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Conformational stability of a model protein (bovine serum albumin) during primary emulsification process of PLGA microspheres synthesis

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Abstract

The goal of this study was to investigate the conformational stability of a model protein, bovine serum albumin (BSA), during the primary emulsification process of poly(D,L-lactide-co-glycolide) (PLGA) microspheres preparation. Differential scanning calorimeter (DSC) was utilized to assess the conformational structure of BSA during primary emulsification in the presence and absence of PLGA. Three excipients [i.e. mannitol, hydroxypropyl-β-cyclodextrin (HP-β-CD) and sodium dodecyl sulfate (SDS)] were investigated for their stabilizing effect on BSA during emulsification process. The DSC profile of intact BSA was best fitted by a non-2-state model with two peaks, which have midpoint temperatures (T_{m1}, 60.9 ± 0.4 °C and T_{m2}, 66.4 ± 1.0 °C), respectively, and a total calorimetric enthalpy ΔH_{tot} of 599 ± 42 kJ/mol. After emulsifying BSA aqueous solution with methylene chloride, an additional apparent peak at a higher temperature was observed. The T_m of this peak was 77.4 ± 0.8 °C. HP-β-CD was able to suppress the occurrence of an additional peak, whereas mannitol failed. SDS increased the thermal stability of BSA dramatically. Furthermore, HP-β-CD increased BSA recovery from 72 ± 8% to 89 ± 7% after extraction from w/o in the presence of PLGA. These results provided evidence that HP-β-CD could be a promising excipient for conformational stability of BSA during synthesis of PLGA microspheres.

Keywords: Emulsification; Conformational stability; Protein; BSA; Poly(D,L-lactide-co-glycolide); Differential scanning calorimetry

1. Introduction

In recent years, biodegradable poly(D,L-lactide-co-glycolide) (PLGA) microspheres have received much attention for their capability to control the release of bioactive macromolecules such as peptides and proteins for a desired period of time (Cohen et al., 1991; Gupta et al., 1997; Cleland, 1998; Crotts and Park, 1998). As a novel delivery system, biodegradable microspheres are suitable for many proteins and peptides which have short biological half-life or poor bioavailability. Protein or peptide-loaded microspheres have been commercially available such as Lupron Depot® (TAP Pharmaceutical Products Inc., Lake Forest, IL) and more recently Nutropin Depot™ (Genentech, Inc., South San Francisco, CA) for long-term delivery of leuprolide acetate and recombinant human growth hormone, respectively.

Water-in-oil-in-water (w/o/w) emulsion technique is commonly used to encapsulate the water-soluble proteins into a polymeric matrix (Jeffery et al., 1993; Yang et al., 2001). In this method, a primary emulsion

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(w/o) is created by emulsifying the protein aqueous solution with a continuous organic phase containing PLGA. The primary emulsion is then mixed in an aqueous solution containing surfactant to produce a double emulsion (w/o/w). Microspheres are formed during solvent removal and can be collected by centrifugation or filtration. However, some drawbacks related to microsphere preparation make it difficult to encapsulate protein into polymer matrix keeping its full native structure and/or biological activity (van de Weert et al., 2000a). Protein stability has been a concern due to exposure to organic solvents, sonication, and mechanical shear forces during microspheres preparation (Morlock et al., 1998; Kim and Park, 1999; Krishnamurthy et al., 2000). All of these stresses can be destructive to protein hierarchy structure as well as biological activity. Aggregation, deamidation and other protein stability problems have been reported during the microencapsulation of proteins (Schwendeman et al., 1996; Jiang et al., 2002). Protein denaturation may lead to the formation of an antibody that gives unwanted immune responses. In addition, protein aggregates have been suggested to lead to incomplete release from microspheres (Park et al., 1998).

The primary emulsification step has been suggested as a major cause for protein denaturation and aggregation (Morlock et al., 1997). 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 (Krishnamurthy et al., 2000). All of these factors can denature proteins. More importantly, sonication produces a large interface between aqueous and organic phases. Proteins may be adsorbed and denatured at the interface (Sah, 1999a,b; van de Weert et al., 2000b). In spite of the wide use of microencapsulation of proteins into PLGA microspheres by 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 (Sah, 1999a,b; van de Weert et al., 2000b). In particular, the current available information is limited to 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 (van de Weert et al., 2000b).

We have recently reported extraction method for protein from PLGA-containing primary emulsion (Kang et al., 2002). Low molecular weight poly(ethylene glycol) (PEG 400) was used in the above extraction method. PEG 400 can break PLGA-containing emulsion by dissolving the polymer. Therefore, protein recovery from PLGA-containing emulsion increased dramatically, which allowed us to study the properties of protein in this system. Differential scanning calorimeter (DSC) measures the change of enthalpy upon thermal denaturation of proteins (ΔH) and the midpoint temperature of the denaturation (T_m). Since DSC allows 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 used in protein aqueous formulation screening studies (Remmele et al., 1998; Kang et al., 2002).

Among all protein stabilization approaches associated with solvent extraction/evaporation technique (van de Weert et al., 2000a), the use of excipients in the protein aqueous phase is one of the effective methods. A number of excipients have been investigated for their effects on protein stability. The most representative types of stabilizing excipients are sugars (Cleland and Jones, 1996), surfactants (Krishnamurthy et al., 2000) and cyclodextrins (Sah, 1999b; Kang et al., 2002). In this study, we investigated the conformational stability of a model protein, bovine serum albumin (BSA), at water/organic solvent interface in the absence and presence of PLGA using DSC. We studied the protein behavior in the presence of PLGA using our recently reported extraction procedure (Kang et al., 2002). We selected mannitol, hydroxypropyl-β-cyclodextrin (HP-β-CD) and sodium dodecyl sulfate (SDS) representing sugar, cyclodextrin and surfactant, respectively. These excipients were studied for their ability to conformationally stabilize BSA during primary emulsification process.

2. Materials and methods

2.1. Materials

BSA, mannitol, PEG 400 and SDS were purchased from Sigma Chemical Company (St. Louis, MO).

HP-β-CD was obtained from Aldrich Chemical Company (Milwaukee, WI). Methylene chloride was purchased from Fisher Chemical Company (Fair Lawn, NJ). PLGA (50:50, inherent viscosity 0.63 dl/g in hexafluoroisopropanol at 30 °C) was obtained from Birmingham Polymer, Inc. (Birmingham, AL). Micro-BCA protein assay reagent kit was received from Pierce Chemical (Rockford, IL).

2.2. Preparation of primary emulsions and their de-emulsifications for protein recovery and stability

Forty milligram of BSA was dissolved in 0.2 ml of phosphate-buffered saline (PBS, pH 7.4, 10 mM). This aqueous protein solution was then emulsified with 3 ml of methylene chloride in the absence and presence of 400 mg of PLGA. The emulsification was carried out by a microtip equipped ultrasonicator (Sonifier® cell disruptor, Model W185, Heat System-Ultrasonics, Inc., Plainview, NY) for 30 s at 40 W. After emulsification, BSA was extracted into aqueous phase by adding 20 ml of PBS and then centrifuged at 3000 × *g* for 20 min to accelerate phase separation.

In case of PLGA-containing emulsion, this extraction process was modified as previously reported (Kang et al., 2002). Briefly, 1 ml of PEG 400 was added to the PLGA-containing emulsion. The clarity of this emulsion increased markedly after gentle shaking. Then 19 ml of PBS was added to this system before further centrifugation. The aqueous phase was subjected to protein quantification and DSC measurement. In some cases, excipients such as mannitol, HP-β-CD, and SDS were added to the aqueous protein solution to test their effects on conformational stability of BSA during primary emulsification. Forty milligram of mannitol or HP-β-CD was used with the aqueous protein solution. However, SDS was used as BSA/SDS molar ratio of 1:10.

2.3. Protein quantification

BSA content in the samples was determined by the MicroBCA protein assay (Smith et al., 1985) using appropriate reagent blank and standard curves. Protein recovery was reported as the percentage of protein extracted into the aqueous phase following centrifugation.

2.4. Conformational stability of BSA by differential scanning calorimeter

DSC measurements were carried out using an ultra-sensitive differential scanning calorimeter (VP-DSC, MicroCal, Northampton, MA). It is especially designed for studies on dilute aqueous solutions of biological macromolecules. All samples and buffers were degassed by stirring under vacuum before loading into the sample and reference cells. The heat flow required to keep the sample cell and reference cell thermally balanced was recorded from 25 to 95 °C, using scan rate of 1.5 °C/min. 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 provided with the instrument.

3. Results

3.1. BSA stability during primary emulsification in the absence of PLGA

To simulate the water/organic solvent volume ratio as in microsphere fabrication, we used 0.2 ml of PBS and 3 ml of methylene chloride in all sample preparations. The BSA concentration was 200 mg/ml. Table 1 summarizes the results on recovery and DSC thermal parameters of BSA recovered in aqueous phase after emulsification with methylene chloride in the absence of PLGA. The recovery of BSA was more than 90%, which is similar to the result obtained by Sah when low concentrations of human serum albumin (0.2–1 mg/ml) were emulsified with methylene chloride (Sah, 1999b). We did not observe any significant difference in BSA recovery between samples in the absence and presence of excipients (i.e. mannitol, HP-β-CD and SDS). However, the recovered BSA showed diverse DSC profiles (Table 1 and Fig. 1). The deconvolution of intact BSA (negative control) curve revealed that the apparent one peak with a shoulder at the right side was best fitted by a non-2-state model with two peaks, which

Table 1
BSA recovery and thermal parameters after emulsifying with methylene chloride in the absence of PLGA (mean ± S.D., $n = 3$)

	Recovery (%)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	ΔH_1 (kJ/mol)	ΔH_2 (kJ/mol)	ΔH_3 (kJ/mol)	ΔH_{tot} (kJ/mol)
Control (−)	–	60.9 ± 0.4	66.4 ± 1.0	–	289 ± 8	310 ± 46	–	599 ± 42
Control (+mannitol)	–	59.7 ± 2.0	67.9 ± 1.5	–	312 ± 42	281 ± 24	–	593 ± 65
Control (+HP-β-CD)	–	60.1 ± 0.1	64.5 ± 1.1	–	385 ± 67	260 ± 63	–	645 ± 20
Control (+SDS)	–	81.8 ± 0.2	–	–	1197 ± 21	–	–	1197 ± 21
No excipient	98 ± 7	62.5 ± 0.6	66.4 ± 2.2	77.4 ± 0.8	276 ± 38	301 ± 59	63 ± 13	624 ± 50
Mannitol	99 ± 7	63.0 ± 2.3	68.1 ± 3.6	77.3 ± 0.5	297 ± 54	306 ± 100	63 ± 17	666 ± 29
HP-β-CD	99 ± 8	62.4 ± 0.6	70.2 ± 0.6	–	490 ± 80	193 ± 38	–	682 ± 71
SDS	94 ± 7	81.1 ± 0.3	–	–	1101 ± 92	–	–	1101 ± 92

represent two independent transitions (Fig. 2A). The transition temperatures of these two peaks were T_{m1} 60.9 ± 0.4 °C and T_{m2} 66.4 ± 1.0 °C, respectively, and the ΔH_{tot} was 599 ± 42 kJ/mol.

Although there was no significant difference among the ΔH_{tot} of all samples except those with SDS, we found some difference in transition temperatures. After emulsifying BSA aqueous solution in the absence of any stabilizer with methylene chloride, an additional apparent peak at higher temperature was observed in recovered BSA thermogram. The T_m of this peak was 77.4 ± 0.8 °C (Fig. 2B). Mannitol failed to stabilize BSA in comparison to the control (without any excipient). A similar additional peak was found at 77.3 ± 0.5 °C in the presence of mannitol.

HP-β-CD affected the DSC profile of recovered BSA to a great extent. Table 1 and Fig. 1 showed that HP-β-CD suppressed the appearance of an additional peak. Transition temperatures T_{m1} and T_{m2} were 62.4 ± 0.6 °C and 70.2 ± 0.6 °C, respectively, slightly higher than those found in controls (intact BSA negative control as well as the intact BSA in the presence of HP-β-CD). However, its thermal profile was comparable to the controls.

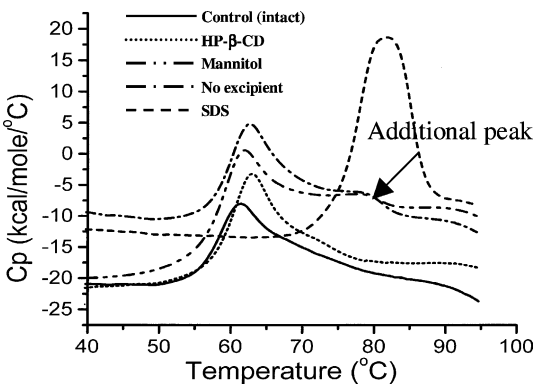

Fig. 1. DSC thermograms of BSA recovered from w/o emulsion in the absence of PLGA. Samples with different excipients were shown by different lines.

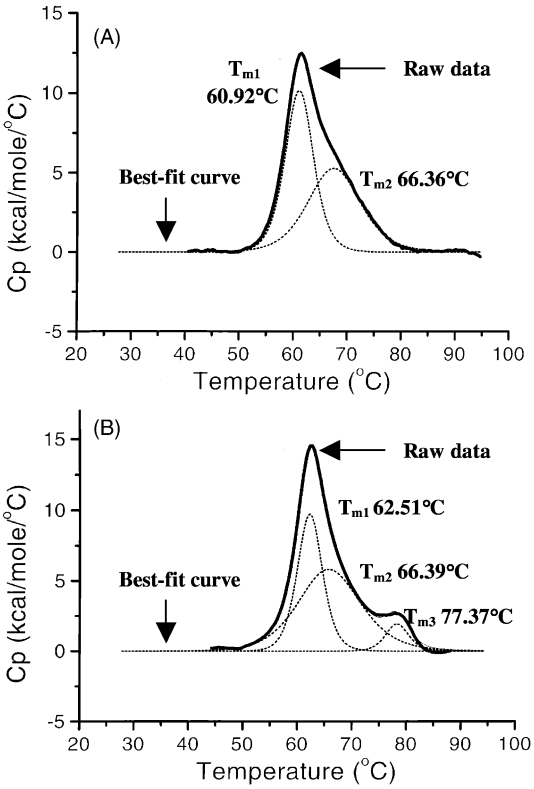

Fig. 2. DSC thermogram of intact BSA (A) and BSA recovered from w/o emulsion without PLGA and any excipient (B). The bold line is experimental raw data and the broken lines represent the best-fit curves deconvoluted using Origin software.

Table 2
BSA recovery and thermal parameters after emulsifying with methylene chloride in the presence of PLGA (mean ± S.D., $n = 3$)

	Recovery (%)	T_{m1} (°C)	T_{m2} (°C)	ΔH_1 (kJ/mol)	ΔH_2 (kJ/mol)	ΔH_{tot} (kJ/mol)
Control	–	58.7 ± 0.7	68.4 ± 0.9	523 ± 75	201 ± 84	724 ± 159
No excipient	72 ± 8	63.4 ± 0.5	71.6 ± 1.9	578 ± 25	126 ± 50	703 ± 75
HP-β-CD	89 ± 7[a]	62.0 ± 2.0	69.6 ± 1.4	565 ± 130	180 ± 67	741 ± 163
SDS	82 ± 5	79.3 ± 0.4	–	1126 ± 46	–	1126 ± 46
Mannitol	31 ± 9[a]	65.4 ± 3.2	73.2 ± 2.0	557 ± 4	180 ± 29	737 ± 29

[a] $P < 0.05$, t-test.

SDS changed dramatically the BSA thermal behavior. Fig. 1 shows an increase of total peak area (ΔH_{tot}) and T_m of BSA recovered from w/o emulsion in the absence of PLGA. Its curve was deconvoluted by a non-2-state model with one peak which has T_m 81.1 ± 0.3 °C and ΔH_{tot} 1101 ± 92 kJ/mol. These values were similar to the BSA control in the presence of SDS, which has T_m 81.8 ± 0.2 °C and ΔH_{tot} 1197 ± 21 kJ/mol.

3.2. BSA stability during primary emulsification in the presence of PLGA

Table 2 summarizes the results on recovery and DSC thermal parameters of BSA recovered in aqueous phase from primary emulsion containing PLGA. DSC thermal profiles are shown in Fig. 3. The baselines of BSA (except the curve with SDS) had a rapid downward shift after occurrence of the first apparent endothermic peak. This downshift of baseline is probably caused by addition of PEG 400 which may lead to precipitation and aggregation of BSA molecules at relatively high temperature range. As a result, the unstable baseline disguised the possible thermal transitions at higher temperature range and made it difficult to detect the third peak as observed in Fig. 1. Interestingly, PEG 400 barely affected the thermal behavior of recovered BSA in the presence of SDS, which showed T_m 79.3±0.4 °C and ΔH_{tot} 1126±46 kJ/mol.

The recoveries of BSA in the presence of PLGA (Table 2) were significantly lower than those in the absence of PLGA (Table 1). The recovery of BSA without excipient was 72 ± 8%. HP-β-CD increased ($P < 0.05$) BSA recovery to 89 ± 7%. SDS did not increase ($P > 0.05$) the BSA recovery. However, mannitol considerably decreased ($P < 0.05$) the recovery of BSA in comparison to the control (BSA recovery without excipient).

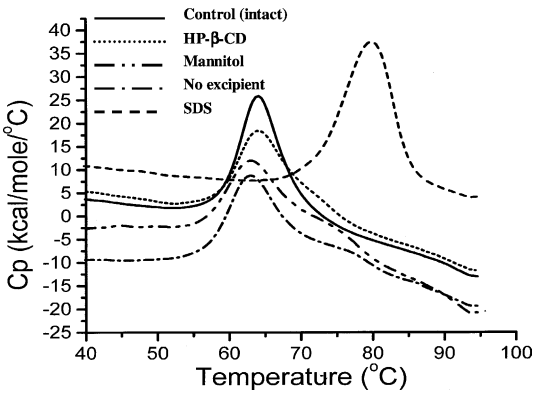

Fig. 3. DSC thermograms of BSA recovered from w/o emulsion in the presence of PLGA. Samples with different excipients were shown by different lines.

4. Discussion

The biological activity of a protein largely depends on its conformational structure (Price, 2000). In this study, DSC was utilized to monitor the conformational structure of a model protein, BSA, during emulsification process. We observed two transition peaks for intact BSA which had T_{m1} 60.9±0.4 °C and T_{m2} 66.4± 1.0 °C, respectively (Fig. 2A). The three-dimensional structure of BSA molecule is similar to that of human serum albumin (HSA), which is made up of three homologous domains that assemble to form a heart-shaped molecule (He and Carter, 1992). It has been suggested that BSA molecule was separated into two thermally independent units due to the crevice in the vicinity of Trp 212 (Yamasaki et al., 1990). It was also hypothesized that the peak of low temperature

transition corresponds to the collapse of N-terminal BSA domain and the high temperature calorimetric domain is due to cooperative unit composed of the other two BSA domains (Giancola et al., 1997).

The third peak observed in BSA thermogram after emulsifying with methylene chloride in the absence of stabilizer is due to either the conformational changes or the formation of water-soluble aggregate of BSA (Sah, 1999b). Formation of a new water-soluble aggregate of BSA after emulsification with methylene chloride is a reason for further aggregation during microsphere synthesis and the overall incomplete release of proteins from PLGA microspheres (Park et al., 1998).

The protective effect of sugars (mannitol, sucrose and trehalose) on proteins during emulsification process of microsphere synthesis is debatable. Stabilizing effect of trehalose and mannitol on human growth hormone was observed during emulsification with methylene chloride (Cleland and Jones, 1996). However, sugars failed to protect lysozyme (Sah, 1999b; van de Weert et al., 2000b; Kang et al., 2002), and erythropoietin (Morlock et al., 1997) from emulsification-induced aggregation. In this study, similar DSC profile was obtained with and without mannitol indicating that mannitol barely prevented the conformational change of BSA induced by emulsification. After adding PLGA to methylene chloride, the BSA recovery in the presence of mannitol decreased sharply to $31 \pm 9\%$, much lower than the control ($72 \pm 8\%$). This may be due to greater adsorption of BSA molecules on PLGA in the presence of mannitol. These observations suggest that mannitol is not a good stabilizing excipient for BSA during emulsification process.

HP-β-CD was found to be a promising stabilizer during primary emulsification in the absence of polymer for proteins such as lysozyme (Sah, 1999b; Kang et al., 2002), ovalbumin (Sah, 1999b) and erythropoietin (Morlock et al., 1997). The stabilizing capability of HP-β-CD was based on its ability to shield hydrophobic amino acid residues of proteins (Matsubara et al., 1997; Sah, 1999b). The hydrophilicity of proteins would increase after the aromatic rings of tryptophan, tyrosine, and phenylalanine inserting into the hydrophobic cavities of cyclodextrins. Such an increase in the hydrophilicity keeps proteins away from methylene chloride/water interface and therefore reduces the aggregation and denaturation of proteins (Sah, 1999b). Our data showed that BSA had almost full recovery from water/methylene chloride interface if PLGA was not involved in this system. Our findings also showed that BSA was very resistant to aggregation associated with exposure to methylene chloride. However, BSA recovery decreased in the presence of PLGA (Table 2). This may be due to BSA adsorption to PLGA during primary emulsification. HP-β-CD prevented this adsorption to some extent by increasing recovery of BSA from $72 \pm 8\%$ to $89 \pm 7\%$.

It has been reported that the BSA has more than 70 binding sites for SDS, the first 10–11 are stronger than the rest (Decker and Foster, 1966). Previous DSC study showed that the SDS increased thermal stability of BSA up to SDS/BSA molar ratio of 10 (Giancola et al., 1997). The ΔH_{tot} increased about 67% and T_m increased about 20 °C due to the strong binding of the first 10–12 surfactant molecules (Giancola et al., 1997). The BSA molecules were most compact because of the rearrangement of higher-order structure of BSA by the binding of 12 SDS molecules (Yamasaki et al., 1996). The SDS has also been reported to significantly prevent insulin aggregation induced by water/methylene chloride interface when present in insulin/SDS molar ratio of 1:10 and 1:20 (Kwon et al., 2001). The SDS and negatively charged insulin at pH 7.4 form insulin–SDS complex which would have a net negative charge. The charge repulsion between the insulin–SDS complex and the negatively charged hydrated SDS polar heads at the interface protected insulin from hydrophobic methylene chloride interface (Kwon et al., 2001). We used BSA/SDS in the molar ratio of 1:10 in our sample preparation. The net charge of BSA at pH 7.1 is −18 (Sivars et al., 1996). BSA–SDS complex should also have a net negative charge as insulin–SDS complex. But, the recovery of BSA in the presence of SDS did not improve ($P > 0.05$) in comparison to the control. However, our data showed about 20 °C increase in T_m (60.9 ± 0.4 °C to 81.8 ± 0.2 °C) and about 100% increase in ΔH_{tot} (599 ± 42 kJ/mol to 1197 ± 21 kJ/mol). These data indicate greater thermal stability of BSA as compared to the control.

We performed recovery experiments with lower concentrations of BSA (5 and 20 mg/ml) from emulsions containing PLGA. We observed 37 and 36% recovery of BSA from 5 and 20 mg/ml solutions,

respectively, in the presence of mannitol. The recovery was 31% from 200 mg/ml BSA solution in the presence of mannitol. However, we did not observe any decrease in the recovery of BSA in the presence of mannitol from emulsion without PLGA. Decrease in the recovery was only observed when PLGA was present in the emulsion. This is due to greater adsorption of BSA molecules with higher concentrations of BSA on PLGA in the presence of mannitol. However, we observed an increase in recovery of BSA with its increasing concentrations in emulsions containing PLGA in the presence of SDS. We found 63, 70, and 82% recovery of BSA from 5, 20 and 200 mg/ml solution, respectively, in the presence of SDS at BSA:SDS molar ratio of 1:10. Thus, the recovery of BSA in the presence of excipients from emulsions containing PLGA would vary with varying concentrations of BSA.

5. Conclusions

A different thermal behavior of BSA after emulsification with methylene chloride was found in comparison to the intact BSA. HP-β-CD showed its ability to prevent this change to a great extent. Also, HP-β-CD increased the BSA recovery from w/o emulsion in the presence of PLGA. These findings made HP-β-CD a promising stabilizer for BSA during microsphere preparation. Mannitol failed to stabilize BSA during primary emulsification. SDS increased the thermal stability of BSA dramatically. Its potential use for conformational stability and biological activity of proteins is under active investigation in our laboratory.

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References

Cleland, J.L., 1998. Solvent evaporation processes for the production of controlled release biodegradable microsphere formulations for therapeutics and vaccines. Biotechnol. Prog. 14, 102–107.

Cleland, J.L., Jones, A.J., 1996. Stable formulations of recombinant human growth hormone and interferon-gamma for microencapsulation in biodegradable microspheres. Pharm. Res. 13, 1464–1475.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H., Langer, R., 1991. Controlled delivery systems for proteins based on poly-(lactic/glycolic acid) microspheres. Pharm. Res. 8, 713–720.

Crotts, G., Park, T.G., 1998. Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J. Microencapsul. 15, 699–713.

Decker, R.V., Foster, J.F., 1966. The interaction of bovine plasma albumin with detergent anions. Stoichiometry and mechanism of binding of alkylbenzenesulfonates. Biochemistry 5, 1242–1254.

Giancola, C., De Sena, C., Fessas, D., Graciano, G., Barone, G., 1997. DSC studies on bovine serum albumin denaturation. Effects of ionic strength and SDS concentration. Int. J. Biol. Macromol. 20, 193–204.

Gupta, R.K., Alroy, J., Alonso, M.J., Langer, R., Siber, G.R., 1997. Chronic local tissue reactions, long-term immunogenicity and immunologic priming of mice and guinea pigs to tetanus toxoid encapsulated in biodegradable polymer microspheres composed of poly lactide-co-glycolide polymers. Vaccine 15, 1716–1723.

He, X.M., Carter, D.C., 1992. Atomic structure and chemistry of human serum albumin. Nature 358, 209–215.

Jeffery, H., Davis, S.S., O'Hagan, D.T., 1993. The preparation and characterization of poly(lactide-co-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique. Pharm. Res. 10, 362–368.

Jiang, G., Woo, B.H., Kang, F., Singh, J., DeLuca, P.P., 2002. Assessment of protein release kinetic, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. J. Control. Release 79, 137–145.

Kang, F., Jiang, G., Hinderliter, A., DeLuca, P.P., Singh, J., 2002. Lysozyme stability in primary emulsification for PLGA microsphere preparation: effect of recovery methods and stabilizing excipients. Pharm. Res. 19, 629–633.

Kim, H.K., Park, T.G., 1999. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. Biotechnol. Bioeng. 65, 659–667.

Krishnamurthy, R., Lumpkin, J.A., Sridhar, R., 2000. Inactivation of lysozyme by sonication under conditions relevant to microencapsulation. Int. J. Pharm. 205, 23–34.

Kwon, Y.M., Baudys, M., Knutson, K., Kim, S.W., 2001. In situ study of insulin aggregation induced by water–organic solvent interface. Pharm. Res. 18, 1754–1759.

Matsubara, K., Irie, T., Uekama, K., 1997. Spectroscopic characterization of the inclusion complex of a luteinizing hormone-releasing hormone agonist, buserelin acetate, with dimethyl-β-cyclodextrin. Chem. Pharm. Bull. 45, 378–383.

Morlock, M., Koll, H., Winter, G., Kissel, T., 1997. Microencapsulation of rh-erythropoietin using biodegradable poly(D,L-lactide-co-glycolide): protein stability and the effects of stabilizing excipients. Eur. J. Pharm. Biopharm. 43, 29–36.

Morlock, M., Kissel, T., Li, Y.X., Koll, H., Winter, G., 1998. Erythropoietin loaded microspheres prepared from biodegradable LPLG-PEO-LPLG triblock copolymers: protein stabilization and in vitro release properties. J. Control. Release 56, 105–115.

Park, T.G., Lee, H.Y., Nam, Y.S., 1998. A new preparation method for protein loaded poly(D,L-lactic-co-glycolic acid) microspheres and protein release mechanism study. J. Control. Release 55, 181–191.

Price, N.C., 2000. Conformational issues in the characterization of proteins. Biotechnol. Appl. Biochem. 31, 29–40.

Remmele, R.L., Nightlinger, N.S., Srinivasan, S., Gombotz, W.R., 1998. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. Pharm. Res. 15, 200–208.

Sah, H., 1999a. Protein behavior at the water/methylene chloride interface. J. Pharm. Sci. 88, 1320–1325.

Sah, H., 1999b. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. J. Control. Release 58, 143–151.

Schwendeman, S.P., Cardamone, M., Klibanov, A., Langer, R., Brandon, M.R., 1996. Stability of proteins and their delivery from biodegradable polymer microspheres. In: Cohen, S., Bernstein, H. (Eds.), Microparticulate Systems for the Delivery of Proteins and Vaccine. Marcel Dekker, New York, pp. 1–46.

Sivars, U., Bergfeldt, K., Piculell, L., Tjerneld, F., 1996. Protein partitioning in weakly charged polymer-surfactant aqueous two-phase systems. J. Chromatogr. B Biomed. Appl. 680, 43–53.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenza, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

van de Weert, M., Hennink, W.E., Jiskoot, W., 2000a. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm. Res. 17, 1159–1167.

van de Weert, M., Hoechstetter, J., Hennink, W.E., Crommelin, D.J., 2000b. The effect of a water/organic solvent interface on the structural stability of lysozyme. J. Control. Release 68, 351–359.

Yamasaki, M., Yano, H., Auki, K., 1990. Differential scanning calorimetric studies on bovine serum albumin. I. Effects of pH and ionic strength. Int. J. Biol. Macromol. 12, 263–268.

Yamasaki, M., Yamashita, T., Yano, H., Tatsumi, K., Aoki, K., 1996. Differential scanning calorimetric studies on bovine serum albumin. IV. Effect of anionic surfactants with various lengths of hydrocarbon chain. Int. J. Biol. Macromol. 19, 241–246.

Yang, Y.Y., Chung, T.S., Ng, N.P., 2001. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22, 231–241.