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Improved activity and stability of lysozyme at the water/CH_2Cl_2 interface: enzyme unfolding and aggregation and its prevention by polyols

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Abstract

Protein inactivation and aggregation at the water/CH_2Cl_2 interface is one of the most detrimental events hindering the encapsulation of structurally unperturbed proteins into poly(lactide-*co*-glycolide) (PLG) microspheres for their clinical application as sustained release dosage forms. We have investigated the inactivation and aggregation of the model protein hen egg-white lysozyme at this interface and devised methods to prevent both events. When lysozyme was exposed to a large water/CH_2Cl_2 interface achieved by homogenization, lysozyme aggregation occurred. Fourier-transform infrared (FTIR) spectroscopic data demonstrated that the aggregates formed contained intermolecular β-sheets. The aggregates were of a non-covalent nature because they slowly dissolved in D_2O and the IR spectral bands typical for the intermolecular β-sheets disappeared at approximately 1617 and 1690 cm^{-1}. The observed loss in specific enzyme activity of soluble lysozyme was caused by the irreversible formation of an unfolded lysozyme species, which was found to be monomeric, and was able to leave the water/CH_2Cl_2 interface and accumulate in the aqueous phase. Polyols were, in a concentration dependent fashion, efficient in ameliorating lysozyme unfolding and aggregation. However, prevention of lysozyme aggregation and activity loss in the various samples were unrelated. Thus, polyols must work by more than one mechanism preventing the two events. For the first time, an excipient effect on the conformational stability of lysozyme has been excluded from contributing to the prevention of lysozyme unfolding and aggregation.

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Introduction

The sustained release of peptides and proteins is an attractive method to administer drugs to patients and to improve the blood level of these drugs, as they often have a short biological half-life (Lee 1995). Most sustained release systems are based on encapsulation of the protein pharmaceuticals into hydrophobic biocompatible polymers (Langer 1993). This requires that the polymer be dissolved in an organic solvent, typically CH_2Cl_2 or ethyl acetate (Van de Weert et al 2000a). The most commonly employed technique to achieve protein encapsulation into the polymer matrix is the so-called water-in-oil-in-water (w/o/w) technique (Van de Weert et al 2000a). Herein, an aqueous protein solution is emulsified into an organic solvent containing the dissolved polymer by probe sonication or homogenization. The resulting primary emulsion is then quickly transferred into a second aqueous phase containing an emulsifier, followed by removal of the organic solvent to effect polymer hardening, collection, washing, and lyophilization of the microspheres. Many of the steps in this process could be detrimental to protein conformation, but

the first emulsion step has been identified as being particularly denaturing in this context (Sah 1999a, b, c; Van de Weert et al 2000a, b).

Some success in developing suitable formulations has been achieved by the use of stabilizing excipients in the aqueous phase before emulsification (Cleland & Jones 1996; Sah 1999b; Van de Weert et al 2000b). In some instances pharmaceutical proteins have been delivered in an unaltered state using the w/o/w encapsulation method, thus preventing chemical and structural changes (Cleland & Jones 1996). However, for many proteins aggregation occurred during the microencapsulation by the w/o/w method (Lu & Park 1995a, b; Morlock et al 1997; Kim & Park 1999), which is pharmaceutically unacceptable because such aggregates might lead to immune reactions. Furthermore, a reduction in the specific biological activity has also been reported for lysozyme, the model protein chosen in this investigation, released from poly(lactide-*co*-glycolide) (PLG) indicating alternative protein inactivation pathways to aggregation (Ghaderi & Carlfors 1997). Some recent investigations have focussed in more detail on how the first emulsification step is linked to protein aggregation (Sah 1999a, b, c; Van de Weert et al 2000b). It has been clarified that interfacial protein adsorption and possibly unfolding is the initial step subsequently causing protein aggregation at the interface. For lysozyme, the resulting aggregates contain large amounts of intermolecular β-sheet structure, supporting the view that they are formed by the non-covalent association of unfolded molecules (Van de Weert et al 2000b). Also, some additives have been identified leading to a reduction in interface-induced protein aggregation and activity loss (Van de Weert et al 2000b). However, many effects are still not quite understood or have not been studied in detail. For example, it has not been established how excipients afford stabilization of proteins against interface-induced inactivation/aggregation. This could be due to competition by the excipients for the interface (Sah 1999b, c) and/or effects of the excipients on the intrinsic protein stability towards unfolding by preferential hydration (Timasheff 1998). Thermal denaturation experiments have not been performed under relevant conditions, and so the latter has never been excluded and has been suggested by some (Cleland & Jones 1996). Furthermore, while it is perceived that unfolded protein species are involved in the formation of non-covalent intermolecular aggregates, no indications for unfolded proteins have been found in most investigations (see Van de Weert et al 2000b and references therein). This implies that all unfolded protein molecules are incorporated into insoluble aggregates. However, one notable exception is the reported formation of an unfolded species of recombinant human growth hormone in the w/o/w method (Cleland & Jones 1996). Both proteins, human growth hormone (Kasimova et al 1998) and lysozyme (Pérez & Griebenow 2000), unfold reversibly in thermal denaturation experiments. Thus, it is conceivable that unfolded lysozyme species could be formed in the procedure also, thus far not detected. We have demonstrated that irreversible lysozyme unfolding indeed occurred upon exposure to the water/CH_2Cl_2 interface. We have investigated further the prevention of detrimental structural changes occurring to lysozyme when using various excipients.

Materials and Methods

Chemicals

Hen egg-white lysozyme (crystallized and lyophilized, EC 3.2.1.17) and all excipients were obtained from Sigma (St Louis, MO) and used as supplied. Anhydrous CH_2Cl_2 (purity 99.8 %) was obtained from Aldrich (St Louis, MO). All other chemicals were of analytical grade and from various commercial suppliers.

Emulsification procedure

The following parameters were defined as standard conditions. Lysozyme was dissolved in 10 mM phosphate buffer to form a stock solution of 25 mg mL^{-1} at pH 5.1 and 2 mL of this solution was placed inside a 20-mL scintillation vial. Experiments in D_2O were carried out at 34 mg mL^{-1} lysozyme concentration and with a pD (= pH read -0.4) value of 5.1. Excipients were co-dissolved with lysozyme at a 1:37 molar ratio of lysozyme-to-additive. After addition of 10 mL CH_2Cl_2 the mixture was homogenized at a speed of 30000 rev min^{-1} for 10 min using a VirTishear 260 W Tempest homogenizer (VirTis, Gardiner, NY), equipped with a 10-mm shaft and a micro-fine rotor-stator type generator for sample volumes of 1–250 mL.

A protein concentration of 25 mg mL^{-1} was chosen in the experiments employing the excipients to exclude conditions where protein aggregation would be inhibited by saturation of the interface with lysozyme causing prevention of aggregation (Sah 1999c). At higher concentrations some self-protection against aggregation was indeed observed (data not shown) and the effect of stabilizing additives might become obscured. When changing the volume ratio of aqueous-to-organic phase we found aggregation to be more pronounced the

smaller the aqueous phase volume. We selected to work with 2 mL aqueous phase and 10 mL CH_2Cl_2 because we observed sufficient aggregation and were able to isolate a sufficient sample to allow for FTIR measurements and other tests. We also studied the effect of pH value on lysozyme aggregation (data not shown). Lowering the pH value of the aqueous solution efficiently reduced lysozyme aggregation. Lysozyme is a very basic molecule with a high isoelectric point of approximately 11 (Roberts & Harris 1998). Increasing the pH value and thus decreasing electrostatic repulsion of lysozyme molecules promoted aggregation. Later on, a pH value of 5.1 was used in the study because sufficient aggregation occurred at this value, which is close to the pH optimum of lysozyme in aqueous solution.

Isolation of insoluble lysozyme aggregates

After homogenization, the aqueous and the organic phase were separated by centrifugation at 3500 rev min^{-1} for 15 min (Sah 1999b). The white precipitate observed at the water/CH_2Cl_2 interface, which consisted of lysozyme aggregates, was carefully collected using a Pasteur pipette and used directly in FTIR experiments as suspension or after subsequent drying with N_2 gas.

Lysozyme recovery

The aqueous phase obtained as described above was carefully collected with a Pasteur pipette and filtered with a 0.20-μm nylon membrane filter. Lysozyme concentration was determined by measuring the absorption at 280 nm employing a standard curve. Recovery of lysozyme (%) after homogenization was determined by measuring the protein concentration before and after sample treatment. Lysozyme loss due to the procedure was excluded by measuring standards in the absence of any homogenization (100 % recovery).

Lysozyme activity assay

Lysozyme activity was determined using freeze-dried cells of *Micrococcus lysodeikticus* as the substrate (Van de Weert et al 2000b). Lysozyme solution (100 μL) was added to 2.9 mL bacterial cell suspension, at concentrations of 0.1–0.3 mg mL^{-1}, in phosphate buffer at pH 5.1. The decrease in absorbance at $\lambda = 450$ nm was followed and the initial rate, V_0 ($\triangle A_{450\ nm}$ mg^{-1} s^{-1}) calculated from the linear part of the curve. The recovered specific activity per mg of lysozyme after homogenization was given in % of the lysozyme activity in aqueous solution before homogenization.

FTIR measurements

FTIR studies were conducted using a Nicolet Magna-IR System 560 optical bench (Carrasquillo et al 1998; Pérez & Griebenow 2000). Each sample was measured at least five times and all spectra corrected for the solvent, excipient and water vapour contributions (Carrasquillo et al 1998; Griebenow et al 1999a, b; Pérez & Griebenow 2000). All spectra were analysed by calculation of the second derivative spectra for their component composition in the amide I (1700–1615 cm^{-1}) band (Byler & Susi 1986). Second derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}). Spectral correlation coefficients (SCC) were calculated from the amide I second derivative spectra of native lysozyme and that of lysozyme to be tested (Prestrelski et al 1993). A SCC value of 1 demonstrates spectral and thus structural identity of two samples, a SCC value < 1 is a measure of overall perturbations in the secondary structure. The SCC was employed as a quantitative measure of homogenization-induced structural perturbations in lysozyme. Thermal denaturation measurements were performed and analysed as described by Pérez & Griebenow (2000). The secondary structure of lysozyme was determined by Gaussian curve-fitting of the resolution-enhanced amide I FTIR spectra in H_2O and amide I′ spectra in D_2O (Byler & Susi 1986; Carrasquillo et al 1998; Pérez & Griebenow 2000).

Size-exclusion HPLC

Size-exclusion HPLC was conducted using a G2000SW-XL1 TSK Gel Column (TosoHaas, PA) and followed by measuring the absorbance at 280 nm. Typically, 50 μL filtered lysozyme sample was loaded onto the column at a flow rate of 0.5 mL min^{-1}. The mobile phase consisted of 0.3 M sodium chloride and 50 mM sodium phosphate, pH 7.0.

Results and Discussion

Interface-induced lysozyme inactivation and aggregation

Hen egg-white lysozyme was used in this investigation because recent investigations have employed this protein and reported substantial aggregation when the protein was exposed to the water/CH_2Cl_2 interface (Sah 1999a, b; Van de Weert et al 2000b). One of our primary goals

Table 1 Loss in specific activity, aggregation, and secondary structure of lysozyme in aqueous solution after emulsification with CH_2Cl_2 at different homogenization velocities.[a]

Sample/state	Specific activity (%)		Lysozyme recovery (%)		SCC[b]	Secondary structure (%)[c]		
	D_2O[d]	H_2O[e]	D_2O[d]	H_2O[e]		α-Helix	Aggregation β-sheet[f]	Intramolecular β-sheet
Aqueous solution 0 rev min^{-1}	100	100	n.d.	n.d.	1.00	36 ± 1	0	14 ± 2
Aqueous phase[g] 20000 rev min^{-1}	87 ± 1	87 ± 1	100[h]	100[h]	0.99 ± 0.01	34	2	17
Aqueous phase[g] 30000 rev min^{-1}	80 ± 3	84 ± 6	84 ± 2	92 ± 2	0.98 ± 0.01	33	5	13
Aqueous phase[g]	51 ± 2	56 ± 1	82 ± 1	87 ± 4	0.91 ± 0.01	29	8	14
Suspension[i]					0.78 ± 0.01	25	19	15

[a]All experiments were performed at least in triplicate and the ± values are the calculated standard deviations. Even though the statistical error was smaller than 1 %, an error of 1 % was assumed for the calculated spectral correlation coefficients (SCC). [b]The SCC values were calculated with respect to the amide I second derivative spectrum of lysozyme as supplied dissolved in D_2O at pD 5.1 (native reference spectrum). [c]The content of secondary structure elements determined for all relevant conditions by Gaussian curve-fitting of the amide I IR spectra after resolution enhancement by Fourier-self deconvolution (see Methods for details). [d]Homogenization conditions: 34 mg mL^{-1} lysozyme in 2 mL D_2O, pD 5.1, 10 mL CH_2Cl_2, 10 min. [e]Homogenization conditions: 34 mg mL^{-1} lysozyme in 2 mL H_2O, pH 5.1, 10 mL CH_2Cl_2, 10 min. [f]The values were corrected for approximately 10 % amino acid side chain contribution at 1617 cm^{-1}. It was assumed that this band in D_2O for native lysozyme was exclusively due to side chains, as it is to be expected. [g]Aqueous phase refers to lysozyme after homogenization under the conditions given followed by centrifugation and filtration to remove insoluble lysozyme aggregates (see Methods for details). [h]All values were normalized to this value to correct for sample loss due to the preparation method. [i]Suspension refers to lysozyme isolated with a Pasteur pipette from the interface. Due to the qualitative nature of these data (see text) only the most extreme case was included in the table.

was characterizing the structural fate of lysozyme in this context by employing FTIR spectroscopy. Due to the strong water absorption band at approximately 1640 cm^{-1} overlapping with the amide I protein IR band it was difficult to measure protein aggregates in H_2O. Accordingly, the following experiments were carried out in D_2O. Previously, the denaturation of proteins at the CH_2Cl_2 interface had been studied mostly in H_2O (Sah 1999a, b, c; Van de Weert et al 2000b). Therefore, we first determined how comparable lysozyme behaviour was when it was dissolved in D_2O or H_2O and then exposed to the CH_2Cl_2 interface by homogenization. The data compiled in Table 1 using different homogenization velocities show that lysozyme behaved similarly under both conditions. Interestingly, in contrast to lysozyme aggregation that did not change significantly when using either 20000 or 30000 rev min^{-1} homogenization speed under both conditions, the recovered specific activity as a measure for the intactness of the soluble fraction of lysozyme did change substantially. Thus, there must be two different deactivation mechanisms for lysozyme upon homogenization. While aggregation is known to be the result of lysozyme adsorption to the interface (Sah 1999a, b, c; Van de Weert et al 2000b), the mechanism causing the significant activity drop in the soluble lysozyme fraction was unknown.

Formation of an unfolded lysozyme species at the D_2O/CH_2Cl_2 interface

The structural investigation of the homogenization step for lysozyme in D_2O employed Fourier-transform infrared (FTIR) spectroscopy. Two measures were taken of dissolved lysozyme obtained by filtering through a 0.20-μm nylon filter to investigate the structure of the soluble fraction, and the unfiltered suspension of lysozyme aggregates mixed with dissolved lysozyme collected near the interface with a Pasteur pipette. To characterize overall structural differences, SCC values were calculated (Prestrelski et al 1993) using the amide I second derivative spectrum of lysozyme in D_2O before treatment and those obtained after homogenization. When lysozyme in solution was exposed to CH_2Cl_2 without any homogenization, the spectra were basically identical to that of untreated "native" lysozyme in D_2O (Figure 1A) and the secondary structure was the same (Table 1). Homogenization at 20000 rev min^{-1} led to insignificant structural changes for the soluble lysozyme fraction (Table 1). In contrast, homogenization at

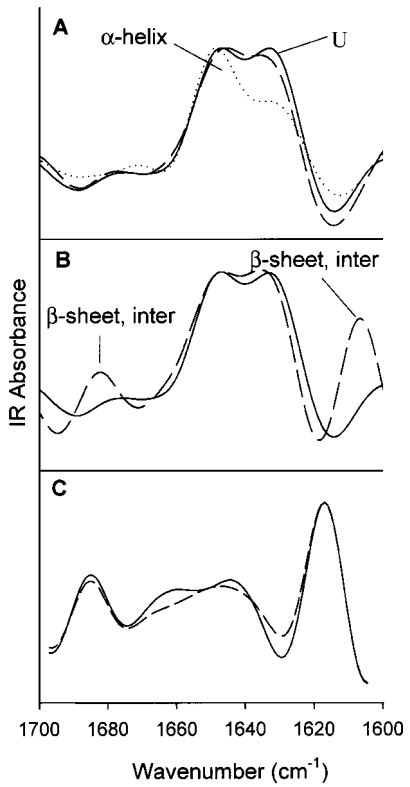

Figure 1 Second derivative amide I infrared spectra of lysozyme. A. Soluble lysozyme fraction after homogenization in D_2O (pD 5.1) for 10 min at 30000 rev min^{-1} obtained by filtration (full line), of lysozyme dissolved in D_2O pD 5.1 at 70°C (broken line), and of native lysozyme before homogenization or thermal denaturation (dotted line). B. Soluble lysozyme fraction as in A (full line) in comparison with aggregated lysozyme collected without filtration close to the CH_2Cl_2/D_2O interface (broken line). C. Aggregated lysozyme collected from the interface by filtration after drying with N_2 gas (broken line) in comparison with the difference spectrum (full line) obtained by subtraction of the two spectra shown in B. Homogenization parameters: 34 mg mL^{-1} lysozyme concentration, pD 5.1, 2 mL D_2O and 10 mL CH_2Cl_2, homogenization at 30000 rev min^{-1} for 10 min.

30000 rev min^{-1} led to some significant changes in the secondary structure of lysozyme (Table 1). Comparison of the second derivative spectrum of the soluble fraction of lysozyme with that of native lysozyme (Figure 1A) showed an increased contribution of the IR band at approximately 1640 cm^{-1} due to unordered secondary structure (Byler & Susi 1986). This showed that a larger fraction of unfolded lysozyme was present in the aqueous solution.

To further validate our finding that a fraction of the soluble lysozyme was unfolded after the homogenization, we performed thermal denaturation experiments with lysozyme at pD 5.1. We determined a melting temperature (T_m) of 70 ± 3°C using FTIR spectroscopy. The spectrum of lysozyme in D_2O at 70°C was similar to that of soluble lysozyme after homogenization (Figure 1A). The thermal unfolding of lysozyme is due to an equilibrium between the native state N and an unfolded state U (Pérez & Griebenow 2000), and this supports the notion that an unfolded species of lysozyme had been formed upon homogenization. At 70°C in D_2O approximately half of the lysozyme molecules were unfolded because the temperature coincided with the T_m. This was in excellent agreement with the drop in the specific activity to approximately 50% of its initial value (Table 1).

The presence of the unfolded species after homogenization could be explained in principle by two phenomena. Firstly, some chemical change might have occurred to the unfolded species, keeping it permanently unfolded at ambient temperature. Secondly, some chemical change could have occurred to all of the lysozyme molecules shifting the T_m towards room temperature. To distinguish both possibilities, thermal denaturation measurements were performed using the soluble lysozyme fraction after homogenization. Even though the thermal denaturation curve was less pronounced than for native lysozyme, a clear T_m was obtained at 51°C. (The lower value in this experiment might have been caused by the presence of the organic solvent CH_2Cl_2.) Thus, both species were not in equilibrium with each other and a new species had been formed which we named U*.

Similarly to our observations, Cleland & Jones (1996) reported the formation of a stable denatured state of recombinant human growth hormone as the result of emulsification at the water/CH_2Cl_2 interface, that contained less α-helix structure than the native molecule. Thus, permanent unfolding might be occurring as a result of the emulsification procedure to more proteins than just lysozyme.

Since Van de Weert et al (2000b) reported that the soluble fraction of lysozyme was structurally unperturbed, we also performed experiments at conditions more comparable with those employed in that work. When we reduced the homogenization time to 1 min, FTIR spectra did not reveal significant structural changes. Thus, the apparent differences between the two sets of data in our case were due to prolonged stress exposure.

Structure of aggregated lysozyme

The FTIR spectrum of lysozyme collected close to the interface after 30 000 rev min^{-1} homogenization speed indicated the presence of aggregated lysozyme, because

a band at approximately 1617 cm^{-1} typically associated with the formation of intermolecular β-sheets (Van de Weert et al 2000b) became significant in amplitude (Figure 1B). The secondary structure composition (Table 1) indicated a significant drop in the α-helix and increase in the intermolecular β-sheet content. However, the "quantitative" numbers derived from FTIR spectra given in Table 1 for the lysozyme suspension should only be taken as a qualitative indicator for the state of the sample because dissolved and aggregated lysozyme molecules were present. Aggregated molecules might not contribute as much to the measured FTIR spectra as dissolved species because scattered IR radiation is excluded from contributing to the spectra collected (Braiman & Rothschild 1988). To obtain an FTIR spectrum of lysozyme aggregates, we isolated aggregated molecules from the interface using a Pasteur pipette, dried the aggregates with N_2 gas, and measured their spectrum (Figure 1C). Except for some band-shifts, probably due to the deuteration of amide backbone groups in our case, the spectrum was similar to that presented by Van de Weert et al (2000b) for lysozyme aggregates obtained from H_2O after drying. Next we investigated whether the increase in band intensity for the "aggregation" β-sheet bands at approximately 1617 and 1690 cm^{-1} for lysozyme isolated from the interface as suspension (Figure 1B) was exclusively due to the formation of insoluble aggregates. We therefore subtracted the spectrum of the soluble lysozyme fraction obtained after filtration from that of the suspended lysozyme (Figure 1B). The resulting difference spectrum (Figure 1C) resembled closely that of the dried aggregates. Thus, the spectral components at 1617 cm^{-1} and approximately 1690 cm^{-1} were indeed exclusively associated with highly aggregated lysozyme molecules.

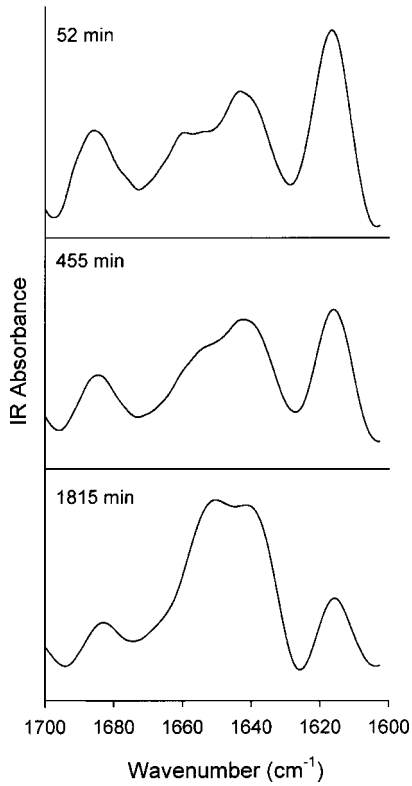

Figure 2 Second derivative amide I infrared spectra of lysozyme aggregates collected from the CH_2Cl_2 interface after suspension in D_2O for various times.

Reversibility of lysozyme aggregation

When working with the isolated aggregates suspended in D_2O we made the astonishing observation that the aggregates slowly and completely re-dissolved and, thus, were of non-covalent nature in agreement with previous data (Van de Weert et al 2000b). FTIR experiments performed at various times after suspension in D_2O showed that the aggregation β-sheet bands at approximately 1617 and 1690 cm^{-1} disappeared slowly, while IR absorption bands characteristic for α-helix and random coil secondary structure between 1656 and 1640 cm^{-1} gained intensity (Figure 2). The spectrum obtained after 1815 min was very similar to that of the suspended lysozyme isolated from the interface without filtration (Figure 1B). However, lysozyme never regained the native structure but instead still showed pronounced signs of unfolded lysozyme U* present. Thus, U* could also be integrated into aggregates. In contrast, the aggregates formed were completely stable in H_2O over a two-day period and no significant amide I IR spectral changes occurred.

We measured the recovery of lysozyme specific activity when it was re-dissolved from aggregates in D_2O. Aggregates were collected from the interface and dried with N_2 gas followed by subsequent stirring in D_2O for 10 h and storage at 4°C for an additional 48 h. The resulting lysozyme solution was diluted to a concentration of 0.038 mg mL^{-1} and the specific activity determined. While no changes due to the procedure were found for native lysozyme stored under these conditions in D_2O, the re-dissolved lysozyme regained only $70 \pm 17\%$ of its specific activity. The exact results varied somewhat for three different batches of lysozyme used. However, these results indicated that while a substantial number of lysozyme molecules refolded correctly leading to the active species, a substantial number did not and remained inactive.

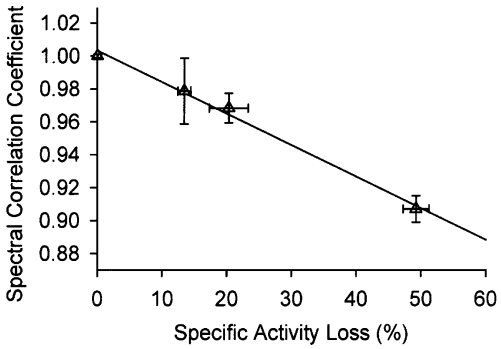

Figure 3 Relationship between structural changes in the soluble fraction of lysozyme after exposure to the water/CH_2Cl_2 interface by homogenization for 10 min at 30 000 rev min^{-1} and the loss in specific activity. The r^2 value for the linear regression was 0.99.

We correlated IR spectral changes with changes in the specific activity to investigate whether the spectral changes observed in the amide I indicated the presence of an unfolded, and thus inactive, form of lysozyme. Figure 3 shows clearly that the activity loss and spectral changes which occurred to the soluble fraction of lysozyme were linearly related ($r^2 = 0.99$). Thus formation of unfolded lysozyme (U*) led to the significant drop in specific activity.

Mechanistic insights on the formation of U*

We performed additional experiments to investigate the formation of U*. Firstly, we homogenized lysozyme in D_2O at pD 5.1 at 30 000 rev min^{-1}. We found no significant spectral changes in the amide I (SCC = 0.97) and no significant change in enzyme activity. We then performed this experiment using D_2O that was saturated with CH_2Cl_2 before the experiment by vigorous shaking for 3 h at 250 rev min^{-1}. Again, the homogenization procedure did not cause structural alterations in lysozyme (SCC = 0.98) excluding the possibility that the level of approximately 2% of CH_2Cl_2 dissolved in D_2O (Castellanos et al 2001) caused the unfolding. Therefore, formation of U* was only observed when the water/CH_2Cl_2 interface was present. We investigated the reversibility of U* formation more closely and produced the species under the standard conditions used earlier (Figure 1A). When we extensively dialysed the lysozyme fraction containing U* against D_2O for 48 h, no structural changes were evident (SCC = 0.99) and thus removal of CH_2Cl_2 from the aqueous phase did not cause refolding of lysozyme. Thus, it was unlikely that non-covalent binding of CH_2Cl_2 to the unfolded lysozyme species stabilized it. Lastly, we performed size-exclusion HPLC experiments to investigate whether soluble lysozyme aggregates were formed. Lysozyme as supplied by the commercial source, lysozyme that had been homogenized for 10 min in buffer and showed no activity loss, and the soluble lysozyme fraction obtained after homogenization (characterized by an approximate 50% activity loss) all eluted at 21.7 min. No formation of soluble lysozyme aggregates occurred, in agreement with other data (Sah 1999b, c; Van de Weert et al 2000b). Thus, U* was not due to formation of soluble aggregates.

Prevention of interface-induced lysozyme unfolding and aggregation

We investigated strategies allowing amelioration of the unfolding and aggregation of lysozyme at the water/CH_2Cl_2 interface. Surface-active components have been shown to prevent interface-induced aggregation of carbonic anhydrase (Lu & Park 1995b), tetanus toxoid (Alonso et al 1994), and lysozyme (Van de Weert et al 2000b). To test another class of compounds we employed various polyols at a 1:37 molar ratio. In addition to being potentially efficient in preventing interface-induced protein denaturation (Cleland & Jones 1996), polyols are also potentially efficient in preventing dehydration-induced protein structural changes (Carrasquillo et al 1998; Griebenow et al 1999a). The latter fact is important since creation of PLG microspheres by the w/o/w technique involved lyophilization as the final preparation step, which is known to perturb protein structure (Prestrelski et al 1993; Griebenow & Klibanov 1995). Employing intense homogenization stress (30 000 rev min^{-1}, 10 min, volume ratio 2:10, 25 mg mL^{-1} protein concentration) we assessed the influence of the polyols on the secondary structure of lysozyme, the specific activity, and the lysozyme recovery.

In the absence of the polyol additives, significant structural changes occurred to lysozyme leading to a drop in the SCC value to 0.83 (Table 2). The recovery of lysozyme was 94% and specific activity dropped to 71% of its initial value. While some additives led to an improvement in lysozyme structure, recovery and specific activity under the conditions employed (e.g. lactulose and fructose), others led to even more denaturation (e.g. sucrose and trehalose). Sah (1999c) reported that sucrose and trehalose were inefficient in preventing lysozyme aggregation. The prevention of lysozyme aggregation and inactivation by the polyols were largely unrelated events. When plotting the data for the loss in specific activity (100 − specific activity (%)) and lysozyme aggregation (100 − recovery (%)) taken from Table 2 against

Table 2 Specific activity, recovery, and secondary structure of lysozyme dissolved in water after homogenization with CH_2Cl_2 in the absence and presence of additives.

Condition, additive	Specific activity (%)	Recovery (%)	Spectral correlation coefficient (SCC)	T_m (°C)[a]
H_2O, pH 5.1	100 ± 1[b]	n.d.	1	74.8
Aqueous phase after homogenization[c]				
None	71 ± 4	94 ± 1	0.83 ± 0.01	74.8
Sucrose[d]	57 ± 4	92 ± 3	0.75 ± 0.01	74.7
Trehalose[d]	63 ± 4	94 ± 1	0.87 ± 0.01	n.d.
Fructose[d]	86 ± 7	98 ± 2	0.97 ± 0.01	n.d.
Maltose[d]	89 ± 6	92 ± 1	0.84 ± 0.01	n.d.
Lactitol[d]	89 ± 1	96 ± 1	0.98 ± 0.01	n.d.
Lactulose[d]	93 ± 7	99 ± 3	0.94 ± 0.01	75.6
Lactose[d]	94 ± 4	96 ± 3	0.96 ± 0.01	74.7
Sorbitol[d]	99 ± 2	95 ± 1	0.92 ± 0.01	75.5

[a]T_m values were determined by UV-vis spectroscopy (for details see Methods section) of aqueous lysozyme samples before exposure to CH_2Cl_2 and homogenization. [b]Specific activity $0.224 \pm 0.001 \triangle A_{450\,nm}\,mg^{-1}\,s^{-1}$. [c]Homogenization conditions: 25 mg mL^{-1} lysozyme in aqueous phosphate buffer, pH 5.1, 30 000 rev min^{-1}, 10 min, 2 mL phosphate buffer, 10 mL of CH_2Cl_2. [d]Additives were co-dissolved with lysozyme in 2 mL aqueous phosphate buffer at a 1:37 mol ratio of lysozyme-to-additive.

each other and calculating a linear regression, the low r^2 value of 0.3 showed that both processes were prevented by the excipients to a different extent. However, we found a reasonable correlation between structural preservation and prevention of lysozyme aggregation (Figure 4A) as well as between structural preservation and prevention in loss of activity (Figure 4B). In general, the better the structure of lysozyme in the aqueous solution was preserved, the better the properties of the sample afterwards.

Polyols are known to stabilize proteins in aqueous solution by being excluded from the protein surface (Timasheff 1998). To investigate the possible mechanism of lysozyme protection by polyols, we determined the melting temperatures T_m for selected samples by UV-vis spectroscopy. The T_m value for lysozyme in aqueous solution at pH 5.1 was determined to be 75°C in agreement with literature values (Hamaguchi & Sakai 1965; Pérez & Griebenow 2000). Only slight variations were found when the additives lactulose ($T_m = 76$°C), lactose ($T_m = 75$°C), sorbitol ($T_m = 76$°C) and sucrose ($T_m = 75$°C) were used at a 1:37 molar ratio of lysozyme-to-additive. This was expected, because polyols typically lead to significant increases in the T_m values at rather high concentrations of the additive (Wimmer et al 1997). The excipient concentration in our experiments was comparably low (< 1 mM in all cases). No correlation was found between this property and recovered specific activity, lysozyme recovery, or lysozyme structure (Table 2), excluding changes in lysozyme stability as being responsible for prevention of interface-induced lysozyme inactivation and aggregation. Thus prevention of lysozyme adsorption to the interface is likely the most important mechanism responsible for preventing detrimental changes to lysozyme structure and function (Sah 1999b, c; Van de Weert et al 2000b).

Finally, we investigated the apparent discrepancy of our results and reported data (Cleland & Jones 1996) to some in the literature (Sah 1999a, b; Van de Weert et al 2000b) for the additive trehalose. This additive improved the recovery of recombinant human growth hormone when its aqueous solution was emulsified in CH_2Cl_2 (Cleland & Jones 1996). In contrast, in experiments with lysozyme and ovalbumin, trehalose (and sucrose) failed to prevent the formation of aggregates (Sah 1999a, b; Van de Weert et al 2000b). In our case sucrose and trehalose, when used at a 1:37 molar ratio of lysozyme-to-additive, did not prevent lysozyme activity loss and aggregation (Table 2). However, we found that increased concentrations of trehalose eventually completely prevented lysozyme aggregation (97 ± 1% and 99 ± 1% lysozyme recovery at a 1:74 and 1:148 molar ratio, respectively). In general, similar tendencies were found for other polyols. The apparent differences compared with the literature data are likely to be due to different experimental conditions.

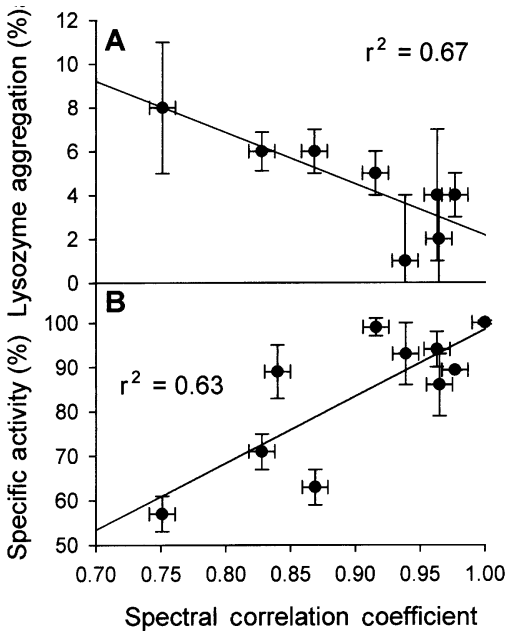

Figure 4 Relationship between the structural perturbations in the soluble lysozyme fraction of the enzyme co-dissolved with various polyols (SCC values from Table 2) and the lysozyme recovery (A) and specific activity (B). The r^2 values are for the linear regressions shown in the plots.

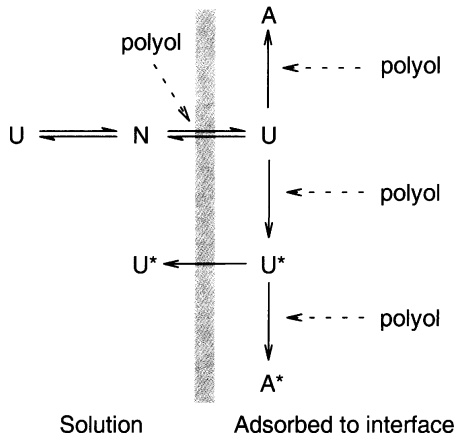

Figure 5 Model of lysozyme aggregation and unfolding at the water/CH_2Cl_2 interface. Native lysozyme (N) adsorbs to the interface and unfolds (U). The unfolded species either aggregates (A) or is transformed in a stable unfolded and inactive form (U*) that is able to leave the interface. U* can also aggregate. Polyols can prevent both detrimental processes, probably by primarily preventing lysozyme adsorption to the interface.

Model for interface-induced lysozyme unfolding and aggregation and its prevention

Based on the results obtained in this and other work we have developed a model for lysozyme inactivation and aggregation at the water/CH_2Cl_2 interface (Figure 5). In aqueous solution, native lysozyme (N) and unfolded lysozyme (U) are in equilibrium with each other. At room temperature the native state N is predominantly found. Due to its amphiphilic character, lysozyme can adsorb to the interface. Aggregation of lysozyme was only observed in the presence of the interface. It can be safely assumed that unfolding occurs as a result of lysozyme adsorption to the interface, followed by the formation of non-covalent aggregates. These do not leave the interface and thus at high protein concentration the aggregation effect levels off. In H_2O aggregate formation is irreversible. In the timeframe of the experiments (10 min), aggregate formation in D_2O can also be considered irreversible, even though reversibility was observed in the time domain of hours. Alternatively, the unfolded species U can be modified by a still unknown event and the species U* is formed. This reaction also requires the water/CH_2Cl_2 interface. The species U* can leave the interface and this in turn leads to the very pronounced drop in the specific enzyme activity in the soluble fraction of lysozyme. Furthermore, U* can also be integrated into aggregates, as evident from the reversibility studies in D_2O. Thus, depending on the exact conditions, eventually all the native lysozyme N will end up as aggregated or irreversibly unfolded and inactive form.

Polyols can prevent the formation of aggregates and formation of the unfolded species U*. However, there must be differences in the mechanism of how this happens because there was no correlation between the prevention of aggregate formation and the specific activity loss. Some details of the model still have to be verified. For example, it is still unclear whether lysozyme, once adsorbed to the interface, can reversibly leave the interface again if not integrated into aggregates. Future studies are certainly needed to clarify details of the proposed model.

Conclusions

It has been demonstrated that two deactivation pathways exist for hen egg-white lysozyme exposed to the water/CH_2Cl_2 interface. In addition to the established pathway leading to lysozyme aggregation, another pathway leads to the formation of unfolded and inactive lysozyme. Polyols are efficient in ameliorating these deactivation pathways but our data point to some variations in the mechanism of how they afford the protection against the two events. The polyol lactulose efficiently prevented both deactivation pathways. How-

ever, in principle all polyols could be employed by properly increasing their concentration to the one needed to achieve the desired effect.

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