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# Molecular parameters of chitosans depolymerized with the aid of papain

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The profiles and positions of the GPC curves are progressively altered during the course of the papain-promoted hydrolysis of chitosan. The chitosan fractions having the highest degrees of polymerization are depolymerized and Mw, $[\eta]$ and $R_G$ values are lowered. Viscometric measurements confirm that the immobilized papain depolymerizes chitosan with high initial velocity, the more acetylated chitosan [0.42] being more susceptible to papain than chitosan [0.22] and chitosan [0.15]. This holds true for chemically reacetylated chitosan [0.49] as well. The behavior of papain in this respect is different from that of lysozyme which is more active on chitosan [0.22] than on more acetylated chitosans. The data suggest that papain acts on the link between glucosamine and *N*-acetylglucosamine units. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

Chitosans have been recently found to be susceptible to the specific hydrolytic activities of several enzymes, namely cellulase (Muraki *et al.*, 1993), hemicellulase, amylase, dextranase, pectinase, lipase (Muzzarelli *et al.*, 1995), and proteases such as pepsin, papain (Muzzarelli *et al.*, 1994; Terbojevich *et al.*, 1993), bromelain, ficin and pancreatin (Pantaleone *et al.*, 1992; Yalpani & Pantaleone, 1994). Some of these enzymes were found to be more efficient catalysts for chitosan hydrolysis than chitinase and lysozyme.

Particular attention has been devoted to papain because of its wide acceptance and low cost, and because preparative-scale operations have been proposed by Yalpani and Pantaleone (1994) at pH 3 and 40°C.

There is interest in using said enzymes in the immobilized form which enables one to use the enzyme repeatedly and supply protein-free chitosan hydrolysates. Thus, Muzzarelli *et al.* (1994) have used papain immobilized on chitin powder, Hayashi and Ikada (1991) used chitosan as a support for covalent immobilization of proteases, while alumina and thermosensitive latex particles have been studied for the immobilization of papain by Hyndman *et al.* (1992) and Kondo *et al.* (1994), respectively. Some of the other enzymes mentioned above have been immobilized on various supports, in particular, lipase which is commercially available in supported form.

In consideration of these facts and because chitinases and chitosanases are presently unavailable in bulk quantities, we have undertaken the present work in view of getting a better insight into the hydrolytic process catalyzed by chitin-supported papain. Moreover, various authors (Nud'ga *et al.*, 1990; Matsumoto *et al.*, 1991; Anthonsen *et al.*, 1994; Rinaudo *et al.*, 1993; Terbojevich *et al.*, 1992; Muzzarelli, 1993) have recently studied the heterogeneous molecular aggregation of chitosans in acidic solutions. Because the presence of supramolecular structures in chitosan solutions disturbs analytical and preparative operations, an additional purpose of this work is to verify the presence of aggregates in the depolymerized chitosan solutions.

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## EXPERIMENTAL

### Chitosans

The following chitosans were used (degree of acetylation in square brackets): chitosan [0.23] (CTA-3 from *Chionoecetes opilio*) supplied by Katakura Chikkarin, Tokyo, Japan; chitosan [0.15] from *Chionoecetes japonicus*, same supplier; shrimp chitosan [0.20] (Seacure 343) supplied by Pronova, Drummond, Norway, and chitosan [0.42] from *Euphausia superba*, manufactured by Rybex, Gdynia, Poland.

Control chitosans were obtained from 7-day old lactate solution of these commercial chitosans; after dialysis against 0.1 M NaOH and then water, they were freeze-dried.

The elemental analysis and the molecular parameters for the control chitosan [0.23] and for the same contacted with immobilized papain for 7 days and isolated as the control sample were found to be the following: Chitosan [0.23]: N = 7.42, C = 40.77, H = 6.91, N/C = 0.182; $[\eta]$ = 11.77 dl/g, Mw = 780,000, $A_2 = 2.10^{-3}$, $R_G$ = 1190 A°. Papain-treated chitosan [0.23] (7 days) P[0.23]: N = 7.31, C = 40.11, H = 6.69, N/C = 0.182, $[\eta]$ = 3.66, Mw = 183 000, $A_2 = 3.10^{-3}$, $R_G$ = 700 A°.

Chitosan standards for GPC calibration were obtained by depolymerization of chitosan [0.15] in 0.6 M HCl at 50°C: the polymer was isolated by precipitation during dialysis against 0.1 M NaOH (Spectra Por, cutoff 3500) and then against water. The characteristics were as follows:

| Hydrolysis time | $[\eta]$ (dl/g) 0.5 M HAc–0.2 M NaAc | Mw (LS) |
|---|---|---|
| 20 min | 10.8 | $8.10^5$ |
| 25 h | 4.0 | $1.8.10^5$ |
| 35 h | 2.2 | $1.1.10^5$ |

Chitin from lobster was a kind gift from Dr O. Bilbao Revoredo, Cuba (Garcia-Alonso & Oviedo Vega, 1990). Chitosans were reacetylated according to Hirano *et al.* (1981).

### Papain

Papain from *Carica papaya* was provided by Calbiochem, La Jolla, CA, USA, and was immobilized on lobster chitin powder as previously described by Muzzarelli *et al.* (1994). The immobilized enzyme was stored at 4°C in the wet state, and used for most experiments with no further treatment. A freeze-dried preparation was also used. The other enzymes were those previously indicated (Muzzarelli *et al.*, 1994, 1995).

Depolymerization experiments were carried out in 1% lactic acid at 20°C in the presence of immobilized papain: at fixed times, aliquots were taken, filtered on filter paper to remove the immobilized enzyme and diluted (10–20-fold) with aqueous solutions of acetic acid and sodium acetate, in such a way as to prepare solutions for $[\eta]$, GPC and LS measurements of the following composition: 0.5 M acetic acid, 0.2 M sodium acetate and 1.0 g/l lactic acid.

### Viscometry

An Ubbelhode viscometer was used for determination of the intrinsic viscosity $[\eta]$ at 25°C. The values of $[\eta]$ were obtained by extrapolation from the $\eta_{sp}/C$ vs $C$ plot.

### Gel permeation chromatography

A chromatographic system consisting of two Biol-Gel TSK columns (ToyoSoda, Tokyo) one 50XL, one Dn XL (both 300 × 7.8 mm, in series), a Knauer HPLC pump Type 6400 with a Rheodyne injector 7125, a degassing device Erma ERC 3312 and a refractive index detector Erma 7512 was used. Eluent and polymer solutions were filtered through 0.45 $\mu$m Millipore filters (HAWPO 1300). The polymer concentration was 1.0 g/l and the injected volume 0.1 ml. Elution volumes were calculated from the flow rate (0.6 ml/min). The total volume was 20.1 ml, as estimated by the external standard ethanol. Calibration was performed by means of chitosan samples, by using the integral molecular weight distribution method (Terbojevich *et al.*, 1993). A number of measurements were also carried out with a Spectra Physics Iso-Chrom chromatographic system, equipped with two TSK Gel GMP WXL columns (300 × 7.8 mm) in series.

### Light scattering

These measurements were performed at 20°C using a Sofica Model 42000 photometer with cylindrical cells immersed in toluene. Non-polarized laser light (633 nm) was used; scattering angles ($\theta$) ranged 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 (Millaud & Strazielle, 1979). Solutions and solvent were clarified by centrifugation at 25 000 rpm for 3 h. The data were treated as previously reported (Terbojevich *et al.*, 1992). The value of $dn/dc$, determined using a Milton Roy KMX-16 refractometer at 633 nm, was 0.205 ml/g.

## RESULTS AND DISCUSSION

### Molecular parameters obtained by gel permeation chromatography

During the course of the papain-promoted hydrolytic process, the elution curves obtained by gel permeation chromatography on the papain-treated chitosans showed a progressive alteration of the elution profile, a shift of the entire curve, and a shift of the elution peak towards higher retention volumes, i.e. lower DP values (Fig. 1).

This is indicative of the disappearance of the chitosan fractions having the highest degree of polymerization, and of the lowering of the average degree of polymerization, as a consequence of the hydrolytic action exerted by the immobilized papain. In all cases the areas of the chromatographic curves were identical, showing that no significant loss of material took place during the preliminary filtration of the polymer solutions, due to opalescence formation. Control solutions (no papain

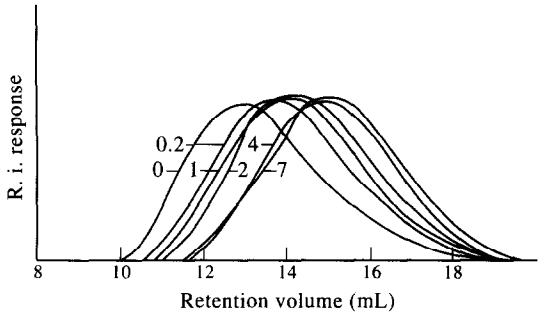


**Fig. 1.** Elution curves for chitosan [0.23] at various times (days) of treatment with immobilized papain.

present) showed a constant position and shape during the 7-day study period.

The molecular parameters thus obtained are listed in Table 1. The DPw and DPn values confirmed that the chemical hydrolytic process due to lactic acid (pH 3.2) is of negligible importance. The kinetic data are diagramatically shown in Fig. 2.

The hydrolytic process yielded narrower distribution curves, as shown in Fig. 3, due to the fact that the high DP chains were more promptly depolymerized by papain, leading to a more marked decrease of Mw than Mn.

The heterogeneity index DPw/DPn should decrease down to a value of 2, which is typical for the most probable distribution, if the enzyme indifferently attacked the $GlcNH_2$–$GlcNH_2$, the GlcNAc–GlcNAc, the GlcNAc–$GlcNH_2$, and the $GlcNH_2$–GlcNAc anhydroglucosidic bonds. Considering that the value 2 is far from being

**Table 1. Variation of the molecular parameters for chitosan [0.23] in the course of the hydrolytic process catalyzed by immobilized papain, and for the controls**

| | In the presence of immobilized papain | | | | |
|---|---|---|---|---|---|
| Time | Mw | Mn | DPw | DPn | DPw/DPn |
| 0 | 784 000 | 195 000 | 4620 | 1150 | 4.03 |
| 1 min | 699 000 | 185 000 | 4120 | 1090 | 3.78 |
| 12 min | 671 000 | 186 000 | 3950 | 1100 | 3.61 |
| 18 min | 654 000 | 168 000 | 3850 | 990 | 3.90 |
| 25 min | 633 000 | 174 000 | 3730 | 1030 | 3.63 |
| 5 h 30 min | 466 000 | 140 000 | 2750 | 820 | 3.33 |
| 1 day | 375 000 | 119 000 | 2200 | 700 | 3.15 |
| 2 days | 344 000 | 104 000 | 2030 | 610 | 3.32 |
| 3 days | 266 000 | 90 000 | 1570 | 530 | 2.96 |
| 4 days | 221 000 | 76 000 | 1300 | 450 | 2.91 |
| 7 days | 201 000 | 67 000 | 1190 | 390 | 3.01 |
| | Controls (papain absent) | | | | |
| Time | Mw | Mn | DPw | DPn | DPw/DPn |
| 0 | 784 000 | 195 000 | 4620 | 1150 | 4.03 |
| 4 h | 733 000 | 177 000 | 4320 | 1040 | 4.15 |
| 1 day | 681 000 | 161 000 | 4010 | 950 | 4.24 |
| 2 days | 774 000 | 187 000 | 4560 | 1100 | 4.14 |
| 3 days | 754 000 | 184 000 | 4440 | 1080 | 4.10 |
| 4 days | 723 000 | 170 000 | 4260 | 1000 | 4.24 |
| 7 days | 678'000 | 169 000 | 3990 | 990 | 4.02 |

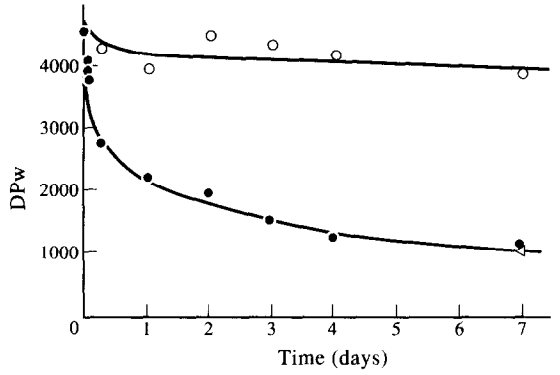


**Fig. 2.** DPw vs time for the chitosan [0.23] in the absence (○) and in the presence (●) of immobilized papain. The values for P[0.23] are also reported (Δ).

reached, the hydrolytic attack should be selective and probably affect the bonds between GlcNAc and $GlcNH_2$ units. Support for this hypothesis comes from the indifference of papain for chitin (to which it can be immobilized for long time) and from available information on the indifference for fully deacetylated chitosan (Yalpani & Pantaleone, 1994). On the other hand, kinetic data relevant to chitosan [0.42] provide an experimental confirmation of such selectivity (Table 2).

## Viscosimetric data

Viscometry also provided evidence of the catalytic activity of papain leading to partial depolymerization of chitosans. Figures 4 and 5 describe the variations of the intrinsic viscosity, $[\eta]$, of chitosan contacted with immobilized papain for the 48-h and 7-day periods, respectively. The intrinsic viscosity value of the chitosan solutions dropped sharply immediately after contact with immobilized papain. After 48 h, a 65% lowering of the initial value of $[\eta]$ took place (control 10%). For contact times longer than 48 h the chitosan [0.23] lactate solutions became opalescent and the $[\eta]$ values,

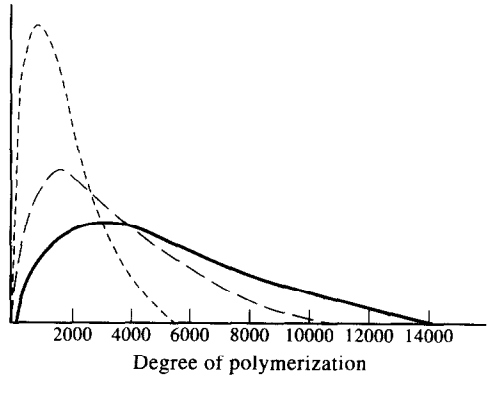


**Fig. 3.** Differential distribution curves obtained from GPC measurements for the chitosan [0.23] in the absence of papain (———), and after contact with papain for 0.2 (—) and for 7 (···) days.

**Table 2. Intrinsic viscosity data (dl/g) for various chitosans contacted with immobilized papain (stored in freeze-dried form and in wet state). Data for controls are given in parentheses**

| Contact time (days) | Chitosan 0.23 | | Chitosan 0.20 freeze-dried | Chitosan 0.40 freeze-dried |
|---|---|---|---|---|
| | wet | freeze-dried | | |
| 0 | 11.77 | 11.77 (11.77) | 11.94 (11.94) | 14.08 (14.08) |
| 1 | 5.71 | 7.66 (10.79) | 8.49 (11.30) | 7.15 (13.10) |
| 2 | 5.22 | 7.22 (10.22) | 8.20 (10.57) | 4.79 (12.56) |
| 5 | 3.14 | 6.53 (6.45) | 6.87 (10.07) | 4.05 (12.25) |
| 7 | 1.38 | | 6.57 (9.90) | 3.54 (11.88) |

measured after filtration through 0.45 $\mu$m filters [$\eta$] were lower than those obtained for isolated P[0.23] sample. This phenomenon however took place also where [$\eta$] reaches a value of 3 dl/g; in both cases such low values could not be accounted for on the ground of a loss on the filters.

For practical purposes the essential information obtained is that the depolymerization of chitosan lactate promoted by immobilized papain proceeds with high initial velocity; longer contact periods (3–7 days) are interesting in terms of oligomer formation.

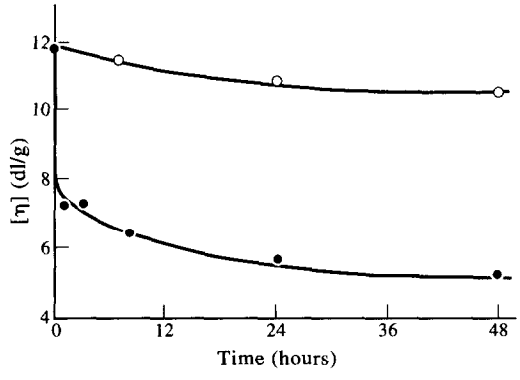


**Fig. 4.** Plots of [$\eta$] vs time (48 h period) for chitosan [0.23] in the presence of immobilized papain (●) and in the absence of papain (○).

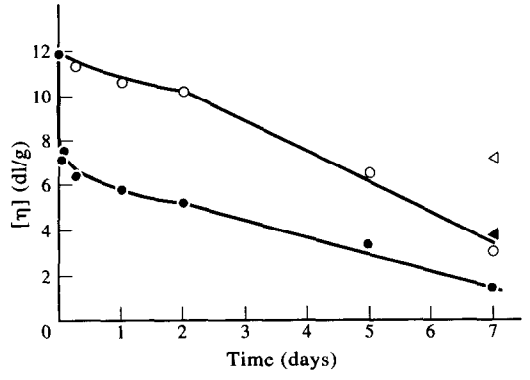


**Fig. 5.** Plots of [$\eta$] vs time (7 day period) for chitosan [0.23] in the presence of immobilized papain (●) and in the absence of papain (○); the values of control [0.23] (△) and P[0.23] (▲) are also reported for comparison.

It is evident that the solutions of certain chitosans undergo viscosity drops unrelated to the depolymerization process, promoted by high chitosan concentration and not revealed by gel permeation chromatography. Viscometry and gel permeation chromatography are both based on the hydrodynamic volume of the polymer which depends on the molecular weight, the chain flexibility, and the interactions with the solvent. The difference between the two techniques is essentially the polymer concentration which is much lower in the case of gel permeation chromatography.

Table 2 summarizes the viscosimetric data for three chitosans in the presence of immobilized papain and for the relevant controls. When stored in wet form, the immobilized papain was found to be more active than freeze-dried immobilized papain, because the [$\eta$] value drop was more marked. The consequences of the enzymatic attack were quite noticeable compared to the controls. The more acetylated chitosan [0.42] was more susceptible to papain than the other two. The chitosans [0.23] and [0.20] had a similar behavior for the initial 48 h of contact, but they behaved differently in the subsequent period of time, because the chitosan [0.23] solution became opalescent and an anomalous decrease of [$\eta$] took place. For one of the chitosans tested, aggregation occurred after 3 days. A further confirmation of the aggregation phenomena revealed by visco-

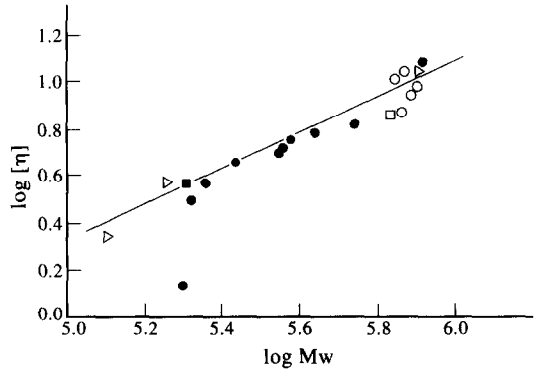


**Fig. 6.** Double logarithmic plot of the intrinsic viscosity and the molecular weight for chitosan samples in 0.5 M HAc–0.2 M NaAc. Standard chitosans (△); chitosan [0.23] in the presence of papain (●); chitosan [0.23] in the absence of papain (○); control [0.23] (□); P[0.23] (■).

metry came from the double logarithmic plot in Fig. 6. The straight line was identified by 3 couples of values for standard chitosans [0.15] previously used for the chromatographic standardization. From slope and intercept, the viscometric parameters for the Mark–Houwink equation were obtained: $a = 0.76$ and $K = 3.5 \times 10^{-4}$ (0.5 M HAc–0.2 M NaAc, in good agreement with Rinaudo *et al.*, 1993).

The data for the papain-treated solutions and controls fitted the curve only when clear solutions were considered; opalescent solutions (treated for more than 48 h) gave values away from the reference curve, because the chitosan aggregates have hydrodynamic values lower than the isolated chains and therefore lower $[\eta]$ values. Opalescence was promoted by high chitosan concentration, and did not take place in 0.1% chitosan solutions. The chitosan [0.20] behaved differently than the chitosan [0.23], insofar as 1.0% solutions did not become opalescent. The difference should reside in the fact that the chitosan [0.23] contains portions of highly acetylated chains, which are more prone to aggregations than randomly acetylated chains; the chemical production treatment of these chitosans was most probably different.

It should be underlined, in any case, that the opalescent solutions, upon filtration, dialysis against NaOH, and freeze drying, yielded partially depolymerized chitosans, which dissolved completely in acid solutions, to give the $[\eta]$ and Mw values reported in Fig. 6 for P[0.23].

### Comparison of papain to lipase and lysozyme

For comparison of the activity of papain, lipase, and lysozyme on chitosan, the three enzymes were used in soluble form at the concentration of 0.05% at the same pH (4.7) at 20°C. Injection of the sample for gel permeation chromatography was done exactly 10 min after mixing the chitosan with the enzyme. The peak of the elution curve for chitosan [0.20] corresponds to 517 000 g/mol: the treatment with papain lowered it to 162 000, with lipase to 225 000 and with lysozyme to 275 000, the reduction values being 68.7, 56.5 and 46.8%, respectively. In all cases the curve was entirely shifted toward lower DP, indicative of the prompt depolymerization of the fractions of highest DP. Therefore, at the pH of the experiment, papain was more effective then lipase and lysozyme.

The peak of the elution curves for Pronova chitosan ([0.20] reacetylated to [0.49]) was also shifted to lower DP values, the percentage reduction values being 80.2% for papain, 71.0% for lipase, and 34.3% for lysozyme. The data for lysozyme indicate that the more acetylated chitosan [0.49] was less susceptible to hydrolysis than chitosan [0.20], in agreement with Muzzarelli (1992) and the literature cited therein. The other results indicate on the contrary that the highly acetylated chitosan [0.49] is more prone to hydrolysis by papain and lipase than the original chitosan [0.20].

## CONCLUSIONS

Chitosans are promptly depolymerized by papain which acts preferably on the longest chains at room temperature. Papain conveniently immobilized on chitin powder can be retrieved from the chitosan solution for further use.

Compared to chemical hydrolysis, the method here proposed offers a number of advantages, mainly slightly acidic pH values and 20°C rather than warm 0.6 M HCl solutions. For instance, the chitosan [0.42] undergoes with papain an intrinsic viscosity drop of 66% within 48 h at 25°C, whilst with 0.6 M HCl at 25°C it would take 20 days to reach the same result (Terbojevich *et al.*, 1992).

The hydrolytic action of papain lowers the average molecular weight of chitosan of at least one order of magnitude, and therefore provides low molecular weight chitosans which are especially sought for use in the medical and pharmaceutical areas.

It would seem that papain cleaves the chitosan chains at defined positions identified by particular sequences of GlcNAc and $GlcNH_2$. Those chitosans which contain long sequences of GlcNAc, 3 days after contact with papain, become opalescent due to aggregation of said fragments if their concentration is high. Nevertheless, the opalescent samples, once freeze-dried and redissolved, are clear and have the correct intrinsic viscosity. This indicates that papain does not promote formation of chemical groups capable of giving rise to permanent cross-linking or similar irreversible reaction.

The anomalous behavior of the solutions of certain chitosans reflects the variability of the raw material and the parameters adopted for its processing. Previous works have indicated that it is difficult to correlate the extent of aggregation with the chemical composition of the chitosans (Anthonsen *et al.*, 1994). For instance, of three chitosans from the same producer, differing for their degrees of acetylation, 0.62, 0.79, and 0.96, only the second gave aggregates (Matsumoto *et al.*, 1991).

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