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Inactivation of lysozyme by sonication under conditions relevant to microencapsulation

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Abstract

Controlled release dosage forms of proteins and other biomolecules can be prepared by microencapsulating them in polymeric microspheres. Proteins are subjected to potentially damaging effects of sonication and exposure to organic solvents during the microencapsulation process. The relatively stable enzyme lysozyme was dissolved in aqueous buffer and sonicated in the presence of methylene chloride to mimic the initial step of the microencapsulation process. The stability of lysozyme was evaluated by determining the enzyme activity before and after sonication, size-exclusion chromatography, native polyacrylamide gel electrophoresis, and by measuring the amount of precipitates formed. Following sonication, the total protein introduced was distributed between a soluble and an insoluble fraction. Sonication of lysozyme solutions in the presence of methylene chloride led to an increase in precipitates. The precipitates were enzymatically inactive, did not dissolve easily, and were held by non-covalent interactions. No fragments or aggregates of lysozyme were detectable in the soluble fraction. Sonicating aqueous lysozyme solutions with and without methylene chloride decreased the specific activity of the enzyme in the soluble fraction. Excipients such as dimethyl sulfoxide (DMSO), mannitol, sucrose, and tween 80 were included in the sonication mixtures containing lysozyme. With the exception of tween 80, the addition of the excipients to aqueous solutions of lysozyme led to a greater decrease in the specific activity of lysozyme when sonicated in the presence of methylene chloride. DMSO caused the greatest loss of enzyme activity following sonication. Sonication of lysozyme with water, methylene chloride, and DMSO yielded methyl radicals, which were trapped with α-phenyl *N*-tert-butylnitrone and detected by ESR spectroscopy.

Keywords: Protein stability; Microencapsulation; Ultrasound; Free radical; Lysozyme

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1. Introduction

Controlled release of proteins and other macromolecules encapsulated in polymeric microspheres is a useful mode of drug delivery that is applicable to a wide range of new products arising from recombinant DNA technology (Bittner et al., 1998; Putney 1998; Pean et al., 1998; Katakam and Banga, 1997; Johnson et al., 1997; Cleland, 1997; Cleland and Jones, 1996). The double-emulsion technique is a widely used method for microencapsulating proteins and other macromolecules (Watts et al., 1990). The primary emulsion is produced by dispersing an aqueous solution of a protein into an immiscible organic solution of the polymer. The primary emulsion is usually unstable and reverts to its immiscible components during the time required to remove the organic solvent. Dispersing the primary emulsion in an aqueous solution containing an emulsifier to form a double emulsion creates a more stable system. The organic solvent is removed by evaporation or by extraction using a different organic solvent and then the microspheres are washed, filtered, and lyophilized for storage.

Owing to the fragile nature of biological macromolecules, it is important to ensure that their conformation and biological efficacy are not altered during the microencapsulation procedure, which involves potentially damaging exposure to organic solvents and ultrasound. Loss of biological activity of the encapsulated protein during microsphere preparation has been demonstrated (Tabata et al., 1993; Alonso et al., 1994; Yang and Cleland, 1997; Pean et al., 1998; Blanco and Alonso, 1998). The pH inside the microspheres during polymer hydrolysis (which may involve cleavage of lactide and anhydride moieties) was measured to be well below the physiological pH (Mader et al., 1996). This decrease in pH may cause acid catalyzed denaturation of the encapsulated protein during release. Denaturation of proteins during lyophilization (Prestrelski et al., 1993) and during storage (Liu et al., 1991; Costantino et al., 1994) can also decrease the potency of the released product.

The primary emulsion is created using a sonicator or a homogenizer. Sonication is preferred since it generates a more homogeneous dispersion with a high encapsulation efficiency (Yan et al., 1994). Sonication can denature proteins through large pressure and temperature gradients (Suslick et al., 1986), high shear forces (Kondo et al., 1989), or by generating free radicals (McLean and Mortimer, 1988; Misik et al., 1995). In addition, sonication exposes the protein to the denaturing action of organic solvent across a very large interfacial area. Stabilization strategies employed during lyophilization or storage of the finished product will not prevent protein denaturation, which might have occurred during earlier steps of the microencapsulation process. Hence, countermeasures that prevent protein denaturation need to be adopted during microencapsulation.

Knowledge of mechanism(s) of enzyme inactivation and protein denaturation relevant to the microencapsulation process can be used to design better dosage forms for controlled delivery of proteins. The capacity of a model protein, lysozyme, to withstand the harsh treatment during microencapsulation was studied by measuring enzyme activity, extent of molecular fragmentation and/or aggregation, formation of precipitates, and change in surface charge. The integrity of lysozyme was assessed using enzyme activity assay, size-exclusion chromatography, total protein assay, and native gel electrophoresis. In addition, the effects of commonly used excipients such as tween 80, sucrose, mannitol, and DMSO on protein stability were also investigated. This study emphasizes the need to understand and prevent the deleterious effects that organic solvents and certain excipients may have on proteins during their microencapsulation and use as controlled release pharmaceutical dosage forms.

2. Methods

2.1. Sonication

Sample solutions were sonicated using a Fisher scientific sonic dismembrator (model F550; 1.27 cm diameter horn and a tapered microtip accessory

of 0.32 cm diameter). The instrument, which can deliver a maximum power of 550 W, was set to deliver 50 W at 20 kHz for all sonication experiments. Ultrasound was delivered to ice cold samples in a pulsed fashion (10, 1 s pulses interspersed with 0.5 s between pulses). Since Tabata et al. (1993) had shown that prolonged sonication led to loss of enzyme activity, the overall sonication time was reduced to 15 s compared to the 30 s or greater sonication time usually employed.

2.2. Sonication of lysozyme and its extraction

Lysozyme (catalog number L-6876) and methylene chloride (catalog number D65100) were obtained from Sigma chemical company, St. Louis, MO. The required amount of lysozyme was reconstituted in 1 ml of phosphate buffer, (66 mM, pH 6.2) and added to 1 ml of methylene chloride (or buffer in the case of controls without organic solvent). Following pulsed sonication for 15 s, 2 ml of buffer were added to the sonicated mixture, and allowed to stand for 10 min. The sample was centrifuged for 5 min in a FISHER benchtop centrifuge (Model 225) at 6500 rpm. Since the aqueous buffer and methylene chloride are immiscible, two distinct layers were obtained; the denser methylene chloride formed the lower layer with the aqueous layer floating to the top. The protein extracted into the aqueous phase was characterized in terms of protein yield, specific activity, and structural integrity.

2.3. Enzyme assay

Lysozyme is an enzyme capable of digesting bacterial cell walls. Taking advantage of this property, the bacteria micrococcus lysodeikticus (Sigma chemical company, St. Louis, MO, catalog number M-3770) was used as the substrate in the standard activity assay for lysozyme as described in the Worthington Manual (Worthington, 1993). A 0.015% (w/v) suspension of micrococcus lysodeikticus was prepared in potassium phosphate buffer (66 mM; pH 6.2). One hundred microliters of an appropriately diluted lysozyme solution were added to 2.5 ml of the bacterial suspension. Since digestion of bacterial membrane causes a decrease in the absorbance at 450 nm, the absorbance at 450 nm was recorded every eight seconds during a total incubation period of 3 min at 25°C. A Hewlett Packard HP8452 diode array spectrophotometer was used for the absorbance measurements. As a blank, 100 μl of buffer were added (in lieu of lysozyme) to the suspension of bacteria. The enzyme activity was calculated by measuring the initial rate of the enzyme reaction in terms of change in absorbance at 450 nm/min (ΔA_{450nm}/min) and assuming that one unit of enzyme activity will produce a ΔA_{450nm} of 0.001 per minute at pH 6.2 at 25°C using a suspension of micrococcus lysodeikticus as substrate in a 2.6 ml reaction mixture.

2.4. Protein determination

The protein content was determined by the Lowry assay using the modified Lowry assay kit P5656 purchased from Sigma Chemical Co. The Lowry assay was performed without precipitation of protein. Appropriate reagent blanks and standard curves were generated using known amounts of lysozyme as well as bovine serum albumin. A Shimadzu UV/VIS 1101 spectrophotometer was used for absorbance measurements.

The specific activity of lysozyme is defined in terms of units of activity per mg of protein. The enzyme activity measurements and the protein concentrations based on the Lowry assay were used to calculate the specific activities of lysozyme in the different samples. The specific activities were normalized with respect to lysozyme that was not sonicated. Protein recovery is reported as the percentage of protein that is extracted into the aqueous phase (soluble fraction) following sonication.

2.5. Size-exclusion chromatography

The sample (20 μl) was introduced into a Waters Protein-Pak SW 125 Column equipped with a U6K injector and a Waters 991 photodiode array detector, and chromatographed at room temperature using pH 7.0 buffer (0.1 M phosphate and 0.15 M NaCl) at a flow rate of 1 ml/min. The eluents were monitored at 220 and 280 nm and

pure native lysozyme eluted 11 min after injection into the column. The retention time of native lysozyme remained constant over the concentration ranges used in this study (average retention time 10.98 min). The performance of the size exclusion column was checked frequently using BioRad gel permeation column calibration standard, which contains lysozyme as one of the components.

2.6. Native polyacrylamide gel electrophoresis

Electrophoresis of lysozyme samples before and after sonication was carried out using a Pharmacia PhastSystem employing a reverse electrode assembly. The electrophoretic run was performed at pH 4.1 for 75 Vh at 200 V, 10 mA and 15°C. The gels were removed and stained according to a Coomassie blue dye protocol (PhastSystem development file No. 200).

2.7. Detection of free radicals

Direct observation of highly reactive and extremely short-lived free radicals such as hydroxyl radicals (·OH) and methyl radicals (·CH_3) by electron spin resonance (ESR or EPR) is difficult. Such radicals are usually detected by the indirect technique of spin trapping. In this technique, a suitable diamagnetic compound (the spin trap) is allowed to react with the short-lived free radical in order to obtain a more stable free radical (the spin adduct), which is more amenable to investigation by ESR (Fig. 1A). The commercially available nitrone PBN was used as the spin trap in this study. The samples were transferred to the sample holder immediately after sonication and the spectra were recorded using a Varian E-4 spectrometer. The data were collected using a program written by Morse (1987).

3. Results and discussion

Protein precipitation occurred when an aqueous solution of lysozyme was sonicated in the presence of methylene chloride. Mass balance indicated that the total protein originally introduced was distributed between two fractions-a soluble fraction, which was extracted into the aqueous phase, and an insoluble fraction, which remained as the precipitate. The methylene chloride phase was devoid of protein. The precipitate formation may be due to an increase in the aqueous-organic interfacial area during sonication (Krishnamurthy, 1997; Krishnamurthy and Lumpkin, 1997; Sah, 1999, 1999a).

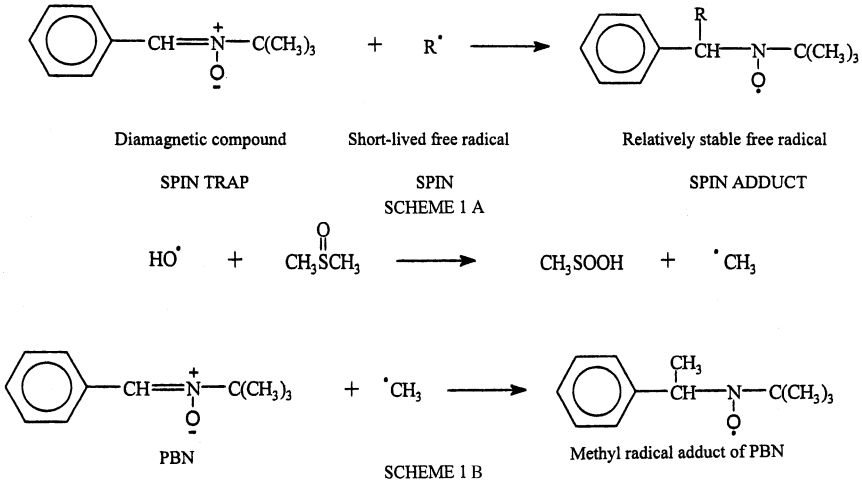

Fig. 1. Example of a spin trapping reaction (A). Generation of methyl radicals by the action of hydroxyl radicals with DMSO and subsequent trapping of methyl radicals by PBN (B).

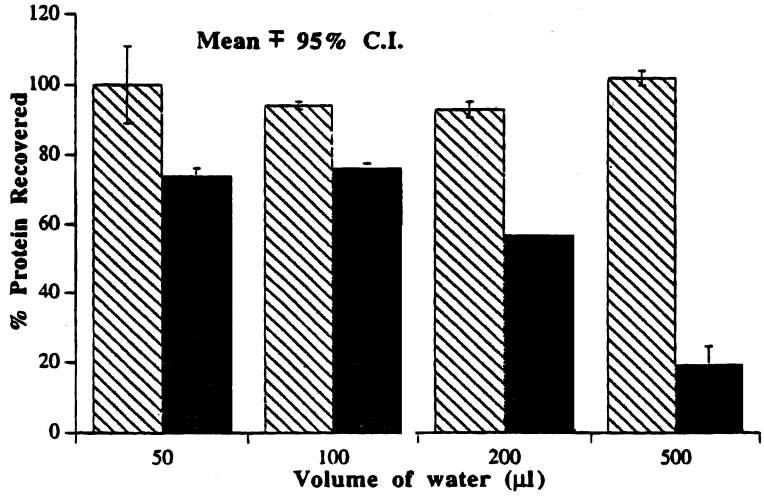

Fig. 2. Effect of the increase in the volume of the aqueous protein solution relative to that of methylene chloride in the sonication mixture. The recovery from sonicated controls without methylene chloride (hatched bars) in the aqueous phase and samples sonicated in the presence of methylene chloride (dark stippled bars) are plotted as a function of increasing increments of volume of aqueous phase in excess of 1 ml.

If this were true, increasing the water/methylene chloride interface should result in increased precipitate formation. To test this possibility, a constant amount of protein was dissolved in different volumes of aqueous buffer and sonicated after the addition of 1 ml of methylene chloride or buffer. Protein recoverable in the aqueous phase progressively decreased (with corresponding increase in precipitation), as the volume of the aqueous solution in the water/ methylene chloride mixture was increased (Fig. 2).

To verify that the decreased recovery was not due to protein adhesion to glass, the glass tubes were rinsed with a solution of sodium dodecyl sulfate (Sigma chemical company, St. Louis, MO, catalog number L-4509) and the rinse was analyzed for protein content. The protein recovered from the rinse amounted to approximately 3% of the total protein introduced. Therefore, the binding of protein to the walls of the glass tube cannot account for the 25–80% of the protein that was not recovered (Fig. 2). To confirm that the extraction process did not induce protein precipitation, 500 µl of protein solution (10 mg/ml) were placed on top of 1 ml of methylene chloride, gently mixed, and extracted as before. No precipitates were formed. This indicated that the extraction process was not responsible for lysozyme precipitation, and that sonication-induced protein precipitation resulted from the exposure of lysozyme to the water–methylene chloride interface.

To understand the nature of the precipitates formed, 1 ml of standard, denaturing and reducing buffers were added to the protein precipitates and agitated overnight at room temperature. The absorbance at 600 nm was measured to determine the extent of dissolution of the precipitates. The basis for this assay is that protein solutions do not absorb at 600 nm while suspensions do. The results are shown in Table 1. The precipitates did not dissolve in phosphate buffer (66 mM; pH 6.2). The insolubility of the precipitate in standard buffer indicated that the protein might be dena-

Table 1
Effect of reconstitution medium on the solubility of precipitated material

Medium used for reconstituting the precipitate	Absorbance at 600 nm
Phosphate buffer (66 mM; pH 6.2)	0.48
Dithiothreitol (10 mM)+disodium EDTA (50 mM)	0.46
Guanidinium chloride (6 M)	0.01

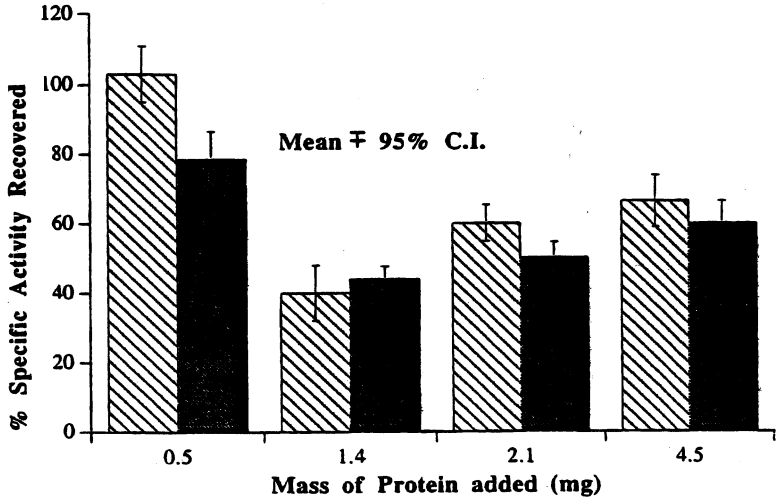

Fig. 3. Effect of protein mass on the amount of protein recovered after sonication. One hundred microliters of protein solutions containing different amounts of lysozyme were sonicated with 1 ml of aqueous buffer (66 mM phosphate; pH 6.2) (hatched bars) or methylene chloride (dark stippled bars) and the amount of enzyme activity recovered in the aqueous phase was determined.

tured in the precipitated form. One common pathway of denaturation is the formation of intermolecular disulfide bonds. Dithiothreitol (Cleland's reagent) is a well-known dithiol capable of cleaving disulfide bonds and dissolving such precipitates. The precipitates did not dissolve in 10 mM DTT containing EDTA (50 mM; to prevent auto-oxidation of DTT), suggesting that intermolecular disulfide bonds were not the cause of protein aggregation and precipitation. Non-covalent linkages can also cause precipitate formation. Such linkages between protein molecules can be disrupted by guanidinium chloride. The precipitates dissolved immediately upon addition of guanidinium chloride (6 M) indicating the presence of non-covalent linkages.

The above results also indicate that it is unlikely that these precipitates would dissolve under conditions encountered in vivo during release from the microencapsulated dosage form. Any precipitate formed during the microencapsulation process is likely to remain inside the microspheres along with native protein. Hence, at the end of the processing step, both native protein and precipitated protein will be encapsulated in polymeric microspheres. This may account for the incomplete release profiles observed with many microencapsulated proteins (Schwendeman et al., 1996), as well as the aggregates observed within microspheres by Lu and Park (1995).

A key indicator of protein stability is its enzymatic activity. The specific activity of the soluble fraction extracted after sonication was measured and normalized with respect to the specific activity of lysozyme before sonication. Fig. 3 depicts the effect of sonication on the activity of lysozyme. This indicates that the decrease in specific activity recovered is independent of the presence of the organic solvent. The decrease in specific activity also varied with increasing amounts of protein. As the protein mass increased, the specific activity recovered initially decreased, and then increased with the maximum loss of activity occurring when lysozyme was sonicated at an initial concentration of 1.4 mg/ml (Fig. 3). We did not observe complete recovery of specific activity in the concentration range studied.

Sonication creates large interfacial areas between the aqueous and organic phases (air and methylene chloride and between air and water) and forms large temperature and pressure gradients (9). Fragmentation of the protein molecule or other structural modifications such as loss of sec-

ondary structure may be induced by the large pressure gradients, while large temperature gradients may lead to thermal inactivation of the protein or pyrolysis of bonds belonging to protein, solvent, or solute molecules generating free radicals.

Size-exclusion chromatography and native gel electrophoresis were used to detect the presence of fragments, aggregates, and changes to the surface charge of lysozyme. There was no evidence for fragmentation or aggregation of lysozyme upon sonication (data not shown). There were no changes in the electrophoretic mobility of native and sonicated lysozyme as well (data not shown). These results suggested that the loss in specific activity upon sonication was not attributable to fragmentation, aggregation, or a change in the surface charge of lysozyme.

Different excipients were added to the aqueous solutions before sonication in order to test their ability to protect against protein loss and function. The excipients were tween 80, sucrose, mannitol, and DMSO. Tween 80 (P-1754), sucrose (S-2395), and DMSO (D-8779) were purchased from Sigma chemical company, St. Louis, MO. Mannitol (25,409-6) was obtained from Aldrich chemicals, Milwaukee, WI. Tween 80 is a nonionic surfactant that can provide a hydrophilic environment around lysozyme and decrease the exposure of the protein to methylene chloride or air. Sucrose is used commonly as an excipient to stabilize proteins by preventing their unfolding (Arakawa and Timasheff, 1985). Since sonolysis of water and aqueous solutions can produce hydroxyl and hydrogen free radicals (Misik et al., 1995; Henglein and Kormann, 1985), the hydroxyl radical scavengers DMSO (Sridhar et al., 1986) and mannitol (Menon et al., 1998; Tsou et al., 1999) were selected. DMSO is also used as a cryoprotectant during lyophilization.

We decided to test the putative efficacy of the excipients under conditions that caused maximum damage to lysozyme in our experiments (Fig. 2), i.e. 500 μl at a concentration of 10 mg/ml (40% decrease in specific activity and an 80% decrease in protein recovery). The effects of the different excipients on specific activity and protein recovery are shown (Figs. 4 and 5). Tween 80 and mannitol prevented the loss of specific activity upon sonication of the protein in phosphate buffer while sucrose and DMSO caused a greater loss compared to that in the control, which did not con-

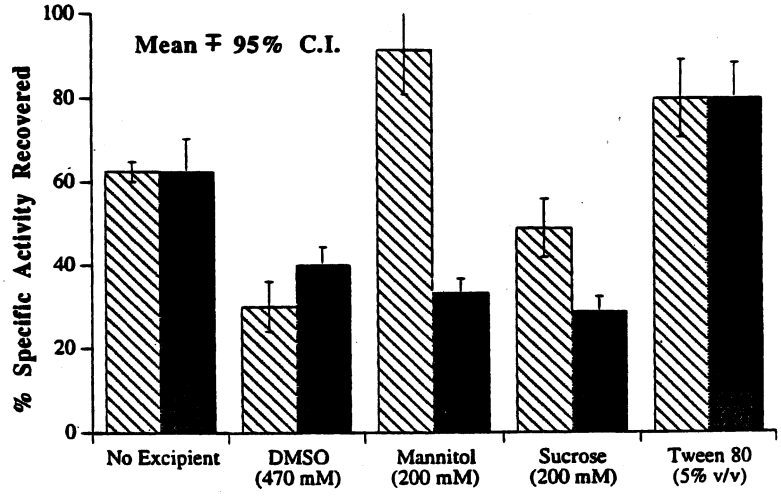

Fig. 4. Influence of different excipients on enzyme activity of aqueous solutions of lysozyme after sonication in the presence and absence of methylene chloride. Five hundred microliters of an aqueous solution containing lysozyme (5 mg) were sonicated with 1 ml of buffer (66 mM phosphate; pH 6.2) (hatched bars) or methylene chloride (dark stippled bars) after addition of each excipient at the denoted concentration. The specific activity of lysozyme recovered in the aqueous phase was determined in each case.

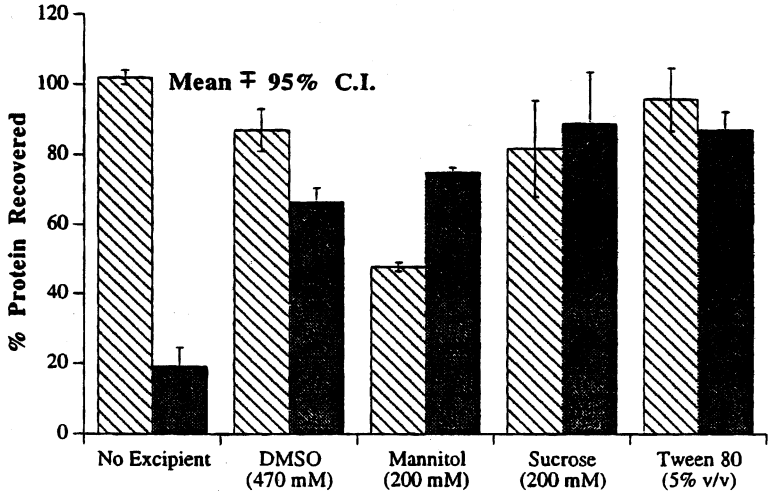

Fig. 5. Excipients protect lysozyme from sonication induced precipitation. Five hundred microliters of an aqueous solution of lysozyme (10 mg/ml) were sonicated with 1 ml of either buffer (66 mM phosphate; pH 6.2) (hatched bars) or methylene chloride (dark stippled bars) and the denoted excipient and then the amount of soluble protein recovered in the aqueous phase was determined.

tain any excipients (Fig. 4). When sonication was performed in the presence of methylene chloride, all of the excipients with the exception of tween 80 led to greater loss of specific activity compared to the control. All of the excipients used increased the percentage of protein recovered in the aqueous phase after sonication in the presence of methylene chloride (Fig. 5). However, protein recovery was low when lysozyme was sonicated in the presence of mannitol and buffer. The reasons for this poor recovery are not clear.

Protection of lysozyme by the nonionic detergent tween 80 but not by other excipients during sonication suggests that the interfacial area between aqueous and organic phases plays a significant role in decreasing the specific activity of the enzyme. Measuring the amount of precipitate formed can monitor the role of the interface in denaturing lysozyme. The fact that all excipients reduced precipitate formation (as evidenced by recovery of protein in the aqueous extract of sonicated mixtures) indicated that the effect of the aqueous–organic interface on denaturation was reduced, if not eliminated. Yet, the decrease in specific activity was greater in the presence of these excipients than the control. The interactions between lysozyme and the excipient during sonication appear to inactivate lysozyme in spite of protection from precipitate formation.

One mechanism of damage could be chemical modification of the substrate binding site or catalytic site by free radicals. Generation of free radicals during sonication due to pyrolysis of solvent and/or solute molecules leading to inactivation of enzymes has been demonstrated (Coakley et al., 1973; Kashkooli et al., 1980; Schmidt et al., 1988). Hydroxyl radicals generated by sonolysis of water or free radicals produced secondarily by the attack of hydroxyl radicals on other molecules can modify the chemical structure of the lysozyme with consequent loss of activity.

Reisz and coworkers (Misik et al., 1995) have demonstrated free radical production (especially hydroxyl radicals) during sonolysis of aqueous solutions by using the spin trapping technique. While these studies demonstrated the formation of free radicals in aqueous solutions and in aqueous–organic solvent mixtures, free radical formation during the microencapsulation process has not been studied previously.

Since sonolysis of water is known to yield hydroxyl radicals (Misik et al., 1995), we tested the

effect of the hydroxyl radical scavenger DMSO as an excipient during sonication of lysozyme. DMSO failed to protect against sonication-induced loss of enzyme activity in the aqueous phase. DMSO is an effective free radical scavenger that reacts with hydroxyl radicals to generate methyl radicals which forms a spin adduct with (Fig. 1B). This adduct can be detected using ESR spectroscopy. Including PBN during sonication of lysozyme dissolved in buffer containing DMSO yielded the methyl radical adduct of PBN resulting from the capture of methyl radicals. The methyl adduct of PBN was identified by ESR spectroscopy (spectrum shown in Fig. 6). PBN (18,027-0) was purchased from Aldrich chemicals, Milwaukee, WI.

Based on the results with DMSO, it appears that free radicals are formed during sonication. Attempts to detect free radicals in the absence of DMSO were not successful. This is not surprising in view of the rather short half life of the hydroxyl spin adduct of PBN (Sridhar et al., 1986). The formation of the methyl spin adduct of PBN when DMSO was included, was strong, albeit indirect evidence for hydroxyl radical formation. It is important that the excipient used in manufacture of the microencapsulated dosage form protects protein against free radical mediated damage and against denaturation leading to precipitation. In addition, the excipient itself should not be a source of deleterious free radicals as is clearly the case with DMSO. More conclusive evidence of free radical mediated structural alterations of lysozyme would require careful analyses of the damaged protein by mass spectrometry and infrared spectroscopy.

A modification of the double emulsion technique has been described (Johnson et al., 1997; Tracy, 1998). In this modification, surfactants and water–organic solvent interface are avoided by using lyophilized protein at cryogenic temperatures. However, the initial step in this technique also relied on sonication of an aqueous solution of the protein. Our investigation shows that sonication induced damage occurs in the presence and in absence of the organic solvent and surfactants. While the presence of trace impurities can destabilize the protein being encapsulated, we believe that this is the first demonstration of damage to the protein by excipients during the primary emulsion formation by sonication. This emphasizes the need to scrutinize the beneficial as well as the harmful effects of standard excipients carefully during microencapsulation.

4. Conclusion

This investigation shows that even a relatively stable enzyme such as lysozyme is denatured and inactivated to a significant extent during the initial step (creation of the primary emulsion) of the microencapsulation process. Loss of enzyme activity occurred during sonication even in the absence of methylene chloride. Sonication of lysozyme solution in the presence of methylene chloride caused a decrease in stability of lysozyme as judged by the loss of enzymatic activity in the soluble fraction and by the formation of an inactive insoluble fraction. The creation of a large interfacial area between the aqueous and organic phases promoted protein precipitation. The precipitates were held together by non-covalent interactions. It is highly likely that the protein precipitate would remain trapped within the microspheres following microencapsulation and not be bioavailable. This could explain the aggregates observed within microspheres (Lu and Park, 1995) and the incomplete release of proteins from microspheres (Schwendeman et al., 1996).

No fragmentation, aggregation, or changes to the surface charge of lysozyme were detected in the soluble fraction, indicating that these changes cannot be the reason for the loss of enzyme activity of the soluble fraction. All the excipients studied, decreased protein precipitation. Only tween 80 prevented the loss of enzyme activity in the soluble fraction. The other excipients led to a greater loss of specific activity than the control. While reducing the air–water interface and the water–methylene chloride interface prevented protein precipitation and possibly even loss of enzyme activity, the interaction between lysozyme and the excipient during sonication damaged lysozyme instead of preserving its stability during sonication.

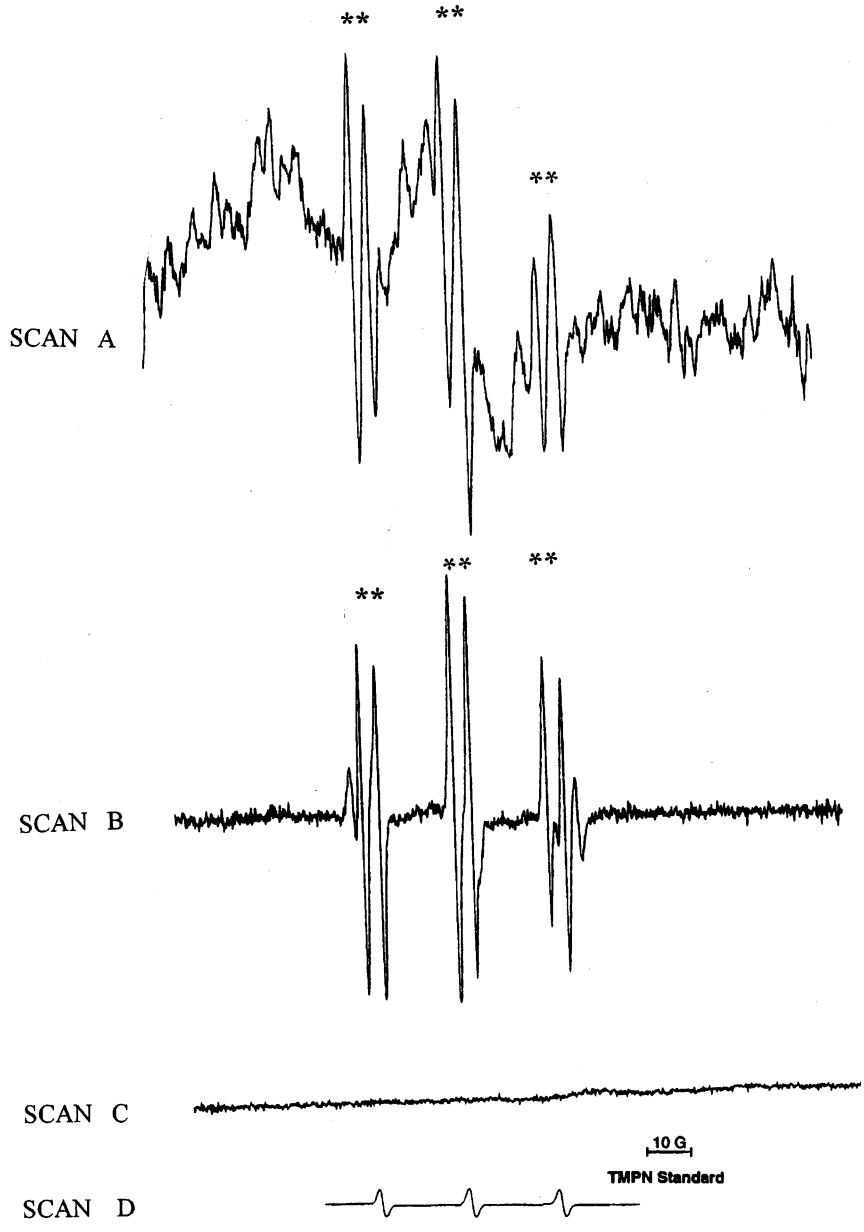

Fig. 6. ESR detection of methyl radicals formed during sonication of aqueous solutions containing DMSO. (A) ESR spectrum of a solution obtained by after sonication (15 s at 50 W) of 1 ml of an aqueous solution of PBN (0. 1 M) mixed with 0.5 ml of DMSO (14.08 M). The asterisks indicate the signals due to the methyl adduct of PBN. (B) ESR spectrum of the methyl adduct of PBN produced by the reaction of dimethyl sulfoxide (1 M) with hydroxyl radicals (produced by the action of 250 μM ferrous sulfate and 2 mM hydrogen peroxide). (C) ESR trace showing that no stable spin adducts are obtained after sonication of 1 ml of an aqueous solution of PBN in buffer. (D) ESR spectrum of a solution of 2,2,6,6-tetramethyl piperidin-4-ol *N*-oxide (TMPN) used as a nitroxyl standard. The horizontal bar represents a scan width of 10 gauss.

Electron spin resonance studies using PBN as a spin trap revealed the formation of methyl radicals during sonication of mixtures containing DMSO. The results suggest that the excipients used to stabilize the protein during the early steps of microencapsulation may inactivate the protein during sonication.

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