Improved α-Chymotrypsin Stability Upon Encapsulation in PLGA Microspheres by Solvent Replacement

Ingrid J. Castellanos[1] and Kai Griebenow[2]

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Purpose. To investigate the potential of different solvents with better biocompatibility to replace CH_2Cl_2 in the encapsulation of α-chymotrypsin in poly (lactic-co-glycolic) acid (PLGA) microspheres without causing protein instability.

Methods. The oil-to-water (O:W) ratio in the emulsification step of the solid-in-oil-in-water (s/o/w) encapsulation process was optimized with respect to maximizing protein stability and encapsulation efficiency for various solvents. Formation of insoluble aggregates and residual enzyme activity were primarily used as stability parameters. Several solvents possessing low toxicity with different water solubility were used to prepare α-chymotrypsin loaded PLGA microspheres.

Results. The O:W ratio in the emulsification step is critical with respect to maintaining protein stability. This was related to the solvents' water solubility. In general, hydrophilic solvents were detrimental to protein stability and encapsulation efficiency. However, after optimization of the O:W ratio for solvents with different water solubility, protein stability was preserved during encapsulation using butyl acetate when poly (ethylene glycol) (PEG) was used as the emulsifying agent (ca. 1% of non-covalent aggregates and 93 ± 10% of residual specific activity).

Conclusions. The s/o/w technique was successfully improved by replacing the ICH class 2 solvent CH_2Cl_2 with the class 3 solvent butyl acetate without compromising α-chymotrypsin stability.

KEY WORDS: microencapsulation; protein aggregation; protein stabilization; protein structure; solid-in-oil-in-water (s/o/w) encapsulation procedure.

INTRODUCTION

In recent years, biodegradable microspheres have gained widespread importance in the delivery of pharmaceutical proteins and drugs. However, the choice of organic solvent for their preparation is critical in developing a successful formulation containing a pharmaceutical agent. The most commonly used microencapsulation techniques use methylene chloride and water as dispersed and continuous phase. The use of halogenated alkanes is not desirable from the viewpoints of environmental and human safety since they are suspect to be carcinogenic and tumorigenic (1). Due to these toxicity issues recently there have been a variety of attempts to replace methylene chloride with a safer solvent in the microencapsulation process. For example, ethyl acetate has been considered a better solvent than methylene chloride and for this reason it has been used to encapsulate some proteins and drugs (1–7). Another solvent that has been used to replace methylene chloride is ethyl formate, which is non-chlorinated and is not classified as a carcinogen (8–10). Investigations relating to the effects of these solvents on microspheres quality have been reported (1). Due to the considerable water miscibility of both solvents, some recent studies have focused on the optimization of the O:W ratio during emulsification to successfully obtain microspheres (1,10,11). An optimum O:W ratio of 1:2.5 (or 8 ml of organic solvent and 20 mL of water) has been chosen so as to saturate the aqueous phase by a small proportion of the dispersed solvent and to form successfully embryonic microspheres. Using less organic solvent could lead to the formation of polymer precipitates due to the rapid solidification caused by the fast diffusion of the solvent into the aqueous phase (10,12). Most of these works have focused on the encapsulation efficiency and quality of the microspheres and the data proved the solvents to be effective for the encapsulation of hydrophobic drugs. However, few works have been reported on protein encapsulation using alternative solvents and the issue of protein stability has practically not been addressed to select a safer solvent. In order to select a less toxic solvent to replace methylene chloride we investigated the effect of the O:W ratio on the stability of α-chymotrypsin during encapsulation in PLGA microspheres using the solid-in-oil-in-water (s/o/w) technique. Our working hypothesis was that O:W ratios that cause slow polymer precipitation would cause protein instability. Under these conditions the majority of the solvent will reside in the polymeric microdroplets and some diffusion of the water into the microdroplets will occur (depending on the solubility of water in the solvent) resulting in hydration of the protein or its exposure to the oil-water interface. To test this, ethyl acetate and methylene chloride were used as solvents to study the effect of different O:W ratios on protein stability, loading efficiency, and microsphere characteristics. After the optimization of the O:W ratio, we investigated some other solvents possessing low toxicity with different water solubility (0.6–26.8 wt%) for their usefulness to prepare biodegradable α-chymotrypsin loaded PLGA microspheres. In addition, we studied the effect of other key parameters in the process, (eg, the emulsifying agent PEG or PVA) on α-chymotrypsin stability and microsphere characteristics using those solvents. The results of this work permit the development of an optimized method to prepare PLGA microspheres with minimal loss of α-chymotrypsin activity, a negligible amount of non-covalent aggregates, and high loading efficiency, considering suitable toxicological parameters.

MATERIALS AND METHODS

Materials

α-Chymotrypsin, poly (vinyl) alcohol (87%–89% hydrolyzed, M_W of 13,000–23,000), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, ammonium thiocyanate, anhydrous ferric chloride and poly(ethylene glycol) (M_W 8,000) were obtained from Sigma. Poly(D,L-lactic-*co*-glycolic) acid with a copolymer ratio of 85:15 [lactide:glycolide] and molecular weight of 35,000 was a generous gift from Alkermes Inc., Cambridge, MA. Methylene chloride (99.9%, ACS HPLC grade) was from Fischer; chloroform, ethyl acetate, butyl acetate and ethyl formate were from Sigma; methyl isobutyl ketone, methyl ethyl ketone and 2-pentanone were from Acros.

[1] Department of Chemistry, University of Puerto Rico, Río Piedras Campus, PO Box 23346, San Juan, PR 00931-3346, USA

[2] To whom correspondence should be addressed (e-mail: griebeno@adam.uprr.pr)

Bicinchoninic acid assay kit was from Pierce. All other chemicals were from various suppliers and the purity of analytical grade or better.

Protein Lyophilization

α-Chymotrypsin was dissolved in deionized water (10 mg/ml, pH 7.8) and PEG was co-dissolved at a 1:4 mass ratio of protein-to-PEG. These samples were frozen in liquid N_2 and lyophilized using a Labconco FreeZone 6L at a condenser temperature of –45°C and a pressure of <60 μm of Hg for 48 h. When the powder thus obtained was suspended in methylene chloride, the particle size was <1 μm as determined by scanning electron microscopy due to the known micronization effect afforded by co-lyophilization with PEG (13,14).

Microsphere Preparation

Two-hundred milligrams of α-chymotrypsin lyophilized with PEG was suspended in 2 ml (or 1 ml) of solvent containing 360 mg of PLGA by homogenization with a VirTis Tempest homogenizer using a 10-mm shaft at 20,000 rpm for 30 s. The resulting suspension was added to an aqueous solution (10% w/v) of the emulsifying agent (PVA or PEG). The oil-in-water emulsion was formed by homogenization (20,000 rpm, 2 min) and the microspheres were collected by filtration through a 0.45 μm cellulose acetate filter, washed with 100 ml of distilled water and dried for 24 h at <60 μm of Hg (15).

Determination of Protein Loading and Encapsulation Efficiency of Microspheres

The actual protein loading of microspheres was determined following the method developed by us (16). Protein aggregates formed during the encapsulation process are taken into account in this procedure. To determine the amount of non-soluble protein aggregates, protein-loaded microspheres were suspended in 1 ml of ethyl acetate to dissolve the PLGA followed by centrifugation. The supernatant was discarded and the pellet was vacuum dried. Next, 1 ml of phosphate buffer was added to the protein powder to dissolve the soluble protein fraction. The buffer-insoluble protein fraction was treated with 1 mL of 6 M urea solution to dissolve non-covalent aggregates. The protein concentration in the solutions obtained was determined from the absorbance at 280 nm or by bicinchoninic acid assay. The encapsulation efficiency of protein in the microspheres was calculated from the actual loading with respect to the theoretical loading of protein (%, w/w) in the microspheres.

Scanning electron microscopy (SEM) was exactly performed as described by us (16).

Determination of Enzyme Activity

Activity of α-chymotrypsin was determined using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate; 60 μl of the enzyme solution was mixed with 240 μl of 0.35 mM substrate in 0.1 M Tris-HCl buffer, 0.01 M $CaCl_2$, pH 7.8 (17). The time-dependent absorbance change at 410 nm was measured and the linear portion of the graph was used to obtain the initial velocities (V_0). To determine the activity of α-chymotrypsin after encapsulation, ethyl acetate was used to dissolve PLGA. It was verified by performing control experiments that exposure of the enzyme to ethyl acetate is not detrimental to the enzyme activity (16).

FTIR Spectroscopy

FTIR studies were performed using a Nicolet Magna-IR 560 optical bench (7,18). The spectra of protein in aqueous solution, in amorphous dehydrated powders, and encapsulated in PLGA microspheres were obtained as described in detail in the literature (17) and corrected for the background. The proper subtraction of the PLGA background from FTIR spectra obtained for α-chymotrypsin in microspheres was performed as described in detail (18,19). All spectra were analyzed for the number and position of components in the amide I region (1700–1600 cm^{-1}) by calculation of the second derivative spectra (20).

The secondary structure of α-chymotrypsin was determined by Gaussian curve-fitting of the amide I band as described by us in detail (21). Fourier self-deconvolution (FSD) was used prior to this procedure as the band narrowing method using the program OMNIC 3.1 and values of 24 for the full-width-at-half-maximum and 2.4 for the enhancement factor (21). Gaussian curve-fitting was performed using the program GRAMS 386 from Galactic Industries using the number of components and their frequencies determined by calculation of the second derivative spectra as starting parameters. All fits were performed without any parameters being kept fixed. The secondary structure contents were calculated from the areas of the individual assigned bands and their fractions of the total area in the amide I region. The band assignment in the amide I region followed exactly that in the literature (21). The secondary structure content was calculated for at least four independently obtained spectra and is reported as the averaged with standard deviation of at least four measurements. Whether differences in the secondary structure content were significant was determined by *t* test analysis for the 95% confidence interval as described by us in detail (15).

Spectral correlation coefficients to quantify procedure-induced protein structural perturbations were calculated as described using the amide I second derivative spectra (22).

Statistical Data Treatment

When differences between two data points are discussed, *t* test analysis was used for the 95% confidence interval to decide whether the differences are statistically meaningful (15).

RESULTS AND DISCUSSION

Stability of α-Chymotrypsin after Encapsulation Using Different Oil:Water (O:W) Ratios During Emulsification

The effect of the O:W ratio on the stability of α-chymotrypsin upon encapsulation in PLGA microspheres using the s/o/w technique was investigated. The formation of non-covalent aggregates, the specific activity of α-chymotrypsin after encapsulation, and the encapsulation efficiency at different O:W ratios were used as parameters. Ethyl acetate was used as solvent in order to replace methylene chloride at the optimum (1:2.5, v:v) O:W ratio reported (10,12). However,

instead of PVA, PEG was used as emulsifier since this improved the stability of γ-chymotrypsin and horseradish peroxidase during encapsulation (23). Figure 1 shows the stability of α-chymotrypsin expressed in terms of the amount of non-covalent aggregates (A) and the specific activity (B) at various O:W ratios (the volume of the oil phase was 2 ml). When the reported 1:2.5 ratio was used (5 ml volume of aqueous phase), severe protein instability was observed. The amount of non-covalent aggregates formed after encapsulation was 23 ± 8%, the residual specific activity was only 25 ± 3%, and the encapsulation efficiency was low with 10 ± 1%. Note that at this O:W ratio the continuous phase was saturated with 0.48 ml of ethyl acetate (24.1%) and a major proportion of ethyl acetate thus resided in the microdoplets. Diffusion of water into the polymer droplets may occur because the solubility of water in ethyl acetate is 65% (v:v). Thus, we hypothesized that larger volumes of the aqueous phase would allow a larger proportion of the solvent to quickly dissolve in the aqueous phase and as a result protein stability would improve. When the volume of the aqueous phase was increased to 50 ml, an increase in the specific activity of α-chymotrypsin (from 25–60%) and a decrease in the amount of non-covalent aggregates formed (from 23–9%) was observed (see Fig. 1). When the volume of the aqueous phase was increased further to 75 and 100 ml, no additional significant differences were observed in terms of the protein stability. The encapsulation efficiency also increased with decreasing O:W ratios from 10–32% (see Fig. 1C). Thus, these results show that the optimum volume of the aqueous phase to obtain the maximum protein stability was 50 ml. This volume is more than twice the volume needed to completely dissolve 2 ml of ethyl acetate in the aqueous phase. The most straightforward interpretation is that protein stability is impaired under conditions of slow microsphere hardening when the solvent is slowly removed by dissolving in the aqueous phase and evaporation. In order to establish whether this was a general solvent effect, methylene chloride was also tested which has a much lower water solubility of only 1.32%. Figures 2A and B show that when the volume of the aqueous phase was sufficient to dissolve 2 ml of methylene chloride (more than 200 ml), α-chymotrypsin had the lowest amount of non-covalent aggregates (3 ± 1%) and the highest specific activity (76 ±

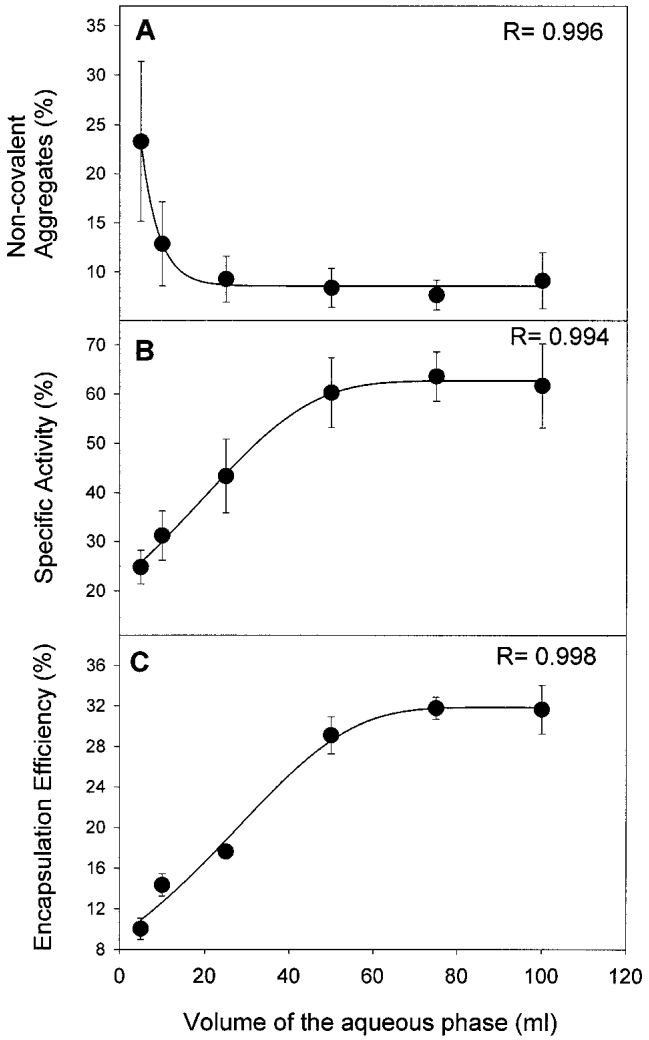

Fig. 1. Effect of the volume of the aqueous phase on α-chymotrypsin stability upon S/O/W encapsulation in PLGA microspheres. Under all conditions, 2 ml of ethyl acetate were used as the organic solvent.

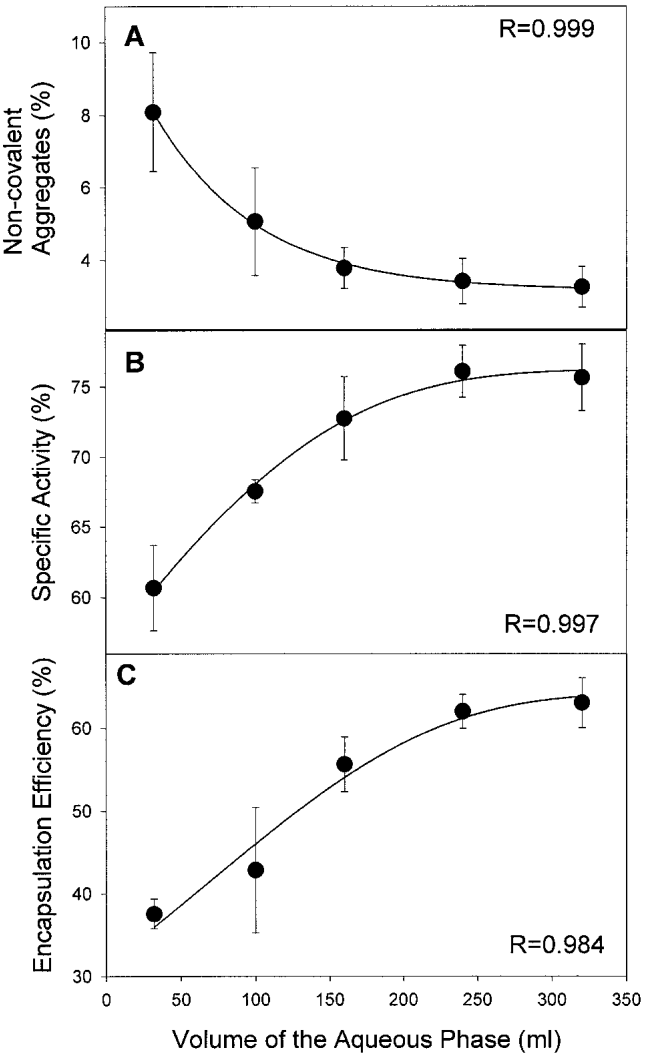

Fig. 2. Effect of the volume of the aqueous phase on α-chymotrypsin stability upon S/O/W encapsulation in PLGA microspheres. Under all conditions, 2 ml of methylene chloride were used as the organic solvent.

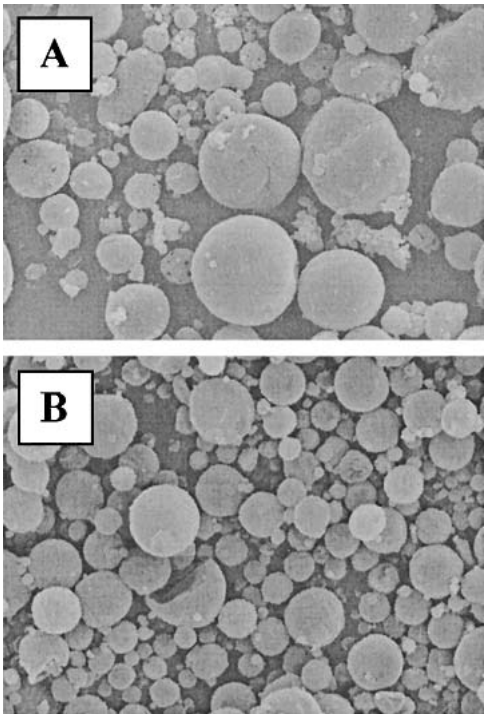

Fig. 3. SEM pictures of α-chymotrypsin-loaded PLGA microspheres prepared using ethyl acetate as the solvent. (A) 2:5 and (B) 2:100 O:W (ml:ml) ratios. The width of the SEM image corresponds to 132 μm.

2%). The encapsulation efficiency was also dependent on the volume of the aqueous phase used and increased from 38 ± 2% to 63 ± 3% when the volume of the aqueous phase was increased from 32 to 240 ml (Fig. 2C). These results confirm that at O:W ratios where only a proportion of the solvent can dissolve in the aqueous phase protein instability and low encapsulation efficiency was observed. The dependence of the protein stability on the volume of the aqueous phase could be explained by the rate of solvent diffusion in the aqueous phase and hence the rate of PLGA precipitation. The lower the volume of the aqueous phase, the slower the rate of polymer precipitation and therefore the droplet is expected to be for a longer period in a liquid state with possible increased influx of water from the aqueous phase. Under such conditions the protein is expected to be exposed to denaturing conditions because protein structure becomes conformationally more mobile (20). In the same way, the volume of the aqueous phase is an important factor in determining the encapsulation efficiency because the protein may partition into the aqueous phase as long as the droplet is in a liquid state. This effect of the rate of polymer precipitation on the drug content has been reported by Bodmeier *et al.* (24). In conclusion, careful selection of the O:W ratio is required to prepare microspheres by s/o/w encapsulation in order to preserve protein stability and achieve high loading efficiencies.

Effect of the O:W Ratio on Microsphere Characteristics

It has been shown that volumes of the aqueous phase higher than those required to saturate it with the organic solvent result in irregular precipitates rather than microspheres due to the rapid solidification of the polymer (6,10). The morphology of the microspheres prepared employing ethyl acetate using the highest and lowest O:W ratios was investigated (Fig. 3). No significant difference in the shape of the microspheres prepared was noted and the size of the microspheres was the same with diameters of 10 ± 7 and 9 ± 4 μm for the 1:2.5 and 1:50 O:W ratio, respectively. This suggests that an O:W ratio that ensures rapid removal of the solvent by dissolving it in the aqueous phase may still be sufficient to allow for formation of microspheres.

α-Chymotrypsin Stability Using Solvents with Different Water Solubility

Table 1 shows some physical properties of the solvents used in this study to replace methylene chloride. We selected an optimum O:W ratio for each solvent based on the results obtained from the aforementioned experiments. The volume of the continuous phase used for each solvent was dependent on the water solubility and it was twice of the volume required to dissolve 1 ml of the solvent used. To reduce the volume of the continuous phase used in these experiments, 1 ml of each organic solvent was used. The volumes chosen for the aqueous phase for butyl acetate, methylene chloride, methyl isobutyl ketone, 2-pentanone, ethyl acetate, ethyl formate, and methyl ethyl ketone are listed in the Table 1. The effect of these solvents on the protein stability and loading efficiency were investigated using PEG or PVA as the emulsifier (note that under all circumstances the protein was colyophilized with PEG prior to encapsulation). Figure 4A shows the amount of non-covalent aggregates formed during the encapsulation process as a function of the water solubility of the solvents. The microspheres prepared with PVA as the emulsifying agent exhibited a slight but statistically significant decrease in the amount of non-covalent aggregates when the water solubility of the solvent increased. Thus, 10 ± 2% of α-chymotrypsin was aggregated during the encapsulation process when butyl acetate (water solubility 0.68 wt%) was used, while 5 ± 2% was aggregated when methyl ethyl ketone (water solubility 26.8 wt%) was used. When PEG was used as the emulsifying agent the amount of non-covalent aggregates was in the range of 0.5–1.3% and it did not show any statistically significant dependence on the water solubility of the solvent. The higher amount of the non-covalent aggregates of α-chymotrypsin in microspheres prepared with PVA using solvents with lower water solubility might be explained by the very hydrophobic interface formed. Protein may adsorb more strongly at the interface of these solvents. For example, Hickel *et al.* (25) showed that Pa-hydroxynitrile lyase adsorbs more strongly at the interface of apolar solvents. It is well

Table I. Properties of the Solvents Used in This Study

Solvent	Water solubility (wt.%)	ρ (g cm^3)	Volume of the aqueous phase (ml)
Methyl ethyl ketone	26.8	0.806	6.0
Ethyl formate	13.6	0.924	14.0
Ethyl acetate	8.70	0.902	21.0
2-Pentanone	4.3	0.812	38.0
Methyl isobutyl ketone	1.9	0.790	84.0
Methylene chloride	1.32	1.325	101.0
Butyl acetate	0.68	0.880	260.0

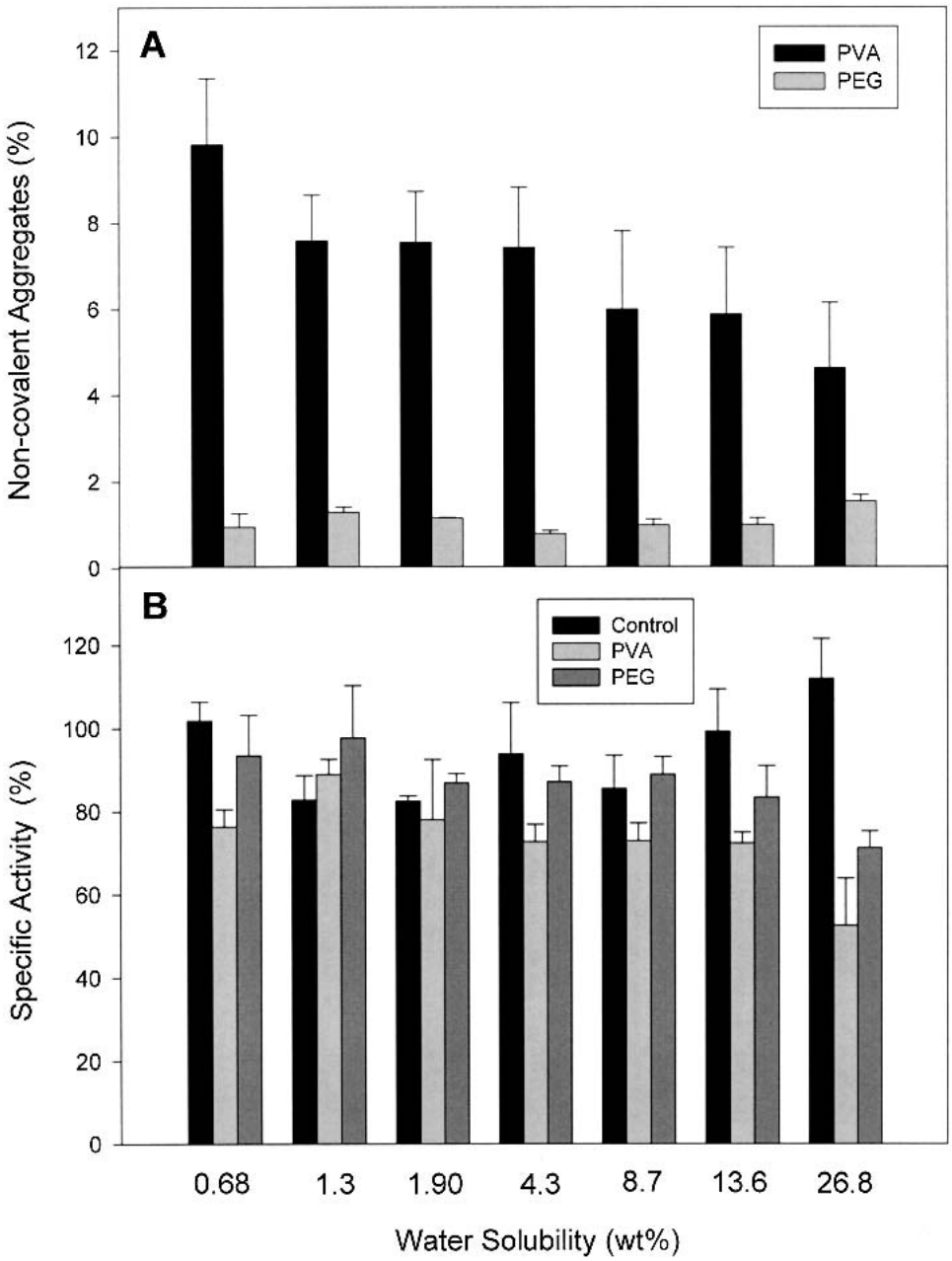

Fig. 4. Effect of the water solubility of the solvent on the α-chymotrypsin stability after encapsulation in PLGA microspheres using PEG and PVA as emulsifying agents. As the control in B the lyophilized powder was exposed to the organic solvent prior to encapsulation to assess solvent-induced enzyme inactivation (see Materials and Methods for details).

known that protein aggregation is caused by the protein adsorption to oil:water interfaces, thus, the difference observed between the amount of non-covalent aggregates formed using PVA or PEG could be due to the effect of these emulsifying agents on the protein adsorption to the interface. It has been shown that PEG adsorption to the O:W interface reduced protein adsorption to the interface and reduced the amount of non-covalent aggregates formed (26). However, more experiments have to be performed to investigate the possible mechanism in more detail. Regardless of this, our stability data clearly demonstrate that PEG is superior to PVA to create protein-loaded microspheres.

Effect of the Solvents on the Activity of α-Chymotrypsin

The specific activity of α-chymotrypsin after encapsulation with the various solvents was also determined. As a control, the effect of the solvents on the activity of α-chymotrypsin was determined (see Fig. 4B). It was notable that exposure of the lyophilized enzyme to some solvents caused a slight drop in enzyme activity. Activity loss upon solvent exposure was not due to the formation of soluble protein aggregates when investigated by SEC-HPLC (data not shown). Comparison with the controls gave some insights into encapsulation-induced inactivation of the enzyme. In general, for all sol-

vents, the use of PVA as emulsifying agent resulted in lower residual enzyme activities than when PEG was used as emulsifier. For solvents with high water solubility, (eg, methyl ethyl ketone and ethyl formate), enzyme activity was significantly impaired after encapsulation. Thus, even though methyl ethyl ketone caused the lowest amount of non-covalent aggregates when PVA was used as emulsifier (Fig. 4A), it caused the highest loss in protein activity. This suggests that α-chymotrypsin may not adsorb strongly to the methyl ethyl ketone:water interface but during the polymer precipitation more water may diffuse into the droplets that may induce conformational changes in the protein leading to a reduction in the catalytic activity. In contrast, when butyl acetate and methylene chloride with low water solubility were used to prepare microspheres with PEG as the emulsifying agent, α-chymotrypsin retained a high bioactivity.

3. Secondary structure of α-chymotrypsin

The secondary structure of α-chymotrypsin was investigated by FTIR spectroscopy. The magnitude of encapsulation-induced structural perturbations using butyl acetate and methylene chloride as solvents was assessed using two methods, Gaussian curve fitting of the Fourier self-deconvoluted amide I spectra and the calculation of the spectral correlation coefficient (22). Utilizing the first method, the secondary structure content of α-chymotrypsin in aqueous solution at pH 7.8 was determined to be 43 ± 1% β-sheet and 11 ± 1% α-helix structure (Table II) in agreement with previous data (21). Lyophilization of α-chymotrypsin caused an increase in the β-sheet content to 47 ± 1% and an apparent increase in the α-helix content to 15 ± 2% (21). These structural changes due to the lyophilization process caused a decrease of the spectral correlation coefficient to 0.886 (Table 2). Co-lyophilization with PEG did not reduce lyophilization-induced structural perturbations (Fig. 5A, see Table II). The structure of α-chymotrypsin after encapsulation in PLGA microspheres using methylene chloride and butyl acetate as solvents and PEG as emulsifier was also investigated. Qualitatively the inverted second derivative spectra after encapsulation of α-chymotrypsin using these solvents did not lead to additional spectral changes in addition to those caused by the lyophilization process (see Fig. 5B). In agreement with this, the secondary structure content of α-chymotrypsin in microspheres prepared with methylene chloride and butyl acetate remained unchanged as is shown in Table II. This demonstrates that butyl acetate was comparable to methylene chloride since it did not cause structural perturbations during encapsulation. However, in contrast to the situation when PEG was used as the emulsifying agent, use of PVA caused additional structural perturbations during encapsulation (see Fig. 5C, Table II). This is in agreement with the data on protein aggregation (see Fig. 4A). In conclusion, these structural data indicate that butyl acetate is an excellent solvent for s/o/w encapsulation of protein in PLGA microspheres, certainly capable of replacing methylene chloride.

Table II. Secondary Structure of α-Chymotrypsin Under Various Conditions

Sample	α-helix (%)	β-sheet (%)	Correlation coefficient
Aqueous solution at pH 7.8	11 ± 1	43 ± 1	0.95 ± 0.01
Lyophilized from pH 7.8	15 ± 2	47 ± 2	0.89 ± 0.01
Co-lyophilized with PEG	16 ± 2	48 ± 1	0.74 ± 0.01
In microspheres			
Methylene chloride (PEG)	17 ± 2	48 ± 1	0.84 ± 0.01
Butyl acetate (PEG)	16 ± 1	46 ± 2	0.82 ± 0.01
Butyl acetate (PVA)	15 ± 1	53 ± 1	0.67 ± 0.02

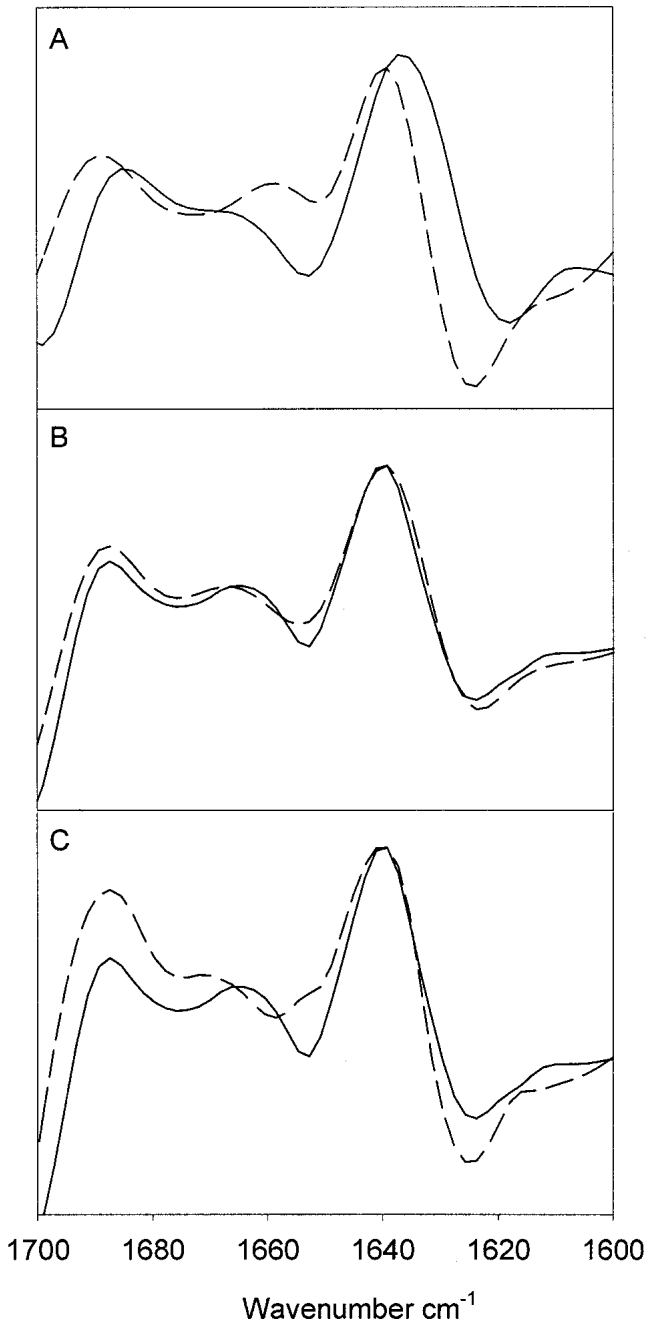

Fig. 5. Inverted second derivative amide I spectra of α-chymotrypsin under various conditions. (A) Aqueous solution at pH 7.8 (solid line) and co-lyophilized with PEG (dashed line); (B) in PLGA microspheres using PEG as emulsifying agent using butyl acetate (solid line) and methylene chloride (dashed line) as solvent; (C) in PLGA microspheres using butyl acetate as solvent and PEG (solid line) and PVA (dashed line) as the emulsifying agent.

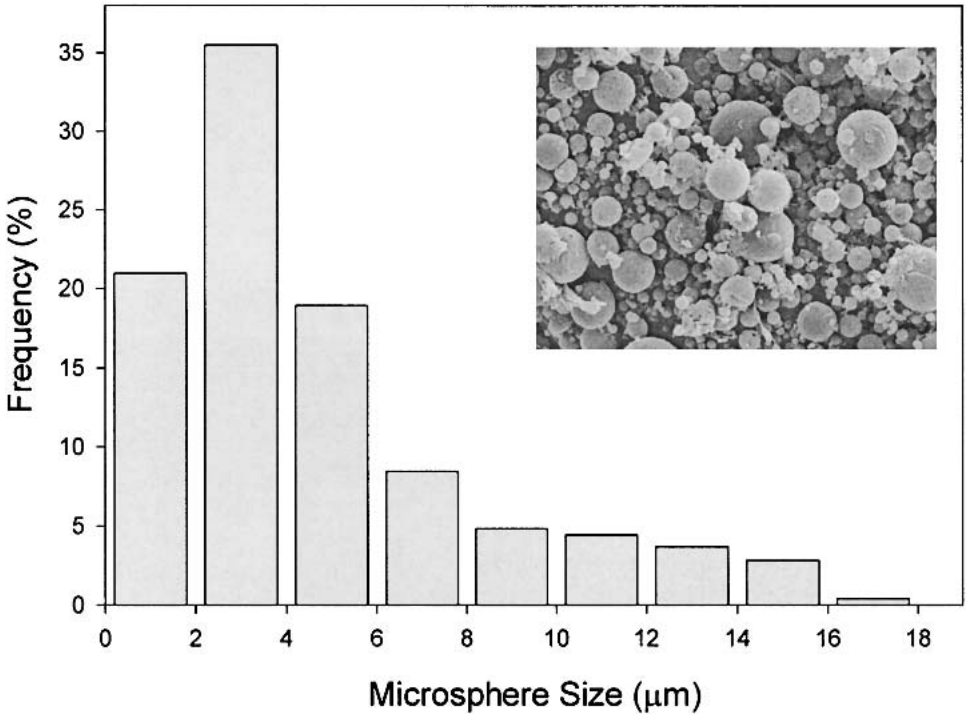

Fig. 6. Size distribution of microspheres prepared using butyl acetate as solvent. Inset: SEM pictures of microspheres.

Characteristics of PLGA Microspheres Prepared with Different Solvents

SEM pictures showed that all of the solvents used produced microspheres with spherical shape, except for those prepared using methyl ethyl ketone, which had an irregular shape. It was expected since it has been shown that large irregular aggregates were formed when this solvent was used (12). Figure 6 (inset) shows that microspheres prepared using butyl acetate as solvent had a smooth surface and approximately 35% of the microspheres were in the range of 2–4 μm. The encapsulation efficiency of α-chymotrypsin for microspheres made with butyl acetate was the highest (83%), while an efficiency of only 33–45% was achieved with methyl ethyl ketone (Fig. 7).

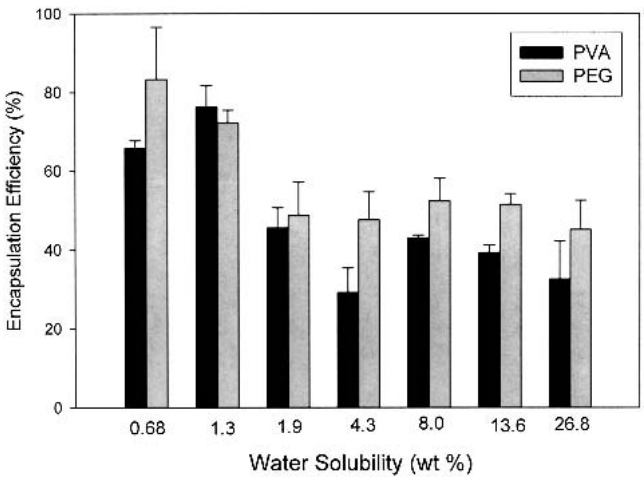

Fig. 7. Effect of the water solubility of the solvent on the efficiency of α-chymotrypsin encapsulation in PLGA microspheres using PEG and PVA as the emulsifying agents.

CONCLUSIONS

For the first time the usefulness of alternative solvents for encapsulation of a model protein in PLGA microspheres was evaluated on the basis of the solvent impact on enzyme integrity during encapsulation. A clear relationship between protein stability and the ratio of organic solvent:aqueous phase was established. It turned out that rapid solvent removal during microsphere formation is key to keeping the protein stable. The results also demonstrate that butyl acetate is an excellent solvent fully capable of replacing methylene chloride in s/o/w encapsulation. Thus, the s/o/w technique was successfully optimized by replacing the ICH class 2 solvent methylene chloride with the ICH class 3 solvent butyl acetate without causing any deleterious effect to the stability of α-chymotrypsin.

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