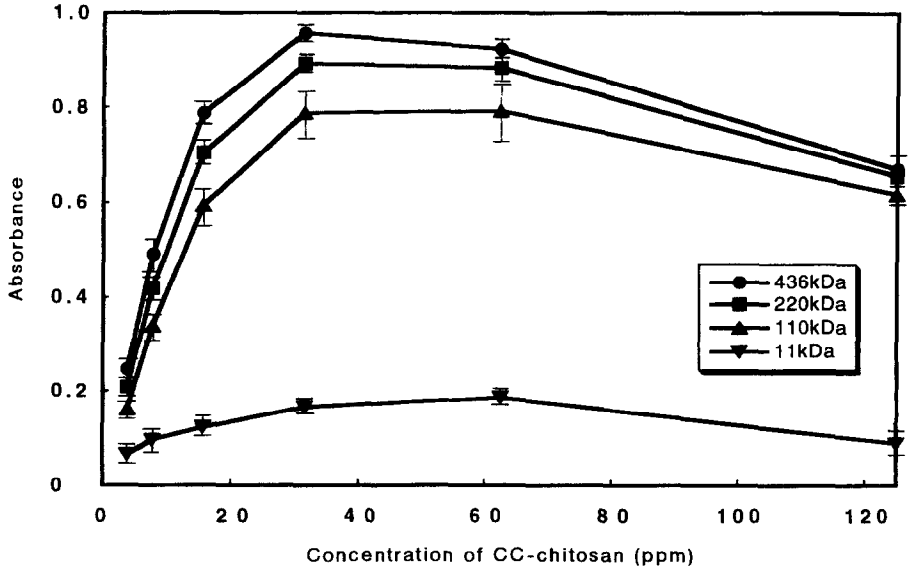

Fig. 8. Dependence of the aggregation of BSA with CC-chitosans on the molecular weight and concentration of chitosan at pH 5.90.

molecular weights has been established starting from β-chitin as well as α-chitin. The progress of partial degradation was followed by GPC, and this preparative method was confirmed convenient to prepare chitosans with fairly narrow molecular weight distributions. Although aggregation of BSA with these two series of chitosans was influenced similarly by some factors, it was quite effective with chitosans of appropriate molecular weight and concentration. The aggregation behavior was similar to each other with SP- and CC-chitosans, but interestingly, SP-chitosans appeared to exhibit slightly higher activity than CC-chitosans of similar molecular weights as suggested from Figs 3–8. This may possibly be interpreted in terms of subtle differences in the molecular weight distribution between the two series of chitosans, although the distributions were not different significantly in the GPC measurements.

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