Lysozyme Stability in Primary Emulsion for PLGA Microsphere Preparation: Effect of Recovery Methods and Stabilizing Excipients

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Purpose. To investigate the conformational stability of a model protein, lysozyme, in the primary emulsion phase of the microsphere preparation process.

Methods. The conformational stability of lysozyme during primary emulsification was assessed by differential scanning calorimetry (DSC) and enzymatic activity assay. PEG 400 was used to separate lysozyme from water-in-oil (w/o) emulsion containing poly(lactide-co-glycolide) (PLGA).

Results. No significant changes in the recovery of lysozyme were observed due to increasing sonication time from 20 to 60 s at 40 W or increasing intensity from 40 to 60 W for 20 s. By using the method involving PEG 400, lysozyme recovery in the presence of PLGA was increased from 11.8% to 70%. Hydroxypropyl-β-cyclodextrin (HP-β-CD) increased lysozyme recovery from 35% to 70% at low lysozyme concentration (20 mg/ml), and from 70% to 77% at high lysozyme concentration (100 mg/ml) in the presence of PLGA. Sugars such as trehalose and mannitol failed to increase lysozyme recovery. DSC results suggested the retention of the conformational structure of the recovered lysozyme, which was supported by an enzymatic activity assay.

Conclusions. HP-β-CD was found to be a promising stabilizer that protected lysozyme during the primary emulsification. Protein recovery method with the help of PEG 400 allowed the study of protein stability in w/o emulsions in the presence of PLGA. DSC provided supplementary information on the conformational changes of lysozyme during emulsification.

KEY WORDS: emulsification; protein stability; lysozyme; poly(lactide-co-glycolide); protein extraction method; differential scanning calorimetry.

INTRODUCTION

Biodegradable poly(lactide-co-glycolide) (PLGA) microspheres have received much attention for the sustained release of bioactive macromolecules such as peptides and proteins. The water-in-oil-in-water (w/o/w) emulsion technique is a commonly used method to encapsulate proteins into a polymeric matrix (1). Peptide-loaded microspheres such as Lupron depot (TAP Pharmaceutical Products, Inc., Lake Forest, Illinois) are already commercially available. However, proteins have been much more problematic to be encapsulated with native structure and/or activity intact. Protein stability has been a concern due to exposure to organic solvents, sonication, and mechanical shear forces during preparation. All of these stresses can be destructive to protein hierarchy structure as well as biologic activity. Aggregation, deamidation, and other protein stability problems have been reported during the microencapsulation of proteins (2–4). Protein denaturation may lead to the formation of an antibody that gives unwanted immune responses. Meanwhile, protein aggregates have been suggested to lead to incomplete release from microspheres (5).

During the microspheres preparation process, the primary emulsification step has been suggested as a major cause for protein denaturation and aggregation (6). In this step, protein is exposed to a number of stresses. Sonication, which is widely used to create the first emulsion, can introduce high pressures, temperature gradients, shear forces, and free radicals (7). All of these factors can denature proteins. More important, sonication produces a very large interface between aqueous and organic phases. Proteins may be adsorbed and denatured at the interface (8–10). Despite the wide use of microencapsulation of proteins/vaccines into PLGA microspheres by the w/o/w emulsion technique, only a limited number of systematic studies have been performed in relation to protein behavior at the water-organic solvent interface (8–10). In particular, the current available information is limited on the protein solution emulsified with methylene chloride or ethyl acetate without the presence of PLGA polymer, which does not exactly simulate the microencapsulation step. The paucity of information may be due to the difficulty of breaking the emulsion in the presence of polymer without introducing any additional stress on protein molecules (10). In this study, a novel protein recovery method was proposed that makes it possible to study protein stability in PLGA-containing emulsions.

It is important to maintain the native conformation of protein during its encapsulation process. The stability of protein has been investigated and monitored using several methods, including gel electrophoresis (8), size exclusion chromatography (8–10), circular dichroism (10), and enzyme-linked immunosorbent assay (11). In this article, differential-scanning calorimetry (DSC) was utilized to study protein stability in the aqueous state during the emulsification process. Calorimetry measures the change of enthalpy upon thermal denaturation of proteins (ΔH) and the midpoint temperature of the denaturation (T_m). Since DSC allows the measurement of the enthalpy, it provides structural information on a macroscopic scale. DSC is an ideal method to study protein thermal stability in solution, and it has been successfully used in protein aqueous formulation screening studies (12).

The objective of this article was to investigate the influence of primary emulsification on the stability of lysozyme, a 14K-model protein, which has a size similar to many therapeutically effective cytokines such as interferons and interleukins. Particular attention was paid to assessing the nature of the protein before and after emulsification with organic solvent. To better mimic the manufacturing process of microspheres, PLGA was also involved in the organic phase. Lysozyme recovery, enzymatic activity, and thermal stability were measured for protein characterization. Protein extraction from the w/o emulsion was optimized to improve protein recovery, which included an assessment of the protective ef-

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fect of several excipients on lysozyme, such as reducing the interface adsorption and denaturation.

MATERIALS AND METHODS

Materials

Lysozyme (from chicken egg white), D-mannitol, D(+)trehalose, poly(ethylene glycol) (molecular weight, 400), and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Company (St. Louis, Missouri). Hydroxypropyl-β-cyclodextrin (HP-β-CD) and poly(vinyl alcohol) (87–89% hydrolyzed; molecular weight, 13,000–23,000) were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Methylene chloride was purchased from Fisher Chemical Company (Fair Lawn, New Jersey). PLGA (50:50, inherent viscosity 0.63 dl/g in hexafluoroisopropanol at 30°C) was obtained from Birmingham Polymer, Inc (Birmingham, Alabama). Micro-BCA protein assay reagent kit was obtained from Pierce Chemical (Rockford, Illinois).

Preparation of w/o Emulsions and De-Emulsification in the Absence of PLGA

A known amount of lysozyme was dissolved in citrate phosphate buffer (pH 4.8, 0.1 M) to make the final concentration of 20 or 100 mg/ml. An aqueous protein solution (0.1 ml) was then emulsified with 3 ml of methylene chloride. The emulsification was carried out by a microtip-equipped ultrasonicator (Sonifier cell disruptor, model W185, Heat System-Ultrasonics, Inc., Plainview, New York) for known time and power level. In most cases, sonication was carried out for 20 s at 40 W. After emulsification, lysozyme was extracted into the aqueous phase by adding 10 ml of buffer and then centrifuged at 3000 g for 20 min to accelerate phase separation. This extraction process was called method 1, differing from the other methods used later. Residual methylene chloride partitioned to the aqueous solution was removed by placing this solution under vacuum for 0.5 h. The aqueous phase was subjected to protein quantification, enzymatic activity assay, and DSC. In some cases, excipients (see Table II) were added to the aqueous protein solution to test their effects on the increasing stability of lysozyme during primary emulsification.

Preparation of w/o Emulsions and De-Emulsification in the Presence of PLGA

The above process was followed with the exception that the methylene chloride contained 200 mg of PLGA. Because of the high viscosity of emulsions in the presence of PLGA, two other methods were investigated for the extraction of lysozyme from PLGA-containing emulsion. In one method (method 2), 1 M NaCl in citrate phosphate buffer was used. In the other method (method 3), 1 ml of PEG 400 was added to the emulsion followed by the addition of 9 ml of citrate phosphate buffer. Both methods utilized centrifugation at 3000 g for 20 min to accelerate phase separation. The aqueous phase was subjected to protein quantification, enzymatic activity assay, and DSC.

Protein Concentration Determination

Lysozyme content in the samples was determined by the MicroBCA protein assay (13) using appropriate reagent blank and standard curves. Protein recovery was reported as the percentage of protein extracted into the aqueous phase following centrifugation.

Enzymatic Activity Assay

Because lysozyme is capable of digesting bacterial cell walls, *M. lysodeikticus* was used as the substrate in the activity assay (10), and the turbidity of the suspension was measured at 450 nm in a Shimadzu UV-1601 spectrophotometer (Riverwood Drive, Columbia, Maryland). Briefly, a 0.01% (w/v) suspension of *M. lysodeikticus* was prepared in potassium phosphate buffer (0.1 M, pH 6.24). One hundred microliters of an appropriately diluted lysozyme solution was added to 2.5 ml of the suspension. The decrease in absorbance was monitored during a total period of 2 min. The activity was calculated from the slope of the linear region of the ΔA_{450nm}/time curve, assuming that one unit of enzyme activity will reduce the ΔA_{450nm} by 0.001/min under the condition employed. Specific activity is defined in terms of units of activity per milligram of protein.

DSC

DSC measurements were carried out using an ultrasensitive differential scanning calorimeter (VP-DSC, MicroCal, Northampton, Massachusetts). It is specially designed for studies on dilute aqueous solutions of biologic macromolecules. All samples and buffers were degassed by stirring under vacuum before loading into the sample and reference cells. The heat flow required keeping the sample cell and reference cell thermally balanced was recorded from 25°C to 95°C, using a scan rate of 1.5°C/min. The cells were then cooled to 25°C and rescanned; the first scan was of native protein, and the following scan was of heat-denatured protein. To ensure that the heat transition in protein is the only source of thermal difference between sample cell and reference cell, the cells were filled with the same buffer vehicle. The buffer background was subtracted from each sample-reference scan during the data analysis process. T_m is the midpoint transition temperature at which folded and unfolded molecules are equally populated. The calorimetric enthalpy ΔH was determined by integrating the area under the transition peak. Data manipulation was performed using Origin software (MicroCal Software, Inc., North Hampton, Massachusetts) provided with the instrument.

RESULTS AND DISCUSSION

Figure 1 shows the thermograms of native (first scan) and heat-denatured (second to seventh scans) lysozyme in citrate phosphate buffer (pH 4.8, 0.1 M). The scan of native lysozyme exhibited a single sharp endothermic transition peak, which had a T_m of 77.36°C and ΔH of 105 Kcal/mol. Similar T_m and ΔH values have been obtained by other researchers (14,15). The scans of the heat-denatured lysozyme showed declining thermal transition peaks, and a new transition peak was observed from the second scan. These results are consistent with that from the loss in native structure that led to less heat

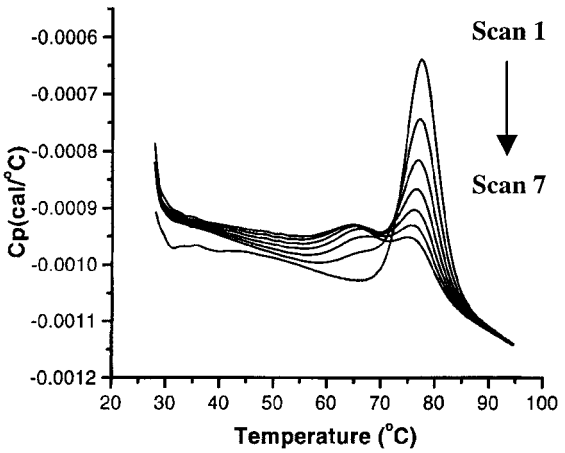

Fig. 1. DSC thermograms of consecutive seven scans of lysozyme in citrate phosphate buffer (pH 4.8, 0.1 M) showed the difference between scans on "native" protein (scan 1) and "heat-denatured" protein (scans 2–7).

absorption during the unfolding transition. Because the ΔH value provides valuable information on protein conformation (16), it was used to assess retention of protein native conformation. A decreased ΔH compared to the control can be consistent with a partially denatured conformation. T_m was also used to evaluate the structure integrity of lysozyme since T_m may indicate the level of protein stability (12).

Table I summarizes the results obtained on recovery, specific enzymatic activity, and two thermodynamic parameters (T_m and ΔH) of lysozyme recovered in the aqueous phase after emulsifying with methylene chloride in the absence of PLGA. No significant change in the recovery of lysozyme was observed by increasing sonication time from 20 to 60 s at 40 W or increasing the intensity from 40 to 60 W for 20 s. The aqueous protein retained its enzymatic activity after emulsification. The thermodynamic parameters, T_m and ΔH, were similar to the intact lysozyme control, suggesting that the native conformation of soluble lysozyme was retained. Sonication is important in microsphere preparation. It has an impact on the morphology of microspheres as well as on the drug release (17). Our study showed that there was no significant difference on lysozyme stability in the studied range of sonication time and power level. This result enabled more choices of process variable to obtain desired microsphere morphology and release profiles.

Higher recovery of lysozyme from primary emulsion indicates that less fraction of lysozyme is adsorbed at the w/o interface. Therefore, we screened several stabilizers on their ability to increase the recovery of lysozyme during the primary emulsification process. Table II shows the effect of some stabilizers such as sugars (trehalose and mannitol) and HP-β-CD on the recovery of lysozyme. The screening was performed by emulsifying the lysozyme aqueous solution containing different excipients with methylene chloride but without polymer. All excipients were examined at both low and high lysozyme concentrations. The enzymatic activity assay and DSC results supported the interpretation that recovered lysozyme had similar calorimetric profile in all formulations. At low lysozyme concentration (20 mg/ml), the recoveries were 83%, 67%, and 59%, respectively, for the control, trehalose, and mannitol. At high lysozyme concentration (100 mg/ml), the recoveries were 84%, 76%, and 68%, respectively, for the control, trehalose, and mannitol. Sugars have been suggested to stabilize protein in aqueous solution (18) and during lyophilization (19), probably by a preferential hydration mechanism (18) and/or hydrogen bonding (19). In this study, however, they did not increase lysozyme recovery at both low and high lysozyme concentrations, which may be due to their lack of affinity to the methylene chloride/water interface (9).

Cyclodextrins (CDs) are molecules with polar outer surfaces and apolar interior cavities through which inclusion complexes are formed with many guest molecules (20). CDs have been used as a stabilizing agent for protein (21). It is suggested that the aromatic rings of tryptophan, tyrosine, and phenylalanine of proteins will insert into the hydrophobic cavities of CDs (22,23). HP-β-CD is one of the modified CDs. It is the most frequently used CD because of its high aqueous solubility and lower toxicity, as well because it has a more hydrophobic cavity compared to the parent CD (24). Several studies have shown that HP-β-CD can reduce organic solvent-water interface-induced protein aggregation and denaturation during the preparation of a w/o emulsion (6,9). In our results (Table II), HP-β-CD was found to increase lysozyme recovery from 83% in the control to 91% at a low lysozyme

Table I. Effect of Sonication on Recovery, Enzymatic Activity, and Thermal Parameters of Recovered Lysozyme after Emulsifying with Methylene Chloride

Sample	Recovery (%)[a]	Specific activity (%)[a]	Tm (°C)	ΔH (Kcal/mol)
Control	—	100 (± 5)	77.36	105
20s, 40W	84 ± 2	104 (± 4)	77.36	106
60s, 40W	84 ± 1	91 (± 2)	77.47	106
20s, 60W	80 ± 3	90 (± 3)	77.45	108

[a] Recovery and specific activity results are expressed as mean ± SD (n = 3).

Table II. Screening of Excipients in Absence of PLGA

Lysozyme (mg/ml)	Excipient (w/v)	Recovery (%)[a]	Specific activity (%)[a]	Tm (°C)	Δ (Kcal/mol)
20	—	83 (± 3)	93 (± 5)	77.45	95.7
20	Trehalose 10%	67 (± 4)	92 (± 2)	77.29	99.4
20	Mannitol 10%	59 (± 1)	99 (± 1)	77.43	94.5
20	HP-β-CD 10%	91 (± 2)	94 (± 1)	77.38	98.2
20	HP-β-CD 5%	66 (± 2)	97 (± 3)	77.43	100
100	—	84 (± 2)	104 (± 4)	77.36	106
100	Trehalose 10%	76 (± 7)	94 (± 3)	77.37	97.3
100	Mannitol 10%	68 (± 2)	104 (± 4)	77.45	96.9
100	HP-β-CD 10%	92 (± 2)	90 (± 2)	77.34	97.1
100	HP-β-CD 5%	86 (± 2)	90 (± 1)	77.33	99.7

[a] Recovery and specific activity results are expressed as mean (± SD) (n = 3).

concentration, and from 84% to 92% at high lysozyme concentration. The effect of HP-β-CD was concentration-dependent. A higher rate of recovery was obtained for 10% HP-β-CD than for 5% HP-β-CD at both lysozyme concentrations. This result suggested that the optimum HP-β-CD concentration needed to be considered based on protein content. The stabilizing effect of HP-β-CD during primary emulsification may be due to shielding of the nonpolar side chains of amino acid residues in proteins, thereby increasing their hydrophilicity. This increased hydrophilicity keeps protein away from the methylene chloride-water interface (9).

The study of emulsification without polymer in the organic phase can provide us with valuable information on the protein stability at the w/o interface. It could be used as a fast and economic method to screen aqueous protein formulations. However, the presence of a polymer, PLGA, cannot be neglected when studying protein stability issues during microsphere preparation. So far, very little research (10) has been found on protein behavior at the water-organic solvent interface with polymer in the organic phase, and there are no satisfactory methods on protein extraction from emulsions containing polymers. One possible reason is that breaking such emulsions is more difficult than those without PLGA because of the high viscosity of emulsions. Another possibility is that PLGA has the trend to precipitate out once the aqueous buffer is added in excess into the emulsion. Encapsulated protein may precipitate together with polymer. This can be observed in Table III. When the same extraction method (method 1) was used, lysozyme recovery was markedly reduced from 84% in the absence of PLGA to 11.8% in the presence of PLGA. To study the properties of the majority of protein in this system, a representative amount of protein should be extracted out. To resolve this problem, two other methods were studied to break the viscous emulsion more completely. In method 2, NaCl was added to promote phase separation. Higher recovery was obtained from 11.8% in method 1 to 23.0% in method 2. Sodium chloride probably acts as channeling agent. It introduces a water outflow from the inner aqueous phase through the polymer layer, which acts as a diffusion barrier. But the disadvantage of this method is that NaCl may induce a pronounced protein denaturation (25). Moreover, the recovery was still relatively low. To further increase the protein recovery, PEG 400 was introduced (method 3). Recovery increased dramatically from 11.8% in method 1 to 70% in method 3. Our preliminary study showed that PLGA could be dissolved in PEG 400. The clarity of PLGA-containing emulsions increased markedly with the addition of PEG 400, indicating that the emulsions were completely broken. As a result, more protein encapsulated in the emulsion droplets was released. DSC results showed that the T_m of recovered lysozyme decreased about 1.5°C compared to the other two methods (Table III) and intact lysozyme control without PEG 400 (Table II). To verify that the extraction process itself of method 3 was not responsible for the decreased T_m, the DSC scan of intact lysozyme was performed in citrate phosphate buffer containing 10% (v/v) PEG 400. This control has the same buffer condition as in final aqueous phase of method 3. The T_m of this control was 75.79°C, which is almost same as the T_m of 75.95°C in method 3 (Table III and Fig. 2). This result suggests that the decreased T_m of 1.5°C is due to the addition of PEG 400, not to the stability loss during the emulsification and extraction process. Despite any interactions between protein and PEG 400, the enzymatic activity of lysozyme was retained. Therefore, this extraction method can be a promising way to study protein behavior at the water-organic solvent interface in the presence of polymer in the organic phase.

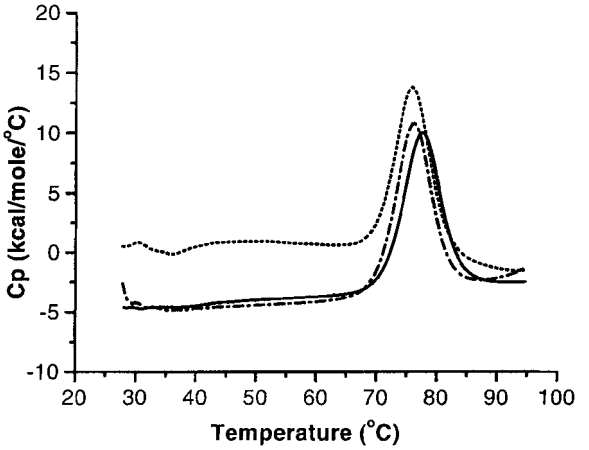

Fig. 2. DSC thermograms of lysozyme in citrate phosphate buffer (pH 4.8, 0.1 M) with and without PEG 400. Key: (solid line) lysozyme control without PEG 400, (dotted line) lysozyme control with 10% v/v PEG 400, and (dash dot line) lysozyme recovered in extraction method 3.

By using method 3, HP-β-CD was further assessed in PLGA-involved emulsions. The results were summarized in Table IV. The recovery of protein increased from 35% to

Table III. Effect of the Extraction Methods on the Protein Recovery, Enzymatic Activity, and Thermal Parameters

Extraction method	PLGA	Recovery (%)[a,b]	Specific activity (%)[b]	Tm (°C)	ΔH (Kcal/mol)
Method 1	No	84 (± 2)	104 (± 4)	77.36	106
Method 1	Yes	11.8 (± 0.1)	110 (± 5)	77.58	102
Method 2	Yes	23.0 (± 0.3)	102 (± 3)	77.59	92.5
Method 3	Yes	70 (± 2)	103 (± 7)	75.95	104
Control[c]	No	—	100 (± 5)	75.79	100

[a] Original lysozyme concentration was 100 mg/ml.
[b] Recovery and specific activity results are expressed as mean (±SD) (n = 3).
[c] Lysozyme 1 mg/ml in citrate phosphate (CP) buffer (containing 10% v/v PEG 400).

Table IV. Protein Recovery, Enzymatic Activity, and Thermal Parameters after Emulsifying with PLGA-Containing Methylene Chloride

Lysozyme (mg/ml)	HP-β-CD (10% [w/v])	Recovery (%)[a,b]	Specific activity (%)[b]	Tm (°C)	ΔH (Kcal/mol)
20	–	35 (± 1)	106 (± 6)	75.83	97.6
20	+	70 (± 1)	111 (± 3)	76.27	93.3
100	–	70 (± 2)	103 (± 7)	75.95	104
100	+	77 (± 2)	115 (± 2)	76.34	96.8

[a] Lysozyme was extracted by using method 3.
[b] Recovery and specific activity results are expressed as mean (±SD) (n = 3).

70% at a low lysozyme concentration of 20 mg/ml, and from 70% to 77% at a high lysozyme concentration of 100 mg/ml. For the same amount of HP-β-CD, the lower lysozyme concentration had a higher magnitude-of-recovery increment than the high lysozyme concentration. This is consistent with the interpretation that the cavity space is limited for a certain amount of HP-β-CD, and it could be saturated when protein concentration increases. The enzymatic activity assay and DSC results of recovered lysozyme did not suggest the denaturation of recovered lysozyme.

CONCLUSIONS

No significant changes in lysozyme recovery were observed by increasing sonication time from 20 to 60 s at 40 W or by increasing intensity from 40 to 60 W for 20 s. HP-β-CD was found to be a promising stabilizer that protects lysozyme during the primary emulsification of microsphere synthesis. The PEG 400-involved extraction method allowed us to study protein stability in w/o emulsions in the presence of PLGA. DSC results showed the retention of the conformational structure of recovered model protein during emulsification, which was supported by the results of enzymatic activity assay.

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