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Effect of lecithin and $MgCO_3$ as additives on the enzymatic activity of carbonic anhydrase encapsulated in poly(lactide-co-glycolide) (PLGA) microspheres

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Abstract

A model enzyme, carbonic anhydrase, was encapsulated and released from poly(lactide-co-glycolide) (PLGA) microspheres (1–3 μm) made by a novel phase inversion technique. Lecithin was used as a surfactant in the encapsulation process and was incorporated in either the organic phase, aqueous phase, both phases, or not at all. Additional microspheres were also made with lecithin incorporated in the aqueous phase and a basic salt, $MgCO_3$, in the polymeric phase. Released carbonic anhydrase, protein extracted from microspheres, or enzyme incubated with lecithin and PLGA were analyzed via HPLC and activity assay to determine the effect of these additives on protein integrity and activity. Lecithin in the aqueous phase appeared to increase the fraction of enzyme in monomeric form as well as its activity for both extracted protein and released protein as compared to the other formulations without $MgCO_3$. Incubation of enzyme with PLGA degradation products indicated that the acidic environment within the microspheres aids in the irreversible inactivation of the encapsulated protein. Addition of $MgCO_3$ further increased the amount of monomer in both the extracted and released protein by decreasing the amount of acid-induced cleavage and noncovalent aggregation, but still greatly decreased the activity of the enzyme.

Keywords: Microsphere; Poly(lactide-co-glycolide); Enzyme activity; Carbonic anhydrase; Acid-induced cleavage

1. Introduction

The encapsulation of proteins within polymeric microspheres for drug delivery has been the focus of many research groups in the past several decades. Proteins such as insulin [1], BMP-2 [2], and ovalbumin [3] have been encapsulated, released, and delivered either locally or to the systemic circulation to produce a desired effect. Perhaps the most important example is the first product introduced into the market, Lupron Depot, which consists of leuprolide acetate encapsulated within a poly(lactide-co-glycolide) (PLGA) matrix, developed by Ogawa et al. [4–7]. Other groups have also studied similar PLGA systems encapsulating leuprolide acetate recently and have observed a controlled release of this LHRH analogue for 120 days accompanied by in vivo efficacy [8]. In addition, delivery of other proteins such as recombinant human epidermal growth factor from PLGA microspheres has resulted in constant plasma levels for as long as 11 days and increased gastric healing over unencapsulated rhEGF solution [9].

While there are many obvious advantages to using a delivery system to administer proteins, numerous hurdles must first be overcome during the encapsulation process. Proteins in aqueous solution are notorious for undergoing hydrophobic interactions and adsorption when in aqueous solution and exposed to polymer surfaces [10–12]. Additionally, proteins can become either cleaved or aggregated [13] when entrapped within the microsphere. PLGA, a bioerodible polymer approved for several drug delivery products, forms acidic degradation products when exposed to physiological fluid. These byproducts form a highly acidic microenvironment within the confines of the microsphere which can affect, not only the conformation, but the activity as well, of the remaining encapsulated protein [10–13]. For this reason, different methods of stabilizing proteins within polymeric microspheres have been employed and have produced positive results. Trehalose, for example, has been used to successfully stabilize BSA so that when released from PLGA microspheres, both the

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monomer content and secondary structure were very similar to unencapsulated BSA [14].

Surfactants have long been used as stabilizers during the protein encapsulation process because they not only decrease the absorption of protein to the polymer surface [15], but also serve to increase the encapsulation efficiencies in drug delivery systems [16]. However, the effect of any additives on the activity of encapsulated molecules must also be considered. Attempts to stabilize proteins by other methods have also been made by Schwendeman [13], including strides to directly counteract the decreasing pH within the interior of degrading PLGA microspheres by the incorporation of basic salts such as $MgCO_3$ and $Mg(OH)_2$. Several other research groups have utilized the incorporation of salts within microspheres to successfully stabilize encapsulated proteins such as recombinant human growth hormone [17] and erythropoietin [18] and to modulate release from these types of microspheres.

In a previous study [19], we have evaluated similar microspheres fabricated from PLGA which were shown to impart controlled release properties to microparticles encapsulating a wide variety of proteins at low loadings ($<2\%$), while at higher loadings (5–6.5%), the more extensive network of interconnecting channels caused the protein to be released more quickly.

In this study, the addition of both a surfactant and basic salt are investigated within the same type of drug delivery system containing carbonic anhydrase, an acid-sensitive model enzyme. The specific aim of this study was to determine the cause of protein inactivation within PLGA microspheres, whether by protein adsorption during the encapsulation process or acid-induced cleavage during hydration and release. In addition, we wanted to study the effect of the addition of lecithin and $MgCO_3$ on the activity of the enzyme. Although the effect of these types of additives on protein activity has been studied by other groups, this study focuses on the evaluation of these additives for small microsphere systems (1–3 μm) with relatively high loadings. The environment within these small particles may not be the same as those described for larger PLGA systems in other studies and so must be investigated separately.

2. Materials and methods

2.1. Materials

Dichloromethane was from Allied Signal and petroleum ether from Mallinckrodt. Bovine carbonic anhydrase was obtained from Sigma-Aldrich and poly(lactide-co-glycolide) (50:50) (PLGA) from Boehringer Ingelheim. The micro-BCA protein assay was obtained from Pierce. Lecithin (USP grade) was obtained from Spectrum Laboratory Products. A Cole Parmer ultrasonic homogenizer, Labconco Lyph-Lock 6 lyophilizer, and Millipore high pressure filtering apparatus were used in the fabrication of microspheres. A Corning pH meter, a Bio-Rad model 3550 microplate spectrophotometer, and a Waters 2690 Alliance separations module and 996 photodiode array HPLC system were used for data collection.

2.2. Microsphere fabrication

Microspheres were fabricated from an overall 2% solution (w/v) of PLGA (MW 12 068) and carbonic anhydrase by a novel phase inversion technique [20]. Briefly, two solutions, 500 μl of a 30 mg/ml solution of carbonic anhydrase in Tris–HCl buffer, pH 8.0, and 9.5 ml of a 38.9 mg/ml polymer in dichloromethane solution, were added together for a final volume ratio of 1:19. This two-phase system was then probe sonicated at an amplitude of 20% for 30 s with an ultrasonic homogenizer at room temperature. Following sonication, the resulting water in oil emulsion was frozen in liquid nitrogen followed by lyophilization at −50°C and 30 μm Hg for 48 h. The dried polymer product was then resuspended with agitation in dichloromethane at an overall concentration of 2% or 20 mg/ml solvent. This suspension of solubilized polymer and insoluble protein particles was then quickly introduced into a nonsolvent bath of petroleum ether at a solvent:nonsolvent ratio of 1:50. The resulting microspheres were then collected with a high pressure ultrafiltration system and stored at 4°C. Theoretical microsphere loading was 7.5%.

For other formulations, lecithin was added to either the aqueous phase, the organic phase, or both phases of the two-phase system prior to the sonication step of the micronization process. For formulation made with lecithin in the aqueous phase, protein was added to a lecithin in water suspension to yield 500 μl aqueous phase with a final concentration of 60 mg/ml lecithin and 30 mg/ml protein. This aqueous phase was then vortexed for 45 s prior to addition to the polymer solution in order to facilitate lecithin protein complexation. For lecithin in the organic phase, 30 mg lecithin was added to the 9.5 ml of polymeric phase prior to combination of the two phases. For lecithin in both phases, protein was added to a lecithin in water solution to make an aqueous phase with final concentrations of 30 mg/ml lecithin and 30 mg/ml protein. This phase was vortexed for 45 s, and an additional 30 mg lecithin was added to the polymer solution prior to combination of the two phases.

2.3. Microsphere fabrication with $MgCO_3$ basic salt

One additional formulation was made incorporating the basic salt $MgCO_3$ within the microspheres. Microspheres were formulated just as with lecithin in the aqueous phase, but with the addition of 10% $MgCO_3$ powder (w/w) to the organic phase. The two phases were sonicated as before, frozen, and lyophilized. Microsphere fabrication then proceeded as above.

2.4. Microsphere characterization

For scanning electron microscopy (SEM), samples were mounted and coated for 2.5 min with a gold and palladium mixture and examined for morphology and size with a Hitachi S-2700 scanning electron microscope.

For particle size distribution, a 3 mg aliquot of unloaded control microspheres suspended at a concentration of 10 mg/ml in a solution of 1% HPMC (hydroxypropylmethylcellulose) and 1% Pluronic F127 was analyzed on a Coulter particle size analyzer small volume module, model LS 230, in order to determine the entire population size distribution.

To determine the degradation rate of the polymer, the average molecular weight of degrading unloaded control microspheres was ascertained via gel permeation chromatography (GPC). For each sample, a 5% solution was made in chloroform and analyzed on a Perkin-Elmer LC pump model 250 gel permeation chromatography system composed of an isocratic LC pump model 250, an LC column oven model 101, and a LC-30 RI detector, and a 900 series interface computer. Samples were eluted through a PL gel 5 μm mixed column and a 5 μm/50 Å column connected in series at a flow rate of 1.0 ml/min and a temperature of 40°C. The system was calibrated with a series of monodisperse polystyrene standards (MW: 600–200 000).

For FTIR (Fourier Transform Infrared Spectroscopy) microsphere samples were analyzed on a Perkin-Elmer model 1725× FTIR spectrometer and compared to unencapsulated lecithin. Samples were ground with a mortar and pestle and mixed with KBr at a ratio of 1:100. The mixture was then pressed into a pellet and spectra analyzed from 400 cm^{-1} to 4000 cm^{-1}. Unique peaks observed for unencapsulated lecithin but not for carbonic anhydrase microspheres without lecithin were compared to spectra obtained for microspheres fabricated with lecithin in each of the phases. The presence of these peaks was used qualitatively to establish the entrapment of lecithin within the microspheres.

2.5. Microsphere protein extraction

Microspheres from each formulation were divided into 10 mg aliquots ($n=2$). Half of the aliquots were incubated in 500 μl of PBS buffer at 37°C for 1 h to allow initial protein release, while the other half were not. At 1 h, incubating samples were centrifuged at $2000\times g$ for 5 min, and the supernatants removed and saved on ice for analysis. Following release, samples were dried and all samples, whether subjected to release or not, were extracted as follows. Microspheres were dissolved in 500 μl dichloromethane and vortexed for 1 min to ensure solubilization of polymer only. An additional 500 μl of acetone was then added to spur protein precipitation (necessary for subsequent extractions once protein has been hydrated) and vortexed for 30 s. Samples were then centrifuged at $11\,000\times g$ for 5 min to precipitate protein and any remaining unsolubilized polymer. Supernatants were removed and discarded and the pellets dried with compressed air. Precipitated protein was then resuspended in Tris–HCl buffer, pH 8.0, and samples centrifuged again to pellet any remaining unsolubilized polymer. The resulting protein-rich supernatant was saved on ice and the pellet subjected to resolubilization in dichloromethane and the entire extraction process repeated. The process was repeated once for samples previously subjected to protein release and twice for those not subjected to release, so that there were three protein fractions for each type of sample. Total loaded protein was extracted for each sample. We assume that all protein was able to be re-solubilized since the extraction was performed until no further particles could be seen. Protein samples were kept on ice whenever possible both during and following the extraction process to maintain protein integrity and activity.

2.6. Protein release from microspheres

Microspheres were divided into 10 mg aliquots ($n=3$) and placed in microcentrifuge tubes. A 1 ml aliquot of PBS buffer, pH 7.4, was added to each vial. The tubes were closed and positioned on their sides so that the maximum surface area of each sample would be available for release into buffer. Samples were incubated at 37°C for a total of 1 week and were assayed at various time points: 10 min, 30 min, 1, 2, 4, 8, 24, 48, and 120 h and 1 week. At each time point, the samples were centrifuged at $2000\times g$ for 5 min, and the supernatants were removed and saved on ice to maintain protein integrity and activity for further analysis. Fresh buffer was replaced in each vial at each time point to allow further protein release. Vials were agitated with each buffer change.

2.7. Protein incubation with additives

Aliquots of carbonic anhydrase (0.1 mg/ml) were incubated at 37°C with various additives in order to determine the effect on both protein form and activity. One sample was incubated for each time point, and each was terminal. Protein was incubated alone, or with the addition of 10, 50, or 100 mg control PLGA microspheres, 3 mg lecithin, or 10 mg control microspheres and lecithin. Incubation times were 10, 20, and 30 min, and 1, 2, 4, 8, 24, and 48 h. The buffer was not changed during these experiments.

2.8. Protein integrity analysis

Aliquots (50 μl) from the release study supernatants ($n=3$) and microsphere protein extractions ($n=1$) were assayed with a micro BCA reagent kit (sensitivity range 1–20 μg/ml) and samples read at 540 nm. A calibration curve was made for carbonic anhydrase and run against

a BSA standard supplied with the assay kit. Values obtained for the release studies were summed to determine the cumulative release and are reported as per cent release of loaded protein based on total protein extraction.

Extracted protein, release study supernatants, and proteins incubated with additives were run on a TosoHaas SEC column with a Waters 2690 Alliance HPLC separations module linked to a 996 photo diode array detector system ($n=1$ for each sample examined via HPLC). The flow rate of the mobile phase (100 mM sodium phosphate with 0.5 M NaCl in HPLC grade water) was set at 0.7 ml/min. Extraction samples, release samples, and additive incubation samples were filtered and injected at a volume of 20 μl with a run time of 30 min and peak areas obtained compared to carbonic anhydrase standards of 0.1, 0.5, and 1.0 mg/ml. Spectra were collected in the range of 200–400 nm and chromatograms extracted at 215 nm using Millennium software.

2.9. Protein sequence analysis

The protein sequence of bovine carbonic anhydrase was examined for amino acid pairings which might promote acid-induced cleavage of the enzyme within the degrading microsphere. The presence of these sequences and the fragments that would be produced by their cleavage were compared to the sizes of protein fragments revealed by HPLC analysis.

2.10. Protein activity analysis

Extracted protein, release study supernatants, and proteins incubated with additives were analyzed ($n=1$) to determine per cent activity of loaded protein using the method of Wilbur and Anderson [21]. Briefly, the pH of a 6 ml aliquot of Tris–HCl buffer (starting pH 8.0 at 23°C) in a 30 ml beaker was continuously measured while being stirred at 4°C. A 4 ml aliquot of water previously saturated with CO_2 gas was added to the beaker and the time taken for the pH to drop from pH 8.3 to pH 6.3 was recorded. This time (T_0) was taken as the blank. To determine enzyme activity, the same procedure was performed with the addition of 100 μl of 0.01 mg/ml enzyme to the reaction mixture. The time for the pH drop was measured in seconds for each sample (T), and active units of enzyme were determined using the following equation:

$$\text{Units/mg} = [2\times(T_0-T)]/[T\times\text{mg enzyme}] \quad (1)$$

3. Results and discussion

3.1. Microsphere characterization

SEM micrographs of carbonic anhydrase loaded PLGA microspheres without lecithin revealed spherical structures fairly uniform in size and ranging from 0.2 to 0.4 μm in diameter (Fig. 1). Particle analysis yielded a size distribution with a median of 2.033 μm, again indicating that these particles are extremely small. The discrepancy between data obtained by the two methods may be explained by a tendency for the microspheres to aggregate when suspended in solution.

GPC of unloaded control microspheres revealed a decrease in polymer MW by 24.7% after 2 days, 55.3% by 5 days and 66.7% by the end of the experiment at 7 days. Previous studies with this polymer have shown that although the polymer molecular weight begins to decrease immediately, mass loss does not commence until 7 days degradation [19]. These results agree with previous observations made for bulk-eroding systems and give an indication as to the amount of degraded material entrapped within the microsphere interior.

Phosphatidylcholine, commonly known as lecithin, was chosen as the surfactant for this study. Lecithin, however, can either be completely solubilized in organic solvent or form micelles in water. Since many encapsulation procedures involve the emulsification of aqueous (water) and organic (oil) phases, we decided to assess the effect of lecithin, when incorporated in each of these phases, on the activity of an encapsulated enzyme.

FTIR spectroscopy of microspheres as compared to unencapsulated lecithin indicated that lecithin is entrapped within microspheres whether incorporated within the organic phase, aqueous phase, or both phases (Fig. 2). The peak corresponding to lecithin at 2852.74 cm^{-1} was found in all microspheres tested but appeared much larger for

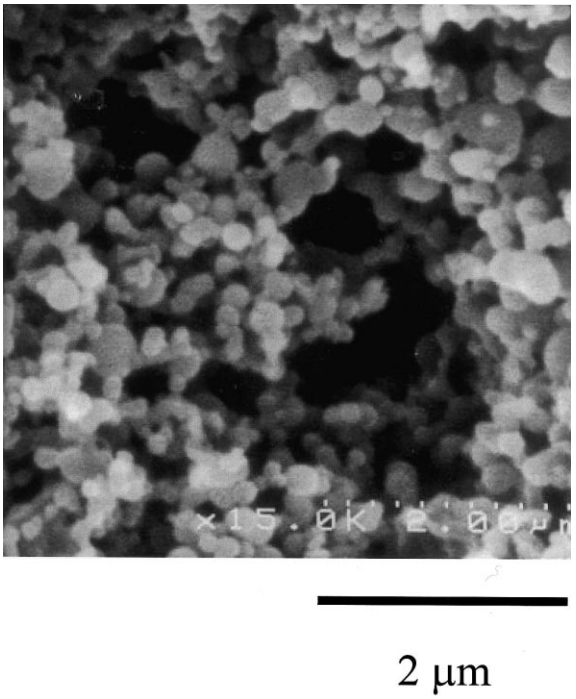

Fig. 1. SEM micrograph of PLGA microspheres loaded with carbonic anhydrase without lecithin.

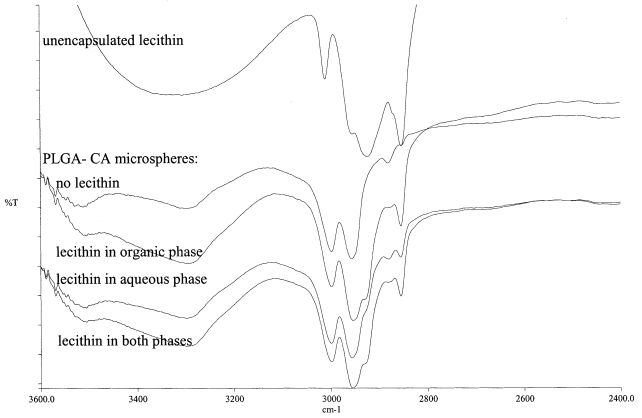

Fig. 2. FTIR spectra of unencapsulated lecithin as compared to PLGA microspheres encapsulating carbonic anhydrase fabricated without lecithin, with lecithin in the organic phase, with lecithin in the aqueous phase, and with lecithin in both phases. Peaks of interest are 2852.74 cm^{-1}, 2923.07 cm^{-1}, and 3287.91 cm^{-1}.

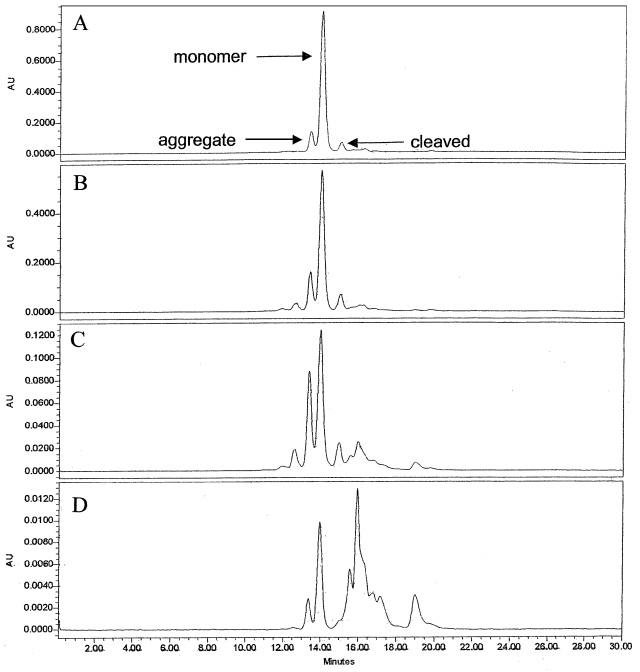

Fig. 3. HPLC traces of carbonic anhydrase with peaks labeled to show the monomeric (active), aggregated, and cleaved forms. (A) Enzyme standard; (B) enzyme extracted from PLGA microspheres; (C) enzyme released from PLGA microspheres for 10 min; (D) enzyme released from PLGA microspheres for 30 min.

each of the microspheres fabricated with lecithin than for those made without lecithin. In addition, a developing small shoulder was observed for microspheres made with lecithin and corresponded to the unique lecithin peak at 2923.07 cm^{-1} not seen for microspheres without lecithin. Finally, the very broad peak observed for unencapsulated lecithin at 3287.91 cm^{-1} was relatively larger for microspheres made with lecithin than for those fabricated without. Interestingly, lecithin in the aqueous phase appeared to be incorporated within microspheres to a lesser degree than lecithin in the organic phase as indicated by the relative size of the observed peaks.

3.2. Microsphere protein extraction

More protein was accounted for via HPLC from microspheres which were subjected to 1 h of release prior to extraction than from microspheres which were extracted directly. Microspheres fabricated without lecithin had an efficiency of encapsulation of 37.1% without prior release but 67.4% with 1 h prior release, based on the theoretical protein loading of 7.5% (w/w). Similar effects were observed for microspheres made with lecithin in the organic phase, aqueous phase, or both phases (Table 1). The higher values obtained from the extraction with prior release were taken as the total protein encapsulated and the actual protein loading calculated based on these values. Actual protein loadings were 5.05% for microspheres made without lecithin, 5.51% for microspheres made with lecithin in the organic phase, 6.23% for microspheres made with lecithin in the aqueous phase, and 6.49% for microspheres made with lecithin in both the organic and aqueous phases. Values for total protein loaded based on extraction preceded by a brief period of release were obtained via BCA protein assay and HPLC, and results for both methods were in close agreement (Table 1). In addition, more protein was able to be extracted from formulations made with lecithin in any phase than that made without lecithin (Table 1), indicating that the addition of lecithin can decrease the aggregation of protein within microspheres.

Table 1
Efficiency of encapsulation for various carbonic anhydrase-loaded PLGA microspheres as determined by protein extraction

Use of lecithin in microspheres	Efficiency of encapsulation (%)			
	HPLC values (direct extraction method)	HPLC values (extraction preceded by release)	BCA assay values (extraction preceded by release)	Actual loading (HPLC values, extraction preceded by release) (theoretical = 7.5%)
No lecithin	37.1	67.4	64.1	5.05
Lecithin in organic phase only	55.5	73.5	81.6	5.51
Lecithin in aqueous phase only	57.1	83.1	76.5	6.23
Lecithin in organic *and* aqueous phase	72.8	86.5	79.6	6.49
Lecithin in aqueous phase only, $MgCO_3$ in organic phase	–	71.6	70.5	5.37

Values obtained using both the BCA assay and HPLC are compared.

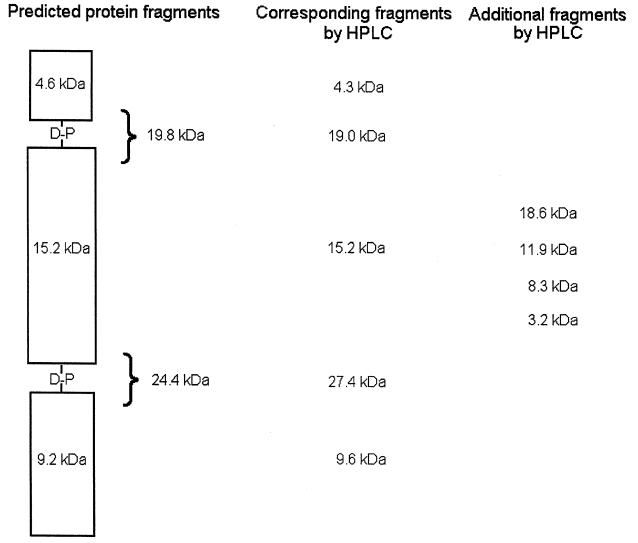

Fig. 4. Predicted protein fragments based on acid-induced cleavage as compared to protein fragments actually observed by HPLC.

Bovine carbonic anhydrase has a 259 amino acid sequence is approx. 29 kDa. The enzyme is active as a monomer, but which can become aggregated or cleaved during the process of encapsulation (Fig. 3). Since protein cleavage products were found within microspheres, an analysis of the protein sequence was conducted in order to determine whether cleavage at any particular amino acid sequence would yield the protein fragments found [22]. Aspartate-proline, a relatively rare sequence in proteins, was suspect since this group is especially sensitive to mild acid, causing the COO^- side chain group of the aspartic acid to attack the H_2N^+ of the proline ring, resulting in protein backbone cleavage [23]. Although relatively rare, two such pairings were found, one at residues 40–41 and one at residues 178–179. The sizes of probable protein fragments due to acid-induced cleavage at these sites were calculated and found to be similar to those revealed by HPLC analysis (Fig. 4). These data support the theory that incubation of protein with the acidic degradation products within PLGA microspheres is a key contributor to protein cleavage in this study [24,25]. Additional peaks not corresponding to these calculated fragments were also observed via HPLC and are believed to be due to cleavage at either a serine or threonine residue (carbonic anhydrase has 16 and 14, respectively), both of which are also somewhat susceptible to acid-induced cleavage [23].

Extraction from microspheres revealed that the amount of protein in aggregated form did not vary much among the types of fabricated microspheres (Fig. 5). Microspheres without lecithin had 25.5% aggregates, while those with lecithin in the organic, aqueous, and both phases had 28.0%, 17.0%, and 13.4%, respectively. Percentage monomer and cleaved forms, however, varied immensely and appeared to be dependent on whether or not any lecithin was introduced into the organic phase. The formulations without lecithin or with lecithin in the aqueous phase had cleaved fractions of 26.1% and 15.6%, respectively, while formulations with lecithin in the organic phase or both phases, had much higher cleaved fractions of 67.5% and 63.6%.

This might indicate that the addition of lecithin to the organic phase lowers the surface tension between the phases to such an extent that carbonic anhydrase in the aqueous phase begins to adsorb onto the polymer in the organic phase. The protein would then either become aggregated or would be trapped within the microsphere where the effect of an acidic microenvironment would cause its cleavage [24,25]. Entrapped protein that was not cleaved to a significantly lower molecular weight fraction by having its covalent peptide bonds broken might become denatured and lose its activity by the breaking of secondary bonds and unfolding in the highly acidic environment. This phenomenon appears to be irreversible since the use of buffers at pH 8.0 during the activity assay did not aid in the recovery of enzymatic activity. The effect of this phenomenon was much lower, though, in a system without surfactant. In a system where lecithin in the aqueous phase is far above its critical micellar concentration of 0.13 mg/ml as it is in this study (60 mg/ml in the aqueous phase) and is in an emulsion with the protein to be encapsulated prior to addition to the polymeric organic phase. In this ease the lecithin might actually be protecting the protein during the emulsification and fabrication process by reducing its contact with the polymer phase [26] either by the formation of liposomes that entrap the protein or by the formation of protein/lecithin complexes.

We can see this effect in the percentage of monomer in each formulation. The monomer fraction was extremely low for the formulation with lecithin in the organic phase (4.54%) and for the formulation with lecithin in both phases (10.0%). Yet, this fraction was greater for formulations without lecithin in the organic phase, with 48.4% and 67.5% monomer for the formulations without lecithin and with lecithin in the aqueous phase, respectively. Since only 75.9% of the original unencapsulated protein solution

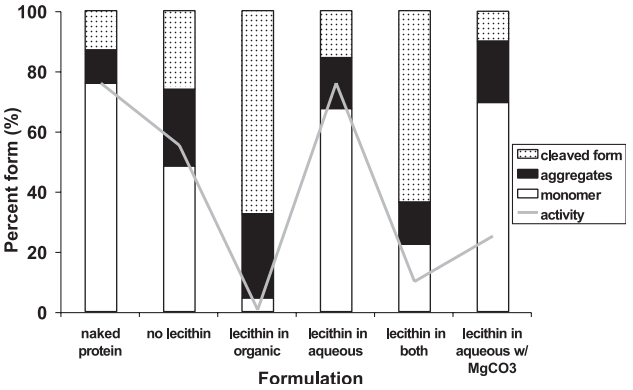

Fig. 5. Percentage of each enzyme form extracted from microspheres as determined by HPLC. Forms included cleaved (dotted bar), aggregated (solid black bar), and monomeric (solid white bar). Line graph represents activity of the total enzyme, inclusive of all forms.

was detected by HPLC to be in the monomer form, the formulation with lecithin in water is thought to be a rather efficient and gentle method of enzyme encapsulation.

Activity of protein extracted from microspheres seemed to correlate well with the per cent of monomer extracted. Microspheres made without any lecithin had an activity of 55.2%. When lecithin was incorporated into both phases, the enzyme activity was only 10.0%, while those made with lecithin in the organic phase only had an activity of 0.62%. The formulation made with lecithin in the aqueous phase had an activity of 75.9%, indicating that all the protein encapsulated retained its activity. Data from the extraction study are summarized in Table 2.

3.3. Protein release from microspheres

Formulations made without lecithin, lecithin in the aqueous phase or both the aqueous and organic phases exhibited similar release profiles to one another (Fig. 6). Each formulation had a fast release of between 81% and 90% up to 8 h. Each then experienced a slowing in the release rate with only another 7–11% released for the duration of the experiment. When the experiment ended at 1 week, each of these microsphere formulations had released between 92% and 99% of the total encapsulated protein.

The formulation made with lecithin in the organic phase only, however, was significantly different from all other formulations at every time point throughout 1 week (Fig. 6). This formulation also exhibited a burst type effect up to 8 h with a release of 64.7 ± 4.73% which slowed after 8 h, releasing only another 5.87% over the remainder of 1 week and bringing the total release up to 70.6 ± 4.95%. Burst effects seen for each type of microsphere were attributed to the relatively high loadings of these tiny particles (5–6.5%) and the consequential formation of interconnecting channels and pores. Lower burst effects have been observed previously for lower loadings (< 2%) in the same type of system [19].

The form of protein released from microspheres seemed to emulate the forms extracted, with the highest content of monomer observed for the formulation made with lecithin in the aqueous phase, followed by the formulation without lecithin, lecithin in both phases, and lecithin in the organic phase (Fig. 7). Microspheres made with lecithin in the organic phase released only aggregates and cleaved forms of the enzyme for the entirety of the experiment (Fig. 7B), and the monomeric fraction was negligible. Aggregates totaled 39.4% of the release at 10 min while cleaved forms made up the remaining 60.6%. The release of aggregates ceased after 30 min, with only cleaved forms being released for the remainder of 1 week and comprising 80.3% of the total protein released. None of the protein released from these microspheres appeared to be active.

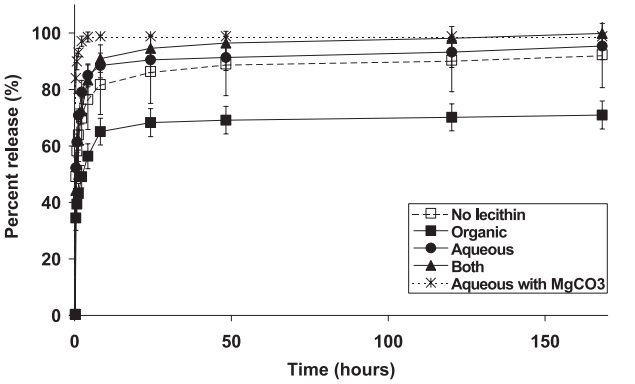

Fig. 6. Release curves for various batches of PLGA microspheres loaded with carbonic anhydrase. Microspheres made without any lecithin (□), with lecithin in the organic phase (■), with lecithin in the aqueous phase (●), with lecithin in both phases (▲), with lecithin in the aqueous phase only and $MgCO_3$ incorporated (*).

The other three formulations, those made without lecithin (Fig. 7A), with lecithin in the aqueous phase only (Fig. 7C), and with lecithin in both the organic and aqueous phases (Fig. 7D), followed a general pattern, releasing all three forms of the enzyme (aggregated, monomeric, and cleaved) for 30 min–1 h, with the vast majority being released during the first 10 min. After this point, the monomeric and aggregated forms were no longer released, and the remainder of the released protein was comprised of only cleaved enzyme. Microspheres made without lecithin released 26.9% monomeric fraction with a total activity of 20.7%. The formulation with lecithin in both phases, however, released about the same amount of monomer (22.7%) but with a lowered activity (3.41%).

Table 2
Percent of each enzyme form encapsulated (determined via extraction) and released (after 30 min) from PLGA microspheres

Enzyme form (%)		Formulation				
		no lecithin	organic phase	aqueous phase	both phases	with $MgCO_3$
Encapsulated	aggregate	25.5	28.0	17.0	13.4	20.4
	monomer	48.4 (55.2)	4.54 (0.62)	67.5 (75.9)	22.4 (10.0)	69.5 (25.1)
	cleaved	26.1	67.5	15.6	63.6	9.96
Released	aggregate	36.1	39.4	36.2	30.0	9.5
	monomer	26.9 (20.7)	0 (0)	46.6 (17.1)	22.7 (3.41)	58.6 (1.74)
	cleaved	37.0	60.6	17.2	47.3	31.9
Total released		91.9 ± 11.6	70.6 ± 4.95	95.0 ± 4.81	99.5 ± 3.56	98.5 ± 0.41

Activity of total protein is indicated in parentheses after monomeric fraction.

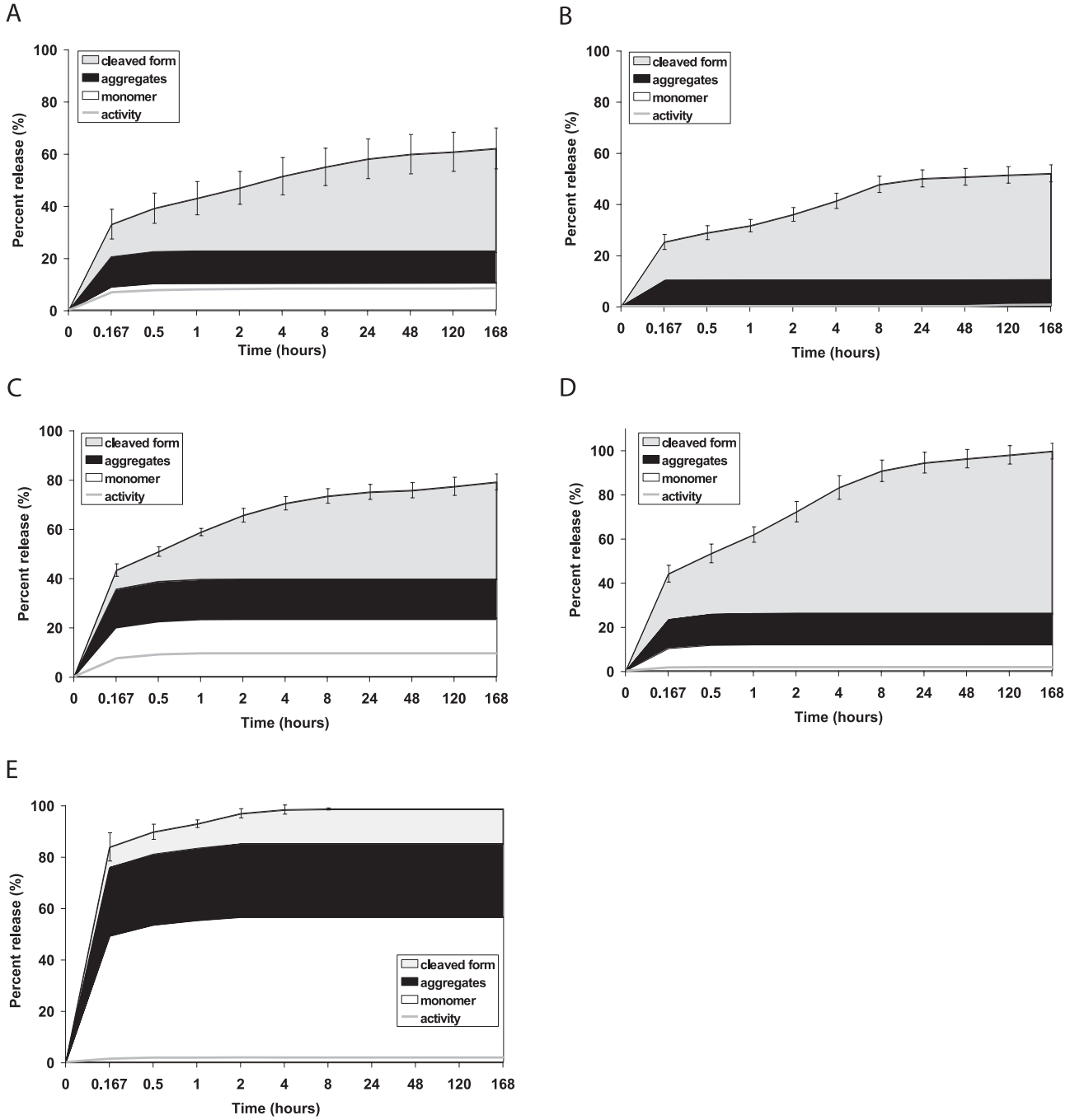

Fig. 7. Release curves for various batches of PLGA microspheres loaded with carbonic anhydrase divided into cleaved (dotted area), aggregated (solid black area), and monomeric (solid white area) fractions. Line graph represents activity of the total enzyme, inclusive of all forms. (A) Microspheres made without any lecithin, (B) with lecithin in the organic phase, (C) with lecithin in the aqueous phase, (D) with lecithin in both phases, (E) with lecithin in the aqueous phase only and $MgCO_3$ incorporated.

Finally, the formulation made with lecithin in the aqueous phase released the highest fraction of monomer (46.6%), but only about 1/3 of the monomer was shown to be active (17.1%) his formulation was, therefore, nearly equivalent to the formulation fabricated without any lecithin. Data from the release studies are summarized in Table 2.

The monomeric fractions released from each microsphere formulation were greatly reduced from those observed in the extractions. In addition, two of the formulations (prepared with lecithin in the aqueous phase and lecithin in both phases) had an even further diminished level of activity. It is believed that the longer the protein remains within the degrading microsphere, the more it becomes cleaved by the acidic degradation products of the polymer. This cleavage-producing effect has been shown previously [24,25].

3.4. Protein incubation with additives

Protein was incubated alone at 37°C or with lecithin or PLGA control microspheres to determine the potential effect of each on the integrity of the protein as it is being released from a microsphere (Fig. 8). Protein incubated alone at 37°C maintained the same general composition of approx. 76% monomer, 11% aggregate, and 13% cleaved form through 4 h incubation (Fig. 8A). Between 8 and 48 h, however, percentage aggregate and cleaved form increased, while percentage monomer decreased to 53.0%. In addition, the activity of the enzyme decreased continuously throughout the experiment and was consistently less than the percentage monomer. The percentage of monomer which was active remained fairly consistent, however, averaging 75.5 ± 9.57% through 8 h.

When incubated with lecithin (Fig. 8B), the percentage aggregated form of the protein increased initially but then

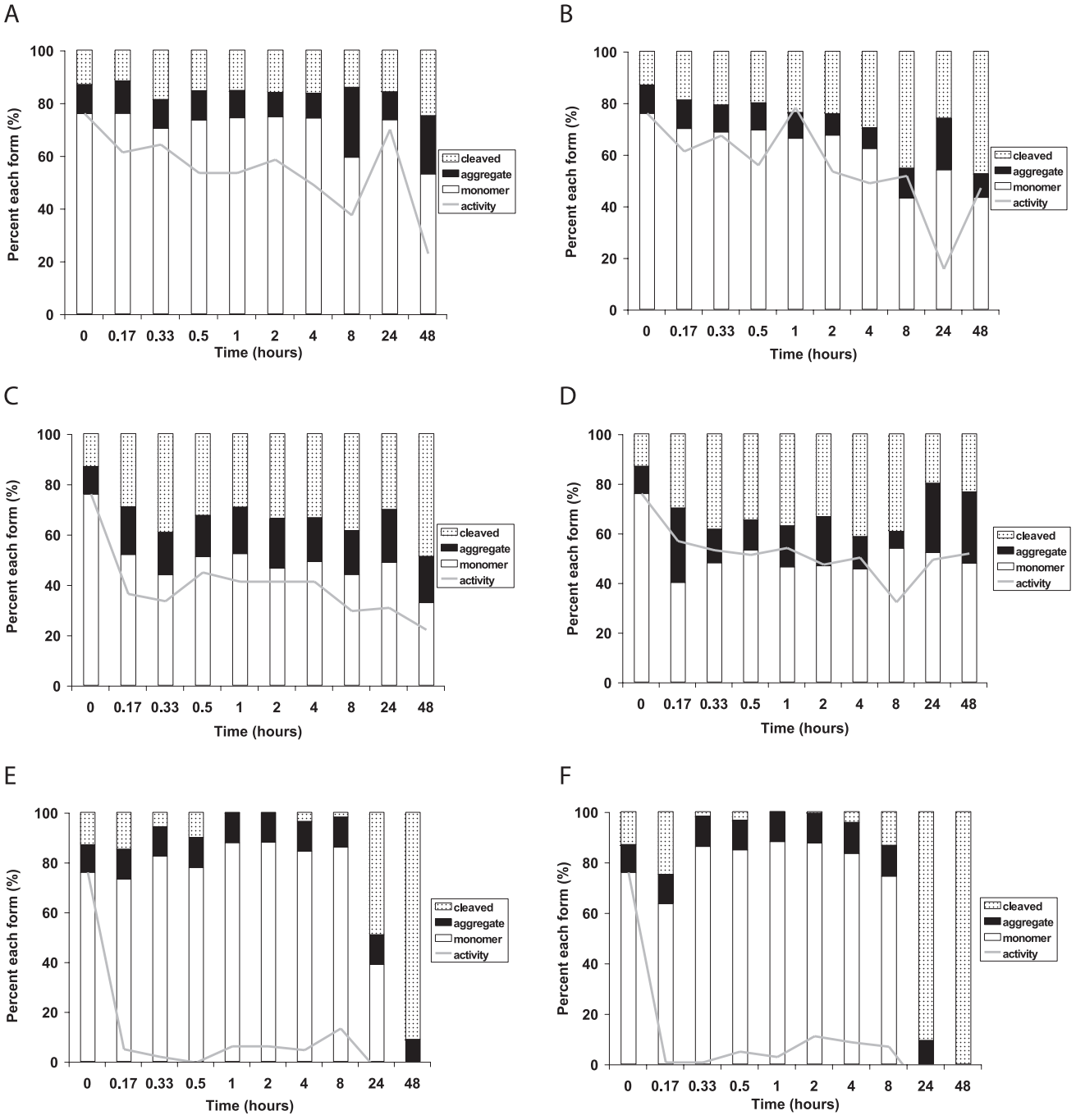

Fig. 8. Percentage of each enzyme form, cleaved (dotted bar), aggregated (solid black bar), and monomeric (solid white bar), as determined by HPLC after incubation with various additives. Line graph represents activity of the total enzyme, inclusive of all forms. (A) Incubation at 37°C, (B) with lecithin, (C) with lecithin and 10 mg blank PLGA microspheres, (D) with 10 mg blank PLGA microspheres, (E) with 50 mg blank PLGA microspheres, (F) with 100 mg blank PLGA microspheres.

remained a constant 18.3 ± 1.48% between 10 min and 48 h, while the cleaved form continuously increased, reaching 48.8% at 48 h and causing a decrease in native monomer to 32.8%. The activity of the monomeric form averaged 75.7 ± 9.50% throughout 48 h.

When incubated with lecithin and 10 mg of PLGA control microspheres (Fig. 8C), the monomeric fraction was noticeably lower than that for incubation with lecithin alone (52.0% by 10 min) and decreased to 32.8% by 48 h. The percentage of active monomer, however, was similar, averaging 75.8 ± 9.49% throughout the 48 h.

Incubation of protein with 10 mg of PLGA control microspheres (Fig. 8D) did not yield results that were significantly different from that of protein alone, with the aggregated form averaging 11.0 ± 3.37% during the entire 48 h experiment. The cleaved form comprised 21.5 ± 5.17% of the total protein for the first 4 h and then increased to 47.5% by 48 h and was accompanied by a decrease in monomeric form from 68.6 ± 4.14% to 43.4%. The activity of the monomeric form was consistently high.

Enzyme incubated with higher concentrations of degrading PLGA microspheres, however, showed very different results than that incubated with only 10 mg microspheres (Fig. 8E,F). While the percent composition of the enzyme did not vary during the first 8 h, significant changes were seen at the 24 and 48 h time points. When incubated with 50 mg microspheres, the monomeric fraction decreased to 38.9% by 24 h, with no monomer remaining by 48 h (Fig. 8E). The majority of the remaining protein had been cleaved (49.2% of total protein). Protein incubated with 100 mg microspheres was cleaved more quickly with a composition of 9.30% aggregate, 90.7% cleaved form, and no monomer at 24 h and 100% cleaved form at 48 h (Fig. 8F). The enzyme activity for these samples did not correlate with the percent monomer remaining but instead averaged approx. 4% of the total protein for samples incubated with either 50 or 100 mg microspheres beginning at 10 min incubation. Activity of both these sample sets was almost identical throughout the experiment.

It was evident that lecithin caused a decrease in the monomeric form of the protein by causing increases in both the aggregated and cleaved forms. The activity of the remaining monomeric form, however, was essentially the same as that of the protein incubated alone at 37°C. The ratio of lecithin to protein in this experiment was 30:1, which is a 15-fold increase from the environment that the protein would experience within a microsphere.

The ratio of additives to protein was increased in this study to be much higher than the ratio used in the fabrication of the microspheres. These higher ratios might help to better simulate the effects of degrading polymer on the conformation and activity of the enzyme within the microenvironment of the microsphere, where protein is held in close contact with polymer at an extremely low pH and has been reported to be as low as pH 1.8 [24,27]. Fig. 9

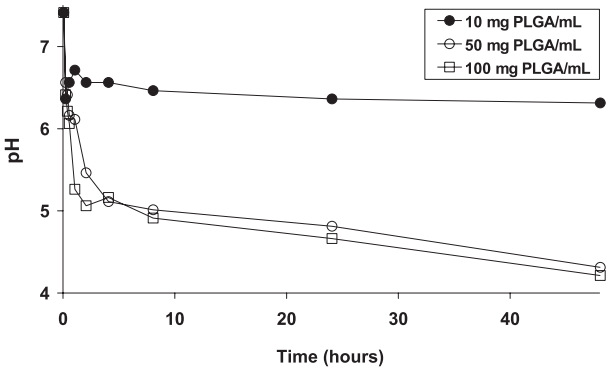

Fig. 9. Decreasing pH of degrading PLGA microspheres in 1 ml PBS buffer at concentrations of 10 mg/ml (●), 50 mg/ml (○), and 100 mg/ml (□).

shows the decreasing pH of the buffer as the blank PLGA microspheres degrade. An 8.1-fold increase in the ratio of PLGA to protein (from 12:1 to 100:1), as seen in the incubations with 10 mg PLGA microspheres (the amount of PLGA microspheres used for our release studies), did cause an increase in the cleaved fraction of the protein, although this increase was not large enough to explain the large fraction of cleaved form being released from microspheres during the release studies. Additionally, this experiment did not cause a decrease in the activity of the remaining monomeric form. Incubation with 50 mg PLGA (40.7-fold ratio increase) and 100 mg PLGA (81.3-fold), however, did show a pronounced increase over the incubation with 10 mg, starting at the 24 h mark. Additionally, the decrease in activity seen for these samples, starting at 10 min, was drastic and did not depend on the amount of monomeric fraction remaining. This might indicate that the enzyme is denatured by the highly acidic degradation products of the polymer prior to its covalent cleavage within the microsphere which correlates with the decrease in polymeric molecular weight observed via GPC. These studies also show that carbonic anhydrase is most likely not being denatured and cleaved after it has been released into the buffered system, but rather is affected detrimentally by the degradation products building up within the microspheres.

3.5. Microspheres with $MgCO_3$ basic salt

$MgCO_3$ was chosen for this study because it is very water soluble and has previously been shown to increase the activity of proteins, such as BSA encapsulated in PLGA, to a greater degree than other less soluble basic salts [27]. Microspheres made with the addition of $MgCO_3$ in the polymer phase were extracted as previously described. The protein extracted consisted of 20.4% aggregate, 69.5% monomeric form, and 9.96% cleaved form, which was similar to the unencapsulated protein and had a slightly higher fraction monomer than microspheres made with lecithin in the aqueous phase alone (Fig. 5).

The enzymatic activity of the extracted protein, however, was much lower at 25.1%.

These microspheres released 98.5% of their contents by 1 week, having released 83.7% by the first 10 min (Fig. 6). This large burst is believed to be due to the incorporation of salts, whose escape from the microsphere causes the formation of large pores [28]. The protein released during the first 10 min, however, was comprised of 58.6% monomer, which was by far the largest fraction of monomeric form released in this study. It is believed that the incorporation of the basic salt helps to maintain a more neutral environment within the PLGA microspheres as they degrade and this most likely aids in maintaining the monomeric form. Additional experiments (data not shown) revealed that the presence of Mg^{2+} does not inhibit the activity of the enzyme.

The activity assay, however, showed very different results. As seen in Fig. 7, the enzymatic activity of the released protein is extremely low at 1.74%. Since the enzyme is mostly in its monomeric form, but inactive, it has most likely been denatured but has not been aggregated or covalently modified due to cleavage. This indicates that although the addition of $MgCO_3$ helps prevent acid-induced cleavage of the protein, it somehow promotes the noncovalent denaturation of the protein. It is possible that the osmotic force of the salts causes the protein to become extremely soluble in the two-phase system during microsphere fabrication, leading to its denaturation at the polymer–aqueous interface. Another simpler explanation is that perhaps that incorporated $MgCO_3$, which is highly water soluble, caused the interior of the microsphere to become too basic and, consequently, denatured the protein.

Additional pH studies were conducted to determine if, in the course of the assay, enough time was allowed for carbonic anhydrase to recover from suboptimal pH incubation. Enzyme was mixed with buffers of various pH values and immediately assayed to determine if the speed of recovery could be interfering with the results of the assay. Enzyme was also incubated with these same buffers for 4 h at 37°C and then assayed. Results showed that enzyme immediately assayed after being combined with buffers of suboptimal pH (pH 4.0–10.0) was as active as the enzyme at its optimal pH, 8.0 (Fig. 10). Enzyme added to buffers at extreme pH values of 12.0 and 2.0, however, showed either reduced activity or no activity, respectively. Only 4 h of incubation with acidic and basic buffers at 37°C severely affected the enzymatic activity of carbonic anhydrase. While enzyme incubated at pH 7.0 retained almost 100% of its activity, a shift to the mildly acidic pH 6.0 caused a sharp decrease of the original activity, while more acidic pH values caused even further decreases. Similar decreases in activity were seen for basic buffers. These decreases indicate that the incubation with acidic or basic species, such as degrading PLGA or $MgCO_3$ salt, could cause a real loss of protein activity which can be adequately measured using the assay in this study.

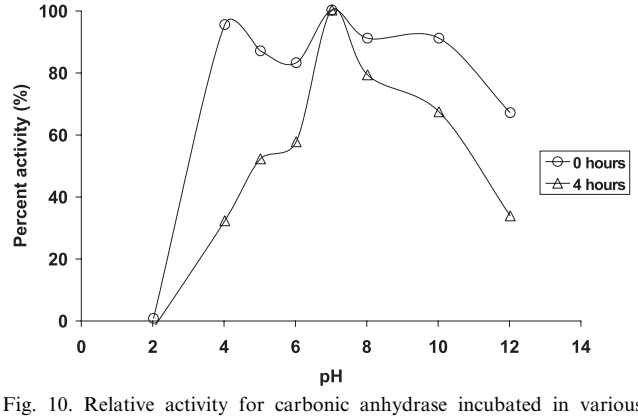

Fig. 10. Relative activity for carbonic anhydrase incubated in various pH buffers, assayed immediately (○) and after4 h incubation at 37°C (△).

4. Conclusion

The goal of this study was to determine the mode of protein inactivation for carbonic anhydrase, an enzyme extremely susceptible to acid-induced cleavage, when encapsulated within PLGA microspheres (1–3 μm). The integrity of carbonic anhydrase was assessed both within formed microspheres and when released. Addition of lecithin to the formulations as a micellar suspension in the aqueous phase aided in the maintenance of the active monomeric form of the enzyme, while fully soluble lecithin in the organic phase caused the inactivation of enzyme via cleavage and aggregation. Addition of a basic salt, $MgCO_3$, which had been thought to be protective against the acidic environment of the interior of the microsphere, actually appeared to cause the inactivation of the monomeric form of the enzyme. More studies with different concentrations of $MgCO_3$ or other less soluble bases might be necessary to determine if lower loadings would still produce a large monomeric fraction, yet enable the protein to retain its activity. Finally, the findings of this study may also be applied to the encapsulation of other protein drugs less susceptible to acid-induced cleavage within PLGA microspheres.

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