

# A study by analytical ultracentrifugation on the interaction between lysozyme and extensively deacetylated chitin (chitosan)

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Analytical ultracentrifugation (sedimentation velocity and sedimentation equilibrium) has been used for the first time to study the extent of complex formation between lysozyme and a chitosan extensively deacetylated to two levels ("C99", 99% deacetylated and "C99.9", 99.9% deacetylated) at pH=4.5 and ionic strength, I=0.17 M. Sedimentation velocity experiments on mixtures of the two reactants (1 mg/ml chitosan and 0.1–0.6 mg/ml lysozyme) give clear evidence of an interaction between chitosan C99 and lysozyme and an indication of an interaction between lysozyme and chitosan C99.9. Sedimentation equilibrium molar mass analyses using the 'M\* function' (Creeth & Harding, 1982) confirmed a clear interaction for C99:lysozyme and a weaker interaction between C99.9 and lysozyme. Further analyses by means of the 'omega function' (Nichol *et al.*, 1976) to determine the fraction of free lysozyme in mixtures with defined total concentration indicated that essentially no free lysozyme (weight fraction <0.05) is present in the mixtures, irrespective of whether the concentration is monitored refractometrically or whether only the lysozyme constituent is monitored by means of the absorption optics. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

Enzymatic degradation of chitin by lysozyme reflects the hydrolytic specificity of this enzyme for  $\beta$ -1,4 linkages between the *N*-acetylglucosamine (GlcNAc) units that comprise the polysaccharide. Chitosan, a partially or fully deacetylated form of chitin, is found (to date only in partially deacetylated form) in the cell walls of fungi (Araki & Ito, 1975; Davis & Bartnicki-Garcia, 1984) which may conceivably have affected the deacetylation as a defence mechanism against hydrolytic attack by enzymes such as lysozyme (Araki & Ito, 1975). Lysozyme has six subsites, in the active cleft, each recognizing a sugar unit in a hexasaccharide sequence, designated conventionally with the letters A

to F. Interaction of GlcNAc residues with subsites C and E of lysozyme is required for catalysis of partially *N*-acetylated oligomers (Amano *et al.*, 1977; Amano & Ito, 1978) Nordtveit *et al.* (1994) concluded that hexamers containing three, four or more acetylated units contribute mostly to initial degradation rate when lysozyme degrades partially *N*-acetylated chitosans, while chitosans with a degree of acetylation higher than 95% were not hydrolysed at all by lysozyme. However, the effect of the GlcNAc residues on the formation of the enzyme-substrate complex with partially *N*-acetylated chitosans has not been studied.

A conclusion drawn from <sup>1</sup>H-NMR studies at pH 4.5 is that the binding of chitotrioses is weakened by the absence of acetyl groups on the residues located at subsites A and B of lysozyme (Fukamizo *et al.*, 1992), with no detectable interaction between the fully deacetylated trimer and lysozyme. Such a finding seemingly correlates with the fact that the primary amino groups of chitosan have an effective pK<sub>a</sub> of 6.2–7.0 (Park *et al.*, 1983; Domard, 1987; Terbojevich *et al.*, 1989; Anthonsen & Smidsrød, 1995).

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To address further the question of the effect of the acetyl group on lysozyme's interaction with extensively deacetylated chitin, analytical ultracentrifugation has been selected as a direct method of establishing the extent of complex formation between lysozyme and a chitosan extensively deacetylated to two levels, with respectively only 1% and 0.1% of the glucosamine residues remaining *N*-acetylated. Sedimentation equilibrium (using general analysis of weight average molar masses) and sedimentation velocity experiments both indicate that the interaction may be weaker for the 0.1% acetylated chitosan. Sedimentation equilibrium using the omega function approach for the analysis of interacting systems rather surprisingly failed to detect the presence of any significant amount of free lysozyme under the conditions examined (pH 4.5, I 0.17 M).

## MATERIALS AND METHODS

### Reactant solutions

#### *Lysozyme*

Crystalline hen egg-white lysozyme obtained from Sigma Chemical Co., St. Louis, MO, USA was dissolved directly in acetate-chloride buffer, pH 4.5, I 0.17M (0.15 M NaCl – 0.02 M sodium acetate, pH adjusted with acetic acid) and dialyzed exhaustively against more of the same buffer. Concentrations of enzyme solutions were determined spectrophotometrically on the basis of an extinction coefficient of 2635 ml g<sup>-1</sup> cm<sup>-1</sup> at 280 nm (Sophianopoulos *et al.*, 1962; Sophianopoulos & Van Holde, 1964)

#### *Chitosan*

A commercial chitosan (Pronova Biopolymer AS, Drammen, Norway) was extensively deacetylated to produce two hybrids ("C99" and "C99.9"). Chitosan C99 had a degree of deacetylation of 99.0% and Chitosan C99.9 a degree of deacetylation of more than 99.9%, as determined by high field NMR spectroscopy (Vårum *et al.*, 1991). The same buffer was used for both chitosans.

### Analytical ultracentrifugation

In a preliminary sedimentation velocity experiment, solutions of chitosan C99 at 1 mg/ml supplemented with lysozyme (at 0.1, 0.2 and 0.3 mg/ml) were subjected to centrifugation at 50 000 rev/min and 20.0°C in a Beckman Optima XL-A analytical ultracentrifuge to allow measurement of the constituent sedimentation coefficient for lysozyme from absorption scans at 280 nm. A second experiment with chitosan C99 (1 mg/ml) control and a C99-lysozyme mixture (1 mg/ml polysaccharide – 0.6 mg/ml enzyme) in 30 mm monosector cells with wedge windows was conducted at 44 000 rev/min and

20°C in the AN-E rotor of a Beckman Model E ultracentrifuge in order that the Schlieren optical system could be used to compare the sedimentation behaviour of the mixture with that of chitosan. A similar set of experiments was then performed with the >99.9% deacetylated chitosan C99.9.

In sedimentation equilibrium experiments on C99 (1 mg/ml) and C99/lysozyme, mixtures (1 mg/ml chitosan and either 0.3 or 0.6 mg/ml enzyme) the six-channel cell was first loaded into the AN-D rotor of the Model E ultracentrifuge and subjected to centrifugation at 15000 rev/min and 20°C for 48 h to obtain an interferometric optical record of the equilibrium solute distribution {expressed in fringe displacement units  $j(r)$  relative to the meniscus ( $r=a$ ), as a function of radial distance  $r$  from the centre of the rotor}. The cell was then transferred to the rotor of the XL-A ultracentrifuge and re-subjected to centrifugation at 15000 rev/min for a further 36 h to obtain the corresponding equilibrium distributions for the mixtures in terms of absorbance at 280 nm. Rayleigh interferograms also recorded sedimentation equilibrium distributions for similar mixtures in which the polysaccharide component was the >99.9% deacetylated chitosan C99.9 (0.5 mg/ml).

The Rayleigh interference patterns have been captured using an Ultrascan XL enhanced laser densitometer (LKB/Pharmacia Instruments, Bromma, Sweden) with appropriately modified software. The fringe shift versus radial position data were evaluated with a Fourier Series PASCAL algorithm 'ANALYSER' to give an accurate record of fringe displacement relative to the meniscus,  $j(r)$ , as a function of radial displacement,  $r$  (Harding & Rowe, 1988; Rowe *et al.*, 1992). Because the experiments were of the low or intermediate type (Van Holde & Baldwin, 1958; Creeth & Harding, 1982) a value of  $J(a)$ , the interferometric concentration at the meniscus, was a prerequisite for converting the measured fringe differences,  $j(r) = J(r) - J(a)$ , to absolute values for the required description of the sedimentation equilibrium distribution,  $J(r)$  as a function of radial distance,  $r$ . On the basis of the meniscus radial position ( $a$ ) and the measured distribution, a plot of  $j(r)/(r^2 - a^2)$  versus  $\int_a^r r j(r) dr / (r^2 - a^2)$  was constructed to evaluate the interferometric meniscus concentration,  $J(a)$  as twice the ratio of the ordinate intercept to the limiting slope (Creeth & Harding, 1982). Both types of equilibrium distributions for mixtures were analyzed by means of the omega function (Millthorpe *et al.*, 1975; Nichol *et al.*, 1976; Jeffrey *et al.*, 1979) to estimate the proportion of lysozyme (the smallest macromolecular species) present at a selected reference total concentration, expressed in terms of Rayleigh fringes or  $A_{280}$ . Both types of sedimentation equilibrium distribution were also analyzed by the  $M^*$  function (Creeth & Harding, 1982) which provides a model-independent procedure of

obtaining the apparent weight average molar mass,  $M_{w,app}$ , for a given solute distribution at sedimentation equilibrium. The routine MSTAR (Harding *et al.*, 1992) recently converted from mainframe FTN77 to a novel interactive version in QUICKBASIC (Cölfen & Harding, 1996) was used. This routine also provides estimates of the local or "point" apparent point weight average molar masses  $M_{w,app}(r)$  as a function of radial position,  $r$  (or corresponding local total solute concentration,  $c(r)$ ) in the ultracentrifuge cell. The MSTAR routine was also used to determine the apparent and ideal molar masses of the chitosan prior to any interaction studies.

## RESULTS AND DISCUSSION

### Molar mass and apparent molar mass (at 1 mg/ml and 0.5 mg/ml) of the chitosan.

It is important to establish not necessarily the "ideal solution" (or zero concentration) molar mass of the chitosan but the apparent (weight average) molar mass at the concentrations employed (1 mg/ml for chitosan C99 and 0.5 mg/ml for C99.9) since at the concentrations employed in the mixtures this highly charged polyelectrolyte is likely to have an apparent molar mass considerably less than the ideal value (see, e.g. Harding *et al.* (1991) for a comparative study of the thermodynamic non-ideality of polysaccharides). A low speed sedimentation equilibrium experiment on the Beckman Model E analytical ultracentrifuge with the Rayleigh interference optical system was therefore performed on the chitosan (C99) at a loading concentration of 1 mg/ml. The apparent weight average molar mass at this loading concentration was determined from the  $M^*$  function using the procedure of Creeth & Harding (1982) where

$$M^*(r) = j(r) / \left\{ \Phi \cdot J(a)(r^2 - a^2) + 2\Phi \int_a^r r j(r) dr \right\} \quad (1a)$$

$\Phi$  is given by

$$\Phi = (1 - \bar{v}\rho_0)\omega^2/2RT \quad (1b)$$

where  $R$  is the gas constant,  $T$  the absolute temperature,  $\omega$  is the angular velocity,  $\bar{v}$  the partial specific volume {taken as 0.565 ml/g (Errington *et al.*, 1993)} and  $\rho_0$  the solvent density (Wills & Winzor, 1992) {taken as 1.0054 ml/g on the basis of measurements in an Anton-Paar density meter (Kratky *et al.*, 1973)}. The  $M^*$  function extrapolated to the cell base = the apparent weight average molar mass  $M_{w,app}$  at the cell loading concentration  $c^0$  (= 1 mg/ml) and a value of (33 000±3000) g/mol is obtained (Fig. 1a).

From a plot of the point or local average molar mass,  $M_{w,app}(r)$  versus the local concentration, (expressed in terms of fringe displacement units,  $J$ ) in the ultracentrifuge cell (Fig. 1b) it is possible to estimate the

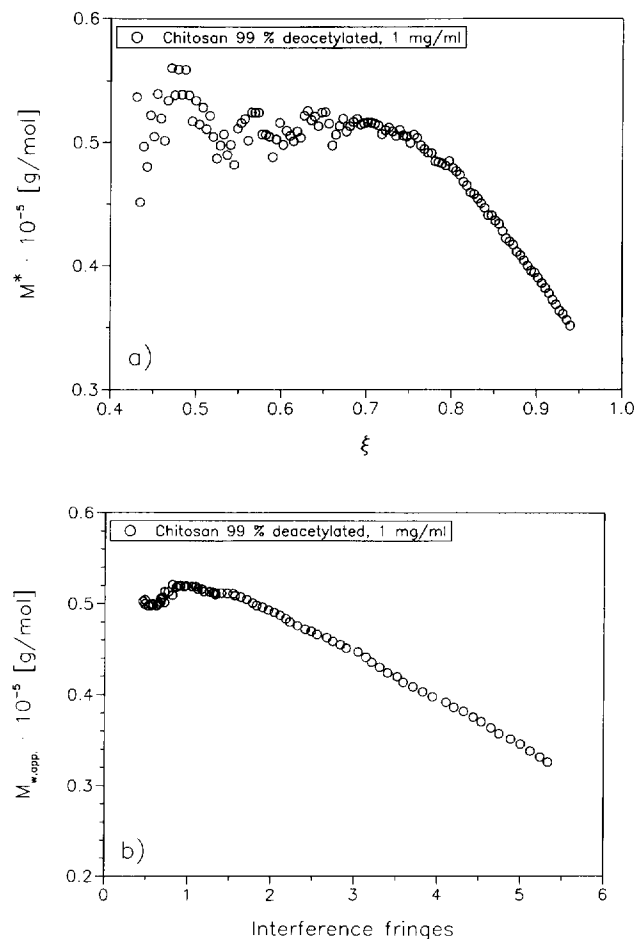


Fig. 1. Molar mass of chitosan (C99) at 1 mg/ml (pH=4.5,  $I=0.17$ ). (a)  $M^*$  (calculated via eq. 1) versus  $\xi = (r^2 - a^2)/(b^2 - a^2)$  with  $b$  = cell bottom,  $M_{w,app} = M^*$ . (b) Point  $M_{w,app}$  versus  $J(r)$ .

ideal weight average molar mass,  $M_w$  from the limiting value at zero concentration ( $J=0$ ) and a value of (52 000±5000) g/mol is obtained, corresponding to a weight average degree of polymerization,  $DP \approx 270$ . Assuming the nonideality is linear in the range 0–1 mg/ml, a value for the apparent molar mass at 0.5 mg/ml of (42 500±6000) g/mol is obtained. These results are summarized in Table 1. All these measurements were done on the chitosan C99: we make the assumption that the extra degree of deacetylation for C99.9 has not significantly affected either its molar mass nor its apparent molar mass at 0.5 mg/ml.

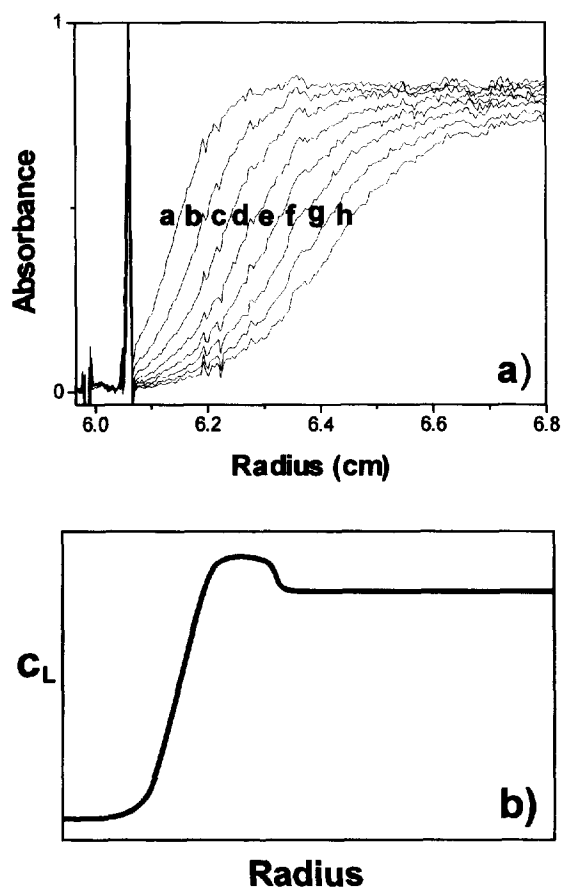
Table 1. Molar mass and apparent molar masses for the chitosans

Weight average molar mass $M_w$ (g/mol)	Apparent weight average molar mass, $M_{w,app}$ at 1.0 mg/ml	$M_{w,app}$ at 0.5 mg/ml
52 000±5000 (DP≈320)	33 000 + 3000	42 500±6000

DP: degree of polymerization.

### Sedimentation velocity of lysozyme-chitosan mixtures

From the preliminary sedimentation velocity experiment conducted in the XL-A ultracentrifuge on chitosan solutions supplemented with lysozyme (0.1–0.3 mg/ml) (corresponding to molar mixing ratios, [substrate]:[enzyme] of  $\approx 3:1$ – $1:1$ ) the weight-average sedimentation coefficient,  $s_{20,b}$ , deduced from the  $A_{280}$  profiles for the enzyme constituent (Fig. 2a) ranged between 1.65 and 1.85S. Correction of these sedimentation coefficients to standard conditions,  $s_{20,w}$  (Tanford, 1961) leads to values of 1.7–1.9S, which compare favourably with the sedimentation coefficient ( $s_{20,w}$ ) of 1.91 for lysozyme monomers (Sophianopoulos et al., 1962). At first sight it might therefore appear that lysozyme is the slower-migrating component of a noninteracting mixture. This is not, however, the case. First cause for doubt comes from the reasonably symmetrical

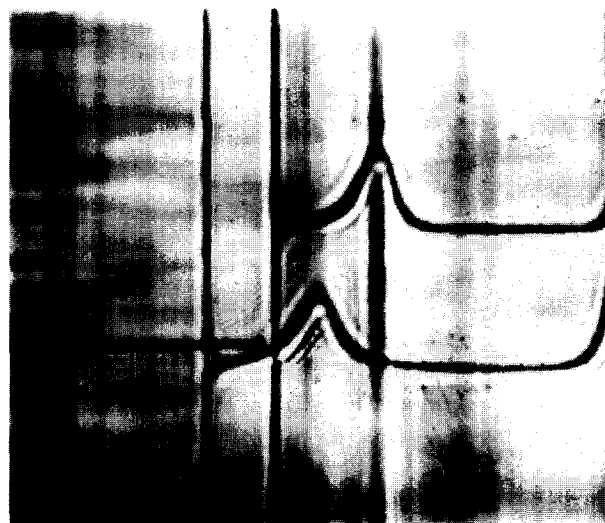


**Fig. 2.** Sedimentation velocity patterns for lysozyme in lysozyme-chitosan mixtures (pH=4.5,  $I=0.17$ ). Spectrophotometric sedimentation velocity patterns for the lysozyme component in a mixture of 99% deacetylated chitosan C99 (1 mg/ml) and lysozyme (0.3 mg/ml) centrifuged at 50 000 rev/min and 20°C in a Beckman Optima XL-A analytical ultracentrifuge (a) Experimental scans ( $A_{280}$ ) at a) = 40 min, b) = 60 min, c) = 80 min, d) = 100 min, e) = 120 min, f) = 140 min, g) = 160 min, h) = 180 min of ultracentrifugation (b) Theoretical form of the profile for a non-interacting mixture with lysozyme as the slower-migrating component.

nature of the sedimentation profile (Fig. 2a). Faster migration of the chitosan, which imparts considerable viscosity to the solution, should lead to the existence of a pronounced Johnston-Ogston effect (Johnston & Ogston, 1946; Nichol & Ogston, 1965) whereby the concentration of lysozyme in the trailing plateau exceeds its concentration in the mixture plateau (Fig. 2b): no such effect is observed experimentally (Fig. 2a).

The theorem of no-interaction is disproved completely from the second sedimentation velocity experiment in which Schlieren optics were used to record both the sedimentation of the chitosan control and the combined concentration gradient arising from the presence of both components. The  $S_{20,b}$  of the chitosan (C99) control is only 1.4S so lysozyme is therefore the slower component in the mixture experiments. For the mixture (1 mg/ml chitosan and 0.6 mg/ml lysozyme, corresponding to a molar ratio of  $\approx 1:1$ , assuming monomeric lysozyme) a sedimentation coefficient  $s_{20,b}$  of 1.9S is obtained, faster than that expected for either the chitosan or the lysozyme: because lysozyme is the faster-migrating component and hence moving in a relatively viscous chitosan environment (which should have slowed it down considerably, if uncomplexed) the  $s_{20,b}$  of 1.9S for the mixture (experiment 2) and 1.7–1.9S for its lysozyme constituent (experiment 1) confirms the existence of chitosan-lysozyme complex(es) in the polysaccharide-enzyme mixtures.

Inclusion of lysozyme in the chitosan C99 solution also has a dramatic effect on the form of the Schlieren pattern (Fig. 3). Whereas the boundary for chitosan



**Fig. 3.** Sedimentation velocity patterns for chitosan and a chitosan-lysozyme mixture (pH=4.5,  $I=0.17$ ). Schlieren patterns obtained for the 99% deacetylated C99 (upper profile) and a C99-lysozyme mixture (lower profile) centrifuged for 115 min at 44 000 rev/min in a Beckman Model E ultracentrifuge. The mixture comprised 0.6 mg/ml lysozyme and 1.0 mg/ml chitosan, the same as its concentration in the run on polysaccharide alone.

alone (upper profile) is relatively sharp because of the pronounced concentration-dependence of the sedimentation coefficient of a polyelectrolyte (Creeth & Pain, 1967; Fujita, 1975), that for the chitosan-lysozyme mixture is extremely broad (lower profile). Similar broad boundaries are seen for mixtures of the lysozyme with the almost fully deacetylated chitosan (C99.9), consistent again with an interaction although the sedimentation coefficients of the mixture and the chitosan are indistinguishable, implying the reaction is weaker. (It should be remembered that in any case, if any complex is present, an increase in mass which tends to increase the sedimentation coefficient could well be compensated for by an increase in asymmetry or hydration, which would tend to decrease the sedimentation coefficient.)

On the grounds that such boundary broadening for mixtures involving both degrees of deacetylation is characteristic of a reaction boundary reflecting coexistence of reactants in rapid association equilibrium (Gilbert & Jenkins, 1959) sedimentation equilibrium experiments have been undertaken in an attempt to characterize quantitatively the interaction of lysozyme with chitosan. We have used two approaches for analysing the solute distribution records at sedimentation equilibrium: the  $M^*$  function approach (Creeth & Harding, 1982), particularly suited for direct molar mass analysis, and the omega approach (Millthorpe *et al.*, 1975), the latter being particularly well suited for the analysis of potentially interacting systems (Wills & Winzor, 1992).

#### Sedimentation equilibrium: $M^*$ analysis of the complex

We repeat the  $M^*$  analysis described above for the chitosan now on the chitosan-lysozyme mixtures. These results, in terms of the effect of the mixture on the apparent weight average molar mass,  $M_{w,app}$  at the solute concentration loaded into the centrifuge cell, are summarized in Table 2.

#### Lysozyme complexing with chitosan C99

$M^*$  analyses were performed on the complexes at a (net) concentration of chitosan of 1 mg/ml mixed with two separate net concentrations of lysozyme of 0.3 mg/ml and 0.6 mg/ml (corresponding to molar mixing ratios,

[substrate]:[enzyme] respectively of  $\approx 1:1$  and  $1:2$ , assuming the lysozyme is in its monomeric form). Similar  $M_{w,app}$  values of  $(48\,000 \pm 2000)$  g/mol and  $(50\,000 \pm 3000)$  g/mol were obtained, well in excess of the apparent value for the chitosan C99 ( $33\,000 \pm 3000$ ) and the predicted apparent weight average values respectively of  $\approx 28\,700$  g/mol and  $\approx 26\,000$  g/mol for unreacting mixtures at the respective lysozyme concentrations of 0.3 mg/ml and 0.6 mg/ml. Even if the lysozyme were in the dimeric form, the predicted apparent weight average molar masses are only  $\approx 32\,000$  g/mol and  $\approx 31\,400$  g/mol respectively. These results are a conclusive proof of an interaction.

#### Lysozyme complexing with chitosan C99.9

Similar results are also obtained although the difference between the measured apparent molar masses is smaller than for case (i), even with an increased molar ratio of lysozyme: this is indicative of a weaker interaction. For a mixture of 0.5 mg/ml (net) chitosan concentration with 0.3 mg/ml lysozyme ([substrate]:[enzyme] $\approx 1:2$ ) an apparent weight average molar mass of  $(48\,500 \pm 2000)$  g/mol is obtained and with 0.6 mg/ml lysozyme ( $1:4$ ), a value of  $(45\,000 \pm 2000)$  g/mol. These compare respectively with predicted apparent weight average values for the unreacting mixtures involving lysozyme monomers of  $\approx 32\,000$  g/mol and  $\approx 27\,200$  g/mol respectively, or, if lysozyme dimers are involved,  $\approx 37\,400$  g/mol and  $\approx 35\,000$  g/mol respectively.

#### Sedimentation Equilibrium: omega analysis of the complex

A more precise description of the interaction process can be obtained by direct modelling of the solute distribution records at sedimentation equilibrium using the omega function. Results derived from omega analysis of the interferometric records of the sedimentation equilibrium experiments on mixtures are summarized in Fig. 4, where the ordinate is the omega function for lysozyme,  $\Omega_L(r)$ , defined (Nichol *et al.*, 1976) as

$$\Omega_L = [J(r)/J(r_F)] \exp[\phi_L M_L (r_F^2 - r^2)] \quad (2a)$$

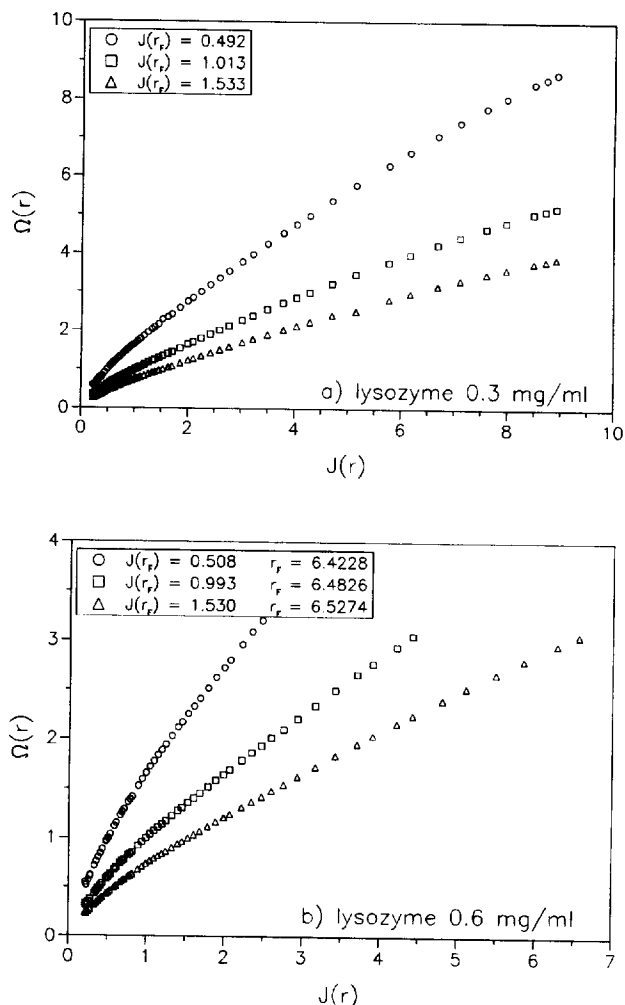
where

$$\phi_L = (1 - \bar{v}_L \rho_o) \omega^2 / (2RT) \quad (2b)$$

Table 2. Apparent molar masses for the chitosan-lysozyme mixtures

	$M_{w,app}$ (g/mol)	$M_{w,app}$ predicted for no interaction
1 mg/ml C99	$33\,000 \pm 3000$	-
1 mg/ml C99 + 0.3 mg/ml lysozyme	$48\,000 \pm 2000$	28 700(m) or 32 000(d)
1 mg/ml C99 + 0.6 mg/ml lysozyme	$50\,000 \pm 3000$	26 000(m) or 31 400(d)
0.5 mg/ml C99.9	$42\,500 \pm 6000$	-
0.5 mg/ml C99.9 + 0.3 mg/ml lysozyme	$48\,500 \pm 2000$	32 000(m) or 37 400(d)
0.5 mg/ml C99.9 + 0.6 mg/ml lysozyme	$45\,000 \pm 2000$	27 200(m) or 35 000(d)

m: lysozyme in monomeric form; d: lysozyme in dimeric form.



**Fig. 4.** Omega analysis of Rayleigh interferometric records of sedimentation equilibrium distributions for chitosan C99-lysozyme mixtures (pH = 4.5,  $I = 0.17$ ). Dependence of  $\Omega_L(r)$ , calculated via eq. 2 for the indicated values of  $J(r_F)$  from sedimentation equilibrium distributions (15 000 rev/min) for solutions of C99 (1 mg/ml) supplemented with (a) 0.3 mg/ml and (b) 0.6 mg/ml lysozyme: the ordinate intercept measures the fraction of uncomplexed lysozyme in a solution with total concentration  $J(r_F)$ .

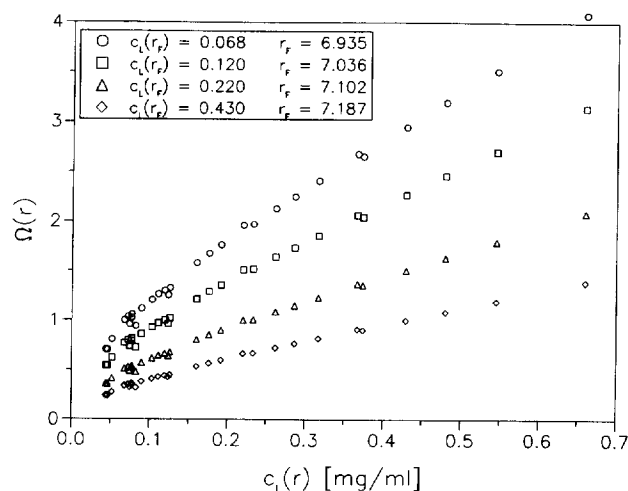
It relates the concentration,  $J(r)$  in Rayleigh fringes, at a radial distance  $r$ , to that,  $J(r_F)$  at a selected fixed radial distance,  $r_F$ , in terms of an expression based on a sedimentation equilibrium equation for a solute with molecular weight  $M_L$  that is centrifuged at temperature  $T$  and angular velocity  $\omega$ :  $v_L$ , the partial specific volume of the lysozyme was taken as 0.702 (Sophianopoulos *et al.*, 1962). The major point of interest in these plots of the concentration dependence of  $\Omega_L$  for the designated reference concentration is the ordinate intercept, which for a thermodynamically ideal system, defines the fraction of free lysozyme in the mixture with total concentration  $J(r_F)$ . Although any of these dependencies could reasonably be assigned a finite but very small value for the ordinate intercept, their collective forms are consistent with a common intercept. On the grounds that a

common, finite value for the fraction of free lysozyme in mixtures with differing total concentrations is incompatible with the law of mass action, we conclude that the various dependencies all extrapolate to the origin; and hence there is no evidence of free lysozyme.

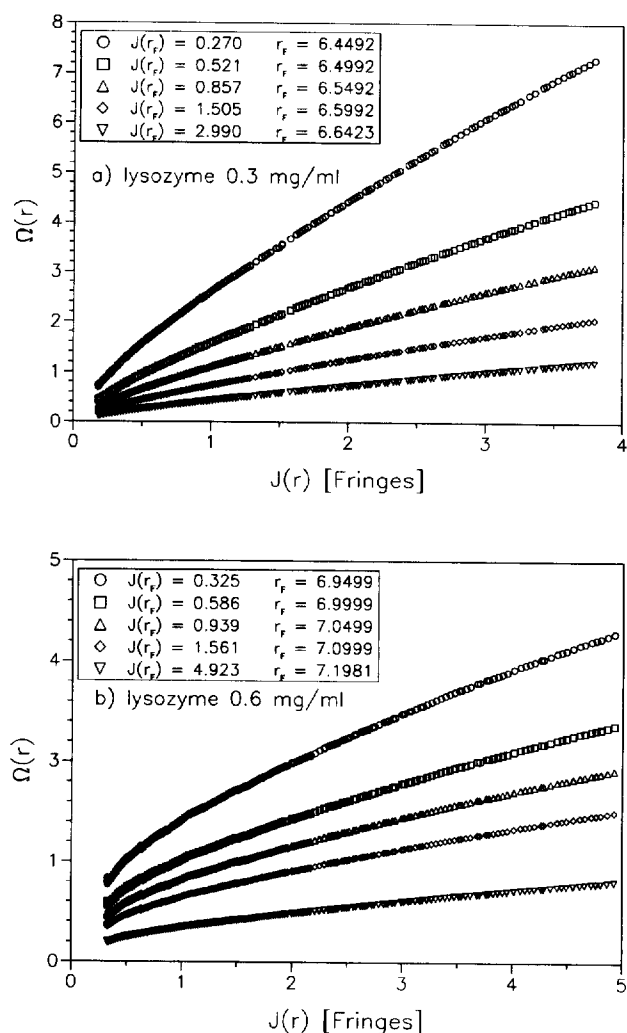
A more stringent test of this conclusion is provided by a corresponding analysis of the spectrophotometric record of the equilibrium distribution of the experiment with lower lysozyme concentration (eq. 2, with  $J(r)$  and  $J(r_F)$  replaced by  $A_{280}(r)$  and  $A_{280}(r_F)$ , respectively). In this situation, where only the lysozyme constituent is being monitored, the ordinate intercept describes the proportion of lysozyme in the free state. Such analysis (Fig. 5) clearly strengthens the conclusion that there is essentially no uncomplexed lysozyme present in the mixture, which thus comprises free chitosan and polysaccharide complexed with enzyme.

Qualitatively similar conclusions emanate from experiments with the almost completely deacetylated C99.9 chitosan preparation. Analysis of the Rayleigh interferometric optical record for a mixture containing 0.5 mg/ml chitosan and 0.3 mg/ml lysozyme (molar ratio  $\approx 1.2$ ), again signifies a very small magnitude for the ordinate intercept of the dependence of  $\Omega_L(r)$  upon concentration (Fig. 6); and hence the effective absence of free lysozyme in the chitosan-lysozyme mixture. The same is valid for the mixture with 0.6 mg/ml lysozyme.

In summary, the omega analyses have shown that the proportion of free enzyme remaining in all of the mixtures examined is very small ( $< 0.05$ ). These results



**Fig. 5.** Omega analysis of the spectrophotometric records of sedimentation equilibrium distributions for chitosan C99-lysozyme mixtures (pH = 4.5,  $I = 0.17$ ). Dependence of  $\Omega_L(r)$  for the indicated concentrations of lysozyme ( $c_L(r_F)$ ) upon  $c_L(r)$ , the constituent concentration of enzyme at radial distance  $r$ , from the absorption record ( $A_{280}$ ) of the sedimentation equilibrium distribution (15 000 rev/min) for a mixture of chitosan C99 (1 mg/ml) and lysozyme (0.3 mg/ml): the ordinate intercept measures the proportion of free lysozyme in a mixture with constituent lysozyme concentration  $c_L(r_F)$ .



**Fig. 6.** Omega analysis of Rayleigh interferometric records of sedimentation equilibrium distributions for chitosan C99.9–lysozyme mixtures (pH = 4.5,  $l = 0.17$ ). Dependence of  $\Omega_L(r)$ , calculated via eq.2 for the indicated values of  $J(r_F)$  from sedimentation equilibrium distributions (15000 rev/min) for a solution of deacetylated chitosan (0.5 mg/ml) supplemented with (a) 0.3 mg/ml lysozyme and (b) 0.6 mg/ml lysozyme: the ordinate intercept measures the fraction of uncomplexed lysozyme in a solution with total concentration  $J(r_F)$ .

cannot be used to comment on the relative strengths of the interactions between lysozyme and the two chitosan preparations which would be expected to differ on the basis of the different extents of acetylation and the known specificity of lysozyme and the two chitosan preparations, which would be expected to differ on the basis of the different extents of acetylation and the known specificity of lysozyme for *N*-acetylglucosamine residues.

#### Limitations of the omega analysis for strongly interacting systems

At the onset of this investigation we had anticipated demonstrating the power of sedimentation equilibrium ultracentrifugation for the quantification of relatively

weak interactions between dissimilar macromolecular solutes. That aim has to some extent been thwarted by the effectively complete extent to which lysozyme bound to the chitosan samples under the conditions examined. This study has therefore instead served to re-emphasize the uncertainty in the use of the omega function (Nichol *et al.*, 1976) to establish the proportion of smallest macromolecular species in a mixture with known total concentration under conditions where this fraction is minute or even zero (Masters & Winzor, 1981). To that end the present selection of several different loading concentrations to infer the essential absence of any free lysozyme in chitosan–lysozyme mixtures (Figs 4 and 6) certainly facilitated that decision. Indeed, the present application of the omega function analysis to the distribution of the small constituent (Fig. 5) is also a further development in that regard. The present study should serve as a general guide for future investigations of heterogeneous macromolecular associations in which the proportion of the smallest reacting macromolecular species remaining is less than (say) 0.05 of the total mixture concentration being examined.

These limitations of the omega analysis should not be construed as a criticism of the method, which ranks as one of the more sensitive procedures for the detection and quantitative characterization of equilibrium interactions between macromolecular species. Any attempt to delineate the system by interpreting the sedimentation distributions in terms of molar mass (via, for example, the  $M^*$  procedure) is less definitive although a qualitative or even quantitative characterisation of an interaction may be possible. Quantitative characterisation of the chitosan–lysozyme interaction requires studies of mixtures with far smaller reactant concentrations than those commensurate with detection by current optical systems available on analytical ultracentrifuges. Indeed the interaction of the intact polysaccharide with lysozyme could be quantified far more readily by techniques such as difference spectroscopy (Dahlquist *et al.*, 1966) or quantitative affinity chromatography (Winzor, 1992; Winzor & Jackson, 1993), or NMR-spectroscopy (Dahlquist & Raftery, 1969).

#### Boundary spreading of chitosan during sedimentation velocity of the mixtures

The only result of the present investigation that still requires rationalization is the Schlieren pattern for the chitosan–lysozyme mixture (Fig. 3, lower pattern) because the initial interpretation of its spread form as an indication of a reaction boundary is now untenable. Inasmuch as there is essentially no free lysozyme present, the pattern must reflect the sedimentation velocity behaviour of a non-interacting mixture of chitosan–lysozyme complexes and excess free chitosan. That realization leads to the conclusion that the spread form of the boundary for the mixture is providing a

more realistic indication of the heterogeneity of the chitosan preparation than the pattern for chitosan alone (Fig. 3, upper profile). Attachment of an occasional (see below) lysozyme molecule to the chitosan chain, presumably disrupts the regular rod-like structure adopted by this polyelectrolyte, and hence reduces the very pronounced concentration-dependence of the sedimentation coefficient that is responsible for hyper-sharpening of velocity patterns for polyelectrolytes (Creeth & Pain, 1967; Fujita, 1975).

### Capacity for binding

Proton NMR spectroscopy studies (Dahlquist & Raftery, 1969; Fukamizo *et al.*, 1992) on the binding of chito-oligosaccharides with lysozyme is strongly indicative that an acetyl group is necessary for binding of a lysozyme molecule to a trisaccharide *N*-glucosamine sequence: fully deacetylated isolated chito-trimers do not interact with lysozyme (Fukamizo *et al.*, 1992). A chitosan with a weight average molar mass of 52 000 g/mol has a weight average degree of polymerisation of  $\approx 270$  (based on a molar mass of 197 g/mol for the anhydroglucosaminehydrochloride monomer) whereupon a 1% extent of acetylation (as in C99) signifies an average of three randomly distributed *N*-acetylglucosamine residues per chitosan chain. On the basis of a maximal value of 0.05 for the free fraction of lysozyme in enzyme-C99 mixtures containing  $60 \mu\text{mol.l}^{-1}$  *N*-acetylglucosamine sites (Fig. 5), specific interaction of the enzyme with those sites would need to be governed by an association constant of at least  $10^5 \text{mol.l}^{-1}$ . Any such explanation based on such high association constants does not extend to the sedimentation equilibrium results for the mixtures of lysozyme and C99.9, which contain, on average, only a single *N*-acetylglucosamine residue per three polysaccharide chains. On the basis that the loaded mixtures would therefore have contained a three- to six-fold molar excess of enzyme ( $20$  and  $40 \mu\text{mol.l}^{-1}$  enzyme cf.  $6 \mu\text{mol.l}^{-1}$  *N*-acetylglucosamine residues), ordinate intercepts greater than 0.2 (Fig. 6a) and 0.3 (Fig. 6b) might reasonably have been predicted: much lower values of the ordinate intercept are indicated by the experimental results.

### CONCLUSION

Although we have shown that sedimentation velocity analyses gives a clear indication of an interaction between the 99% deacetylated chitosan and lysozyme, and stronger than that involving the 99.9% deacetylated material, the rather surprising observation was found from sedimentation equilibrium 'omega analysis' that there is virtually no free lysozyme ( $< 0.05$  by weight fraction) in *both* cases. Further investigations by other experimental methods are clearly required in order to

ascertain the molecular explanation of the present finding that the extent of lysozyme binding by extensively deacetylated chitosan preparations is too great for quantitative characterization by sedimentation equilibrium.

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