Complex Coacervation of Lysozyme and Heparin: Complex Characterization and Protein Stability

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Purpose. To characterize complex coacervates/flocculates of lysozyme and heparin in terms of binding stoichiometry and to determine the effect of complexation on protein structure and stability.

Methods. Insoluble lysozyme-heparin complexes were formed at pH 7.2 and the binding stoichiometry determined using a solution depletion method. Protein structure was determined by infrared spectroscopy and intrinsic fluorescence. Protein stability was evaluated using differential scanning calorimetry and followed in a 12-weeks storage stability study at 37°C.

Results. Binding stoichiometry between heparin and lysozyme was found to be dependent on ionic strength of the solution. At low ionic strength (I ≈0.01) about 11 lysozyme molecules could bind to a 17 kDa heparin chain, 3 to a 6 kDa chain, and less than 2 to a 3 kDa chain. At higher ionic strength (I ≈0.1), only 7 lysozyme molecules could bind to a 18 kDa heparin chain.. Above ionic strengths of approximately 0.32 M, no insoluble complexes were observed. Infrared spectroscopy and intrinsic fluorescence did not show any major changes in protein structure upon complexation to heparin. In contrast, differential scanning calorimetry showed a large decrease in the melting temperature of the protein, from 77°C to 61°C. Moreover, after 12-weeks storage at 37°C, only 60% protein recovery was observed for the complexes, with no loss of protein for the uncomplexed protein.

Conclusions. Heparin has multiple binding sites for lysozyme, amounting to at most one lysozyme molecule per 3 disaccharide units of heparin. Complexation decreased lysozyme stability, suggesting that heparin has a higher affinity for the unfolded state than the native state. Similar destabilization may occur for other proteins upon interaction with highly charged polymeric compounds or surfaces.

KEY WORDS: complex coacervation; DSC; fluorescence spectroscopy; FTIR; protein stability.

INTRODUCTION

Complex coacervation is the binding of two oppositely charged molecules, often both of them macromolecules, resulting in a phase separation between the complex and the bulk solution (1). In some cases, the complexation results in a precipitate, known as a complex flocculate. Complexation coacervation has been used in the formulation of various drug delivery systems (2–5), where the protein may be either one of the polyions in the complex coacervation processes or the intended drug compound. It has also been used in protein separation processes (6). However, little attention is paid to the effect of complex coacervation on the physicochemical stability of the protein, even though it is known that complex coacervation may result in protein denaturation (1). Moreover, it is known that proteins may be destabilized by adsorption to highly charged surfaces (7).

Various proteins are known to form complexes with glucosaminoglycans (GAGs) like heparin, for example, alcohol dehydrogenase (8), lipoprotein lipase (9), and acidic fibroblast growth factor (10). This binding often results in an increase in thermal stability. However, the above-mentioned proteins all have a specific heparin binding site. This prompted us to investigate the potential of using heparin as an excipient for proteins that do not have a well-defined heparin binding site, but which do form a complex (coacervate) upon addition of heparin. The model protein in our study was lysozyme, a 14 kDa protein with a pI of ~10.5. It is known to interact with heparin between pH values of 2 and 10, forming a flocculate (11). Analysis of the protein surface reveals a close to homogeneous distribution of positive charges over the surface, suggesting that the interaction with heparin does not occur at a very specific site on the lysozyme molecule. In the human body, lysozyme is known to interact with chondroitin sulfate, but the function of this interaction is as yet unknown.

MATERIALS AND METHODS

Materials

Heparin sodium salt fractions from porcine intestinal mucosa with molecular weights of 3, 6, and 17 kDa (data provided by supplier), hen egg white lysozyme, triethylamine and all buffer salts were obtained from Sigma (St. Louis, MO, USA). MilliQ water (Millipore, Billerica, MA, USA) was used in all preparations.

Binding Stoichiometry

Upon mixing of lysozyme and heparin, insoluble complexes are formed. Assuming that at excess protein concentration all heparin molecules are complexed and irreversibly precipitate, and that the concentrations are high enough above the affinity constant such that all (major) binding sites are saturated, the average number of lysozyme molecules per heparin chain can be calculated using formula 1:

$$\frac{[\text{lysozyme}]_{\text{free}}}{[\text{lysozyme}]_{\text{initial}}} = 1 - n * \frac{[\text{heparin}]_{\text{initial}}}{[\text{lysozyme}]_{\text{initial}}} \qquad (1)$$

in which $[\text{lysozyme}]_{\text{free}}$ is the concentration of lysozyme in the supernatant, $[\text{lysozyme}]_{\text{initial}}$ and $[\text{heparin}]_{\text{initial}}$ are the starting concentrations of lysozyme and heparin, respectively, and n is the average number of lysozyme molecules per heparin chain.

To determine the stoichiometry, various amounts of a heparin were added to a fixed amount of lysozyme (2.4 mg/ml) in 10 mM Tris buffer, pH 7.2, with a variable ionic strength. An insoluble, white complex was formed immediately, and the mixture was allowed to incubate for at least one hour before the insoluble material was pelleted using centrifugation (18,000 rpm for 10 min). The protein concentration in the supernatants was subsequently determined by size-exclusion chromatography (Tosoh Biosep TSK-gel

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G2000SW$_{XL}$, Stuttgart, Germany) using a LC-Module 1 (Waters, Milford, MA, USA). The running buffer consisted of 10 mM Tris buffer pH 7.2 with 500 mM NaCl, at a flow rate of 0.7 ml/min. Sample (75–100 μl) was injected and the protein detected using a UV detector set to 280 nm.

Ionic Strength Required for Dissociation

The ionic strength required for dissociation was determined by monitoring the scattering of a suspension of complexes as a function of ionic strength. To this purpose, the optical density at 500 nm was monitored (Cary 1-Bio, Palo Alto, CA, USA) upon addition of increasing amounts of a 4 M NaCl solution (also containing 10 mM Tris pH 7.2) to 8:1 heparin-lysozyme complexes (2.4 mg/ml protein) in 10 mM Tris pH 7.2.

Fourier-Transform Infrared Spectroscopy

Infrared spectra were acquired on a Bomem MB-100 series FTIR spectrometer (Bomem, Quebec, Canada) in transmission mode using calcium fluoride windows with a predrilled 6 μm depression (BioTools, Wauconda, IL, USA). Lysozyme solutions and 5:1 lysozyme-heparin complexes of at least 20 mg/ml protein concentration were analyzed by co-adding 1024 scans at 4 cm^{-1} resolution. The spectra of an appropriate blank solution (without the heparin) and of water vapor were subtracted using previously established criteria (12). Subsequently, the second derivative was determined in the Amide I region using a second-order 9-point Savitzky-Golay derivative function. For practical purposes, the second derivative spectra were multiplied by –1. For some samples, the spectral area-overlap was determined using the procedure described by Kendrick *et al.* (13).

Fluorescence Spectroscopy

Fluorescence emission spectra of lysozyme and lysozyme-heparin complexes were acquired on a Spex Fluorolog 3-22 fluorescence spectrometer (Jobin-Yvon Horiba, Longjumeau, France) equipped with a 450 W Xenon lamp. Ten scans were averaged using an excitation wavelength of 295 nm to selectively excite tryptophan residues, and a protein concentration of approximately 130 mg/l was used. The scan range was 300–450 nm, the step size 0.5 nm, the measurement time per step 0.1 s, and the slits were 2 and 4 nm for the excitation and emission slits, respectively. A spectrum of an appropriate blank solution (without the lysozyme) was subtracted. Note that the spectra are not corrected for instrumental characteristics.

Differential Scanning Calorimetry

Differential scanning calorimetry was performed on a VP-DSC (Microcal, USA). Thermograms were obtained by scanning at 90°C per hour from 15 to 100°C (gain 1, zero feedback mode). All samples were first degassed for 5 min using a vacuum. The protein concentration was approximately 5 mg/ml, with a 5:1 lysozyme-heparin ratio for the complexes. Origin software was used to determine melting temperatures and unfolding enthalpies. The raw thermograms were all corrected for the baseline signal, normalized for concentration, and subsequently fitted using a non-2-state unfolding model.

Long-Term Stability Study

Lysozyme-heparin (5:1) complexes and a lysozyme control in 10 mM Tris buffer pH 7.2 (also containing 0.02% sodium azide to prevent microbiological contamination) were stored in eppendorf tubes at 37°C in a heating cupboard for 12 weeks. The starting protein concentration was approximately 0.5 mg/ml. After 12 weeks, the complexes were dissociated by the addition of sodium chloride up to a final concentration of 1 M. Any remaining insoluble material was removed by centrifugation (18,000 rpm, 5 min). A control lysozyme solution was treated equally. The supernatant was subsequently analyzed by heparin-affinity (HAC) and size-exclusion (SEC) chromatography on a Waters LC-Module 1 with UV detection at 215 nm for HAC and 280 nm for SEC. For the HAC a Tosoh Biosep TSK-gel Heparin-5PW column was used at a flow rate of 1 ml/min. The running buffer consisted of 10 mM phosphate buffer, pH 7.4, and the protein was eluted by a gradient from 0 to 800 mM NaCl in the buffer in 35 min. For SEC, a Tosoh Biosep TSK-gel G2000SWXL was used at a flow rate of 0.5 ml/min. The running buffer consisted of 50 mM phosphate buffer, pH 7.4, also containing 400 mM NaCl and 0.1% triethylamine. For both HAC and SEC, 25 μl of sample was injected in triplicate.

RESULTS AND DISCUSSION

Binding Stoichiometry with Heparin

At sufficiently high protein concentrations and low ionic strength, one may assume that addition of small amounts of heparin results in complete saturation of all binding sites on heparin. High micromolar concentrations of lysozyme (~170 μM) were used to fulfill this saturation criterion. Under these conditions a white precipitate was observed, which is the insoluble heparin-lysozyme complex. Table I shows a summary of the average number of lysozyme molecules per heparin chain as determined using formula 1, while Fig. 1 shows two graphs obtained by measuring the free lysozyme concentration in the supernatant. The obtained curves are close to linear, and above a critical heparin concentration no lysozyme could be observed in the supernatant for the 6 and 17 kDa heparin chains. This suggests that the assumptions leading to formula 1 are indeed correct in this concentration range.

The data shows that about 11 lysozyme molecules can bind to a heparin chain of approximately 17 kDa at low ionic strength (0.01 M), with a reduction in binding sites to 8 and 7 at ionic strengths of 0.05 and 0.1 M, respectively. A 6 kDa chain apparently has slightly less than 3 binding sites (2.7), and a 3 kDa chain approximately 1.5 binding sites. Assuming

Table I. Binding Stoichiometry Between Lysozyme and Heparin Under Various Solution Conditions at pH 7.2 as Determined by Solution Depletion Studies and UV Spectroscopy (n = 3)

Buffer	Ionic strength	Heparin type	Mole lysozyme per mole heparin
10 mM Tris	0.01	17 kDa	11.3 (±0.2)
		6 kDa	2.7 (±0.1)
		3 kDa	1.5 (±0.0)
10 mM Tris + 40 mM NaCl	0.05	17 kDa	8.1 (±0.1)
10 mM Tris + 90 mM NaCl	0.1	17 kDa	7.1 (±0.2)

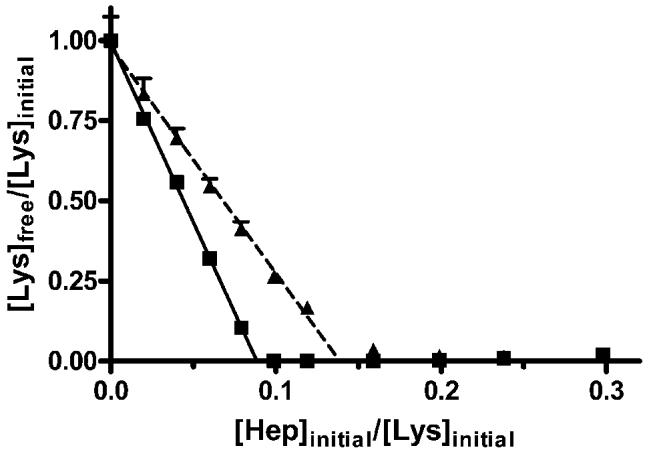

Fig. 1. Solution depletion graphs of lysozyme-heparin complex coacervates obtained by addition of known amounts of heparin to a lysozyme solution and subsequent protein concentration determination in the supernatant using UV spectroscopy. The observed points correspond to the depletion in 10 mM Tris buffer pH 7.2 (■, solid line) and in 10 mM Tris buffer pH 7.2 with 90 mM NaCl (▲, dotted line). The lines are linear curves fitted through the points at which the solution depletion is not yet completed.

an almost complete sulfatation of heparin, a 17 kDa heparin chain consists of approximately 30–32 disaccharide units. Thus, each lysozyme molecule requires about 3 disaccharide units to bind. Three disaccharide units of heparin have a length of approximately 2.6 nm (14), while lysozyme can be approximated by a sphere with a diameter of 3.2 nm (15). This suggests that there is some overlap of lysozyme molecules on the heparin binding sites. The overlap appears to be smaller for the 6 and 3 kDa fractions, which consist of about 10 and 5 disaccharide units, respectively.

Insoluble complexes were also observed at equimolar concentrations of heparin 17 kDa and lysozyme, with no evidence of any lysozyme in the supernatant. To investigate whether these complexes are equimolar complexes, additional lysozyme was added to the supernatant of the original mixture. New insoluble complexes were formed, indicating the presence of non-complexed heparin in the supernatant. This procedure could be continued until the total amount of added lysozyme resulted in a ratio of approximately 8:1 lysozyme/heparin. Apparently, multiple lysozyme molecules preferentially fill up one heparin chain first, rather than distribute between the chains. This apparent *sequential* rather than *parallel* filling of the heparin chain suggests that binding of the first lysozyme molecule is energetically less favorable than binding of subsequent lysozyme molecules. A possible explanation is that the first lysozyme molecule to bind to heparin requires a structural change of the heparin chain, whereas binding of subsequent molecules require less or no further changes of the heparin conformation. Note that this behavior is in contrast to most typical complex coacervation processes, which usually show a clear optimum ratio for complex formation (1).

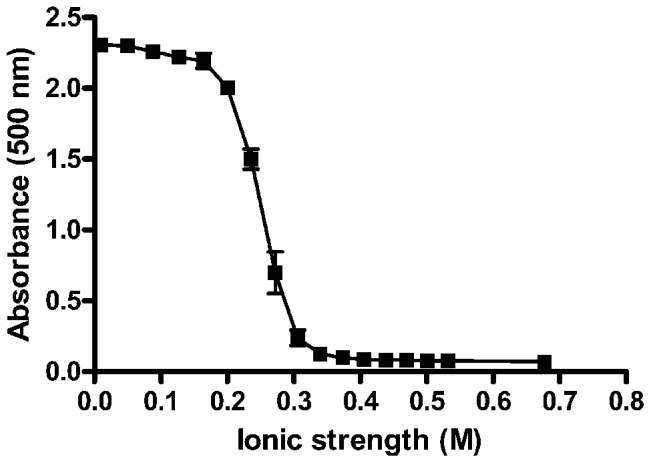

Fig. 2. Optical density measurements at 500 nm of dispersions of lysozyme-heparin complexes (n = 3) at varying ionic strength in 10 mM Tris buffer pH 7.2. The curve through the points is made by smoothed connections between the actual data points and is for guiding purposes only.

Influence of Ionic Strength, and Ionic Strength Required for Dissociation

Table I shows that at increasing ionic strength the number of binding sites on heparin decreases. This is typical be-

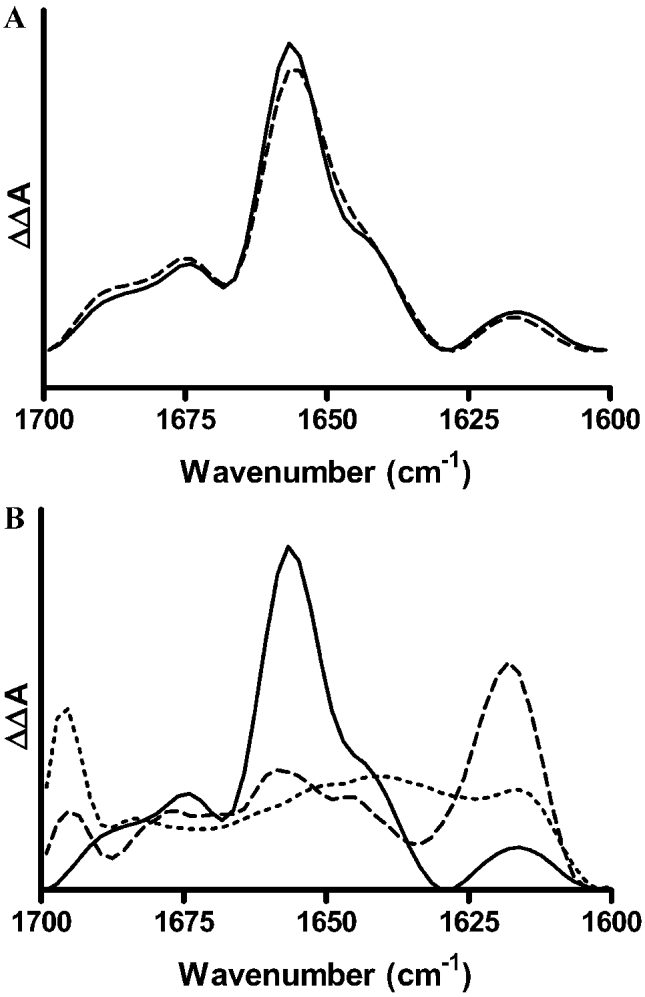

Fig. 3. (A) Inverted second derivative FTIR spectra of lysozyme (solid line) and lysozyme-heparin complexes (dashed line) in 10 mM Tris buffer pH 7.2. (B) Inverted second derivative spectra of lysozyme (solid line), heat-aggregated lysozyme (dashed line), and the insoluble solid material after 3-months storage of lysozyme-heparin complexes (dotted line).

Table II. Area Overlap of Second Derivative FTIR Spectra (n = 3)

Sample comparison	Area overlap
Lysozyme vs. lysozyme	0.98 (±0.01)
Complex vs. complex	0.98 (±0.00)
Lysozyme vs. complex	0.95 (±0.01)*

* Differs statistically significant ($p < 0.05$) from the other area overlap values.

havior for binding that is governed through electrostatic interactions, and suggests that van der Waals interactions are of only minor importance in the binding.

The ionic strength required to "dissolve" the complexes was determined using UV spectrometry. Figure 2 shows the graph constructed from the optical density measurements as a function of ionic strength. Apparently, about 0.32 M NaCl is required to "dissolve" the complexes. It should be noted that this does not necessarily imply that the complexes are dissociated completely, as soluble complexes may be present. Heparin-affinity chromatography showed that lysozyme elutes at about 0.35 M NaCl (data not shown), which is slightly higher than the value obtained from UV spectrometry.

Structural Analysis of the Protein in the Complexes

The effect of complexation on the protein structure was determined using FTIR and intrinsic fluorescence. Figure 3A shows the inverted second derivative FTIR spectra of lysozyme free in solution and in complexes with heparin. The area of overlap (Table II) between lysozyme and lysozyme-heparin complexes is close to 1 and does not differ much, allbeit statistically significant, from the in-sample area of overlap. This indicates that only very small changes in the secondary structure occur upon complexation.

The normalized intrinsic fluorescence spectra are shown in Fig. 4. Upon complexation, there is a minor blue shift (1 nm) of the fluorescence, as well as a small reduction in the fluorescence intensity. The latter may be partially caused by scattering; scattering is significant as evidenced by the high "fluorescence" intensity around 300 nm. This suggests, in turn, that there are only minor changes in the tertiary structure of lysozyme upon complexation to heparin.

Differential scanning calorimetry showed a large difference in thermal behavior between complexed and free lysozyme (Fig. 5, Table III). The DSC thermogram of lysozyme (Fig. 5B) could be fitted using one single transition with a

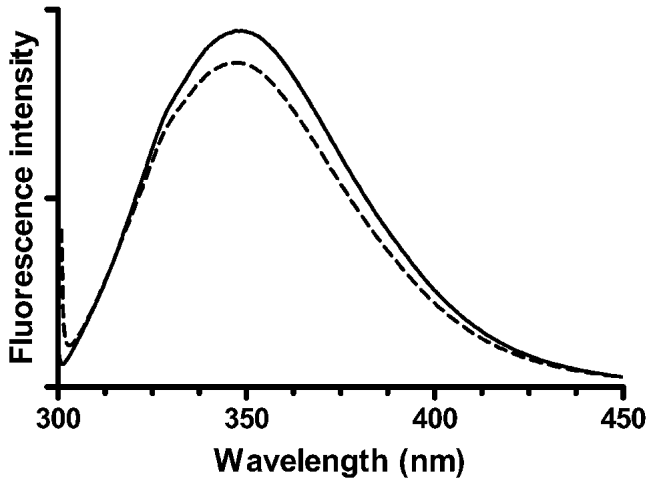

Fig. 4. Normalized intrinsic fluorescence spectra of lysozyme (solid line) and lysozyme-heparin complexes (dashed line) in 10 mM Tris buffer pH 7.2.

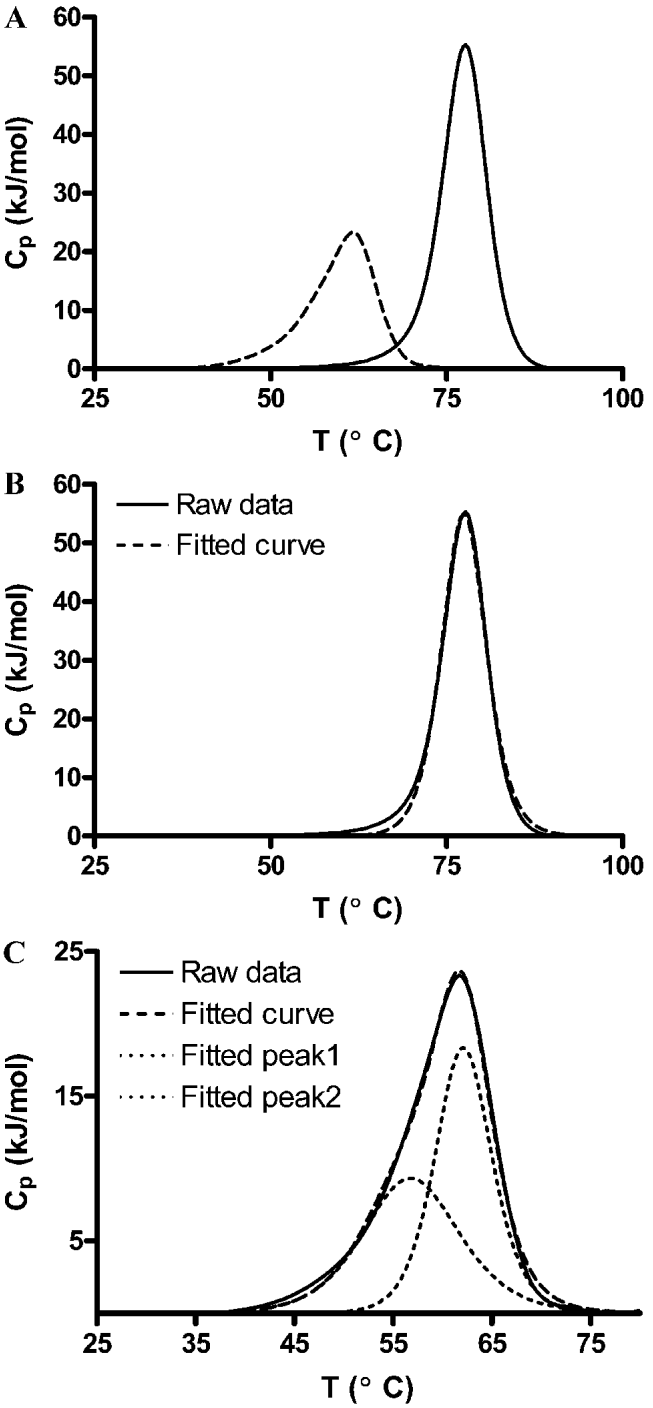

Fig. 5. (A) Normalized DSC thermograms of lysozyme (solid line) and lysozyme-heparin complexes (dashed line) in 10 mM Tris buffer pH 7.2. (B) Fitted DSC thermogram of lysozyme. The original data is shown as a solid line, whereas the fitted curve is shown as a dashed line. (C) Fitted DSC thermogram of lysozyme-heparin complexes. The original data is shown as a solid line, whereas the fitted curve is shown as a dashed line, and the two fitted peaks as dotted lines.

Table III. Calorimetric Data Obtained from Curve-Fitting DSC Thermograms of Lysozyme and Lysozyme-Heparin Complexes in 10 mM Tris Buffer, pH 7.2

Sample	Melting temperature (°C)	Enthalpy of unfolding (kJ/mol)
Lysozyme[a]	77.53 (±0.07)	461 (±7)
Complexes[b]	57.1 (±0.2)	135 (±8)
	62.0 (±0.4)	118 (±14)

[a] n = 4.
[b] n = 3.

melting temperature (T_m) of approximately 77.5°C and an overall unfolding enthalpy of 461 kJ/mol. The ratio of the calorimetric and van't Hoff enthalpy (data not shown) was close to 1, suggesting that lysozyme unfolds as a single cooperative unit. Fitting the curve using a 2-state model expectedly resulted in an almost identical T_m and unfolding enthalpy (data not shown).

In contrast to the plain lysozyme solution, the thermogram of the complexes (Fig. 5C) was best fitted using two peaks, with calculated T_m's of about 62 and 57°C and a total unfolding enthalpy of 253 kJ/mole. Also, a non-2-state model had to be applied to achieve a reasonable fit of the data. However, our knowledge on the exact structure of the complexes is too limited to speculate on the nature of these two unfolding peaks, and this is also beyond the scope of this paper. Attempts to determine the melting temperature of the plain lysozyme and the complexes using fluorescence spectroscopy failed. No clear transition was observed under our experimental conditions, probably due to the fact that lysozyme contains six tryptophan residues resulting in a complex fluorescence spectrum.

The results above show that heparin binding significantly decreases the physical stability of lysozyme. This observation is contrary to the increase in thermal stability of proteins with a specific heparin-binding site (8–10). Simulated DSC studies by Waldron and Murphy (16) have shown that a decrease in melting temperature upon ligand binding indicates a higher affinity of the ligand for the unfolded state than for the native state. This should then also be the case for the lysozyme-heparin binding.

Protein Stability upon Storage

The 12-weeks stability study indicated a significantly reduced physical stability of lysozyme when complexed to heparin (Fig. 6). Both size-exclusion (SEC) and heparin affinity (HAC) chromatography show a significant loss of lysozyme recovery in the solutions containing the complexes, amounting to approximately 40% protein loss. In addition, a precipitate was formed over time that did not dissolve upon addition of 1 M NaCl. Infrared spectroscopic analysis of this precipitate (Fig. 3B) showed that its secondary structure is markedly different from the native protein, and better resembles the structure of heat-aggregated lysozyme. Both the heat-aggregated lysozyme as well as the precipitate show bands typically assigned intermolecular β-sheets at 1695 and 1618 cm^{-1} (12) although the relative intensity of the two peaks differs between the two samples. This suggests that the insoluble, non-dissociable precipitate consists of aggregated

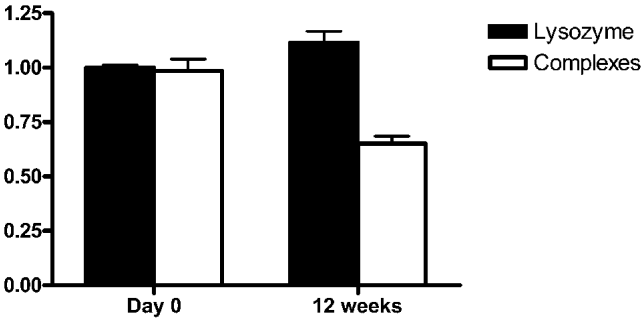

Fig. 6. Relative recovery of lysozyme as determined using SEC and HAC after 0 (fresh samples) and 12 weeks storage at 37°C (n = 6). Noncomplexed lysozyme is shown as a black bar, complexed lysozyme as a white bar.

protein, possibly still complexed with heparin. This reduced storage stability fits well with the observed reduced thermal stability in the DSC studies, whereas fluorescence and IR spectroscopy did not show any major changes in protein structure upon heparin binding.

In conclusion, this study shows that complex coacervation may potentially be deleterious to protein stability. The use of complex coacervation with a protein as one of the components must therefore be carefully evaluated in terms of the protein long-term stability. Moreover, this study also shows that structural analysis of the complex coacervates or flocculates may be insufficient to predict the protein's stability. However, differential scanning calorimetry or, if possible, thermal stability studies using spectroscopic techniques, are viable alternatives to long-term stability studies for screening protein (physical) stability.

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