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Triggered release of proteins from emulsan–alginate beads

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Abstract

Emulsan/alginate beads were studied for protein adsorption and stability in the context of controlled release. The beads, 400 ± 80 μm diameter with approximately 10% emulsan content, offer unusual opportunities for delivery of proteins due to the natural ability of emulsan to bind proteins, coupled with the selective biological activation features of this complex lipoheteropolysaccharide. The binding capacity of azo-bovine serum albumin by the emulsan/alginate beads was 0.637 ± 0.004 vs. 0.170 ± 0.007 μg/mg for beads formed from alginate alone. In additional protein adsorption experiments, the lipase and subtilisin maintained activity when adsorbed to the emulsan/alginate beads albeit with lower specific activity when compared to the enzyme free in solution. However, the half life of the adsorbed enzyme was significantly higher than the free forms. To explore functional utility of this system, two types of triggered release were studied in the context of these bead systems. First, azo-BSA as a model protein was physically bound to emulsan/alginate beads and then selectively released by triggering with subtilisin, a serine protease, which cleaves the azo dye, sulfanilic acid, from the bound protein. In absence of subtilisin no triggered release was observed. Second, azo-BSA as a prodrug model, was adsorbed to the emulsan/alginate beads and then release of the dye was demonstrated by lipase treatment which cleaves the fatty acid esters from the emulsan structure to release the bound protein. The results establish the versatility and utility of emulsan-based beads for protein binding and triggered release.

1. Introduction

Emulsan is an amphipathic lipoheteropolysaccharide of $\sim 1 \times 10^6$ Da produced and secreted by *Acinetobater venetianus* strain RAG-1. The complex polymer is released into the medium in large amounts during stationary phase growth. The main chain consists of a repeat of three amino sugars: D-galactosamine, D-galactosamine uronic acid, and 2,4-diamino-6-deoxy-D-glucosamine, and the amphipathic properties of the polymer are conferred by fatty acid side chains appended via *N*- and *O*-acyl bonds to the sugar backbone [1]. In recent studies we have demonstrated that emulsan microspheres provide unique and important attributes in terms of protein

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adsorption and controlled release [2]. Emulsan–alginate beads displayed different functional features compared to those composed of alginate alone. For example, high adsorption of bovine serum albumin (BSA) was observed over a range of pH, ionic strength, and temperatures when compared with alginate alone. In addition, emulsans are nontoxic to bacterial or mammalian cells while demonstrating biological activation of macrophages [3]. Emulsan naturally 'carries' up to 23 wt.% protein that is strongly adsorbed, requiring hot phenol extraction for removal [4]. These findings suggest that emulsan microspheres could be utilized in various biomedical applications without toxic side effects.

A range of polymers has been used to generate microspheres for delivery of pharmaceuticals and other compounds [5]. For protein delivery, polymer systems including chemically synthesized/modified 4-[4-[(2-hydroxybenzoyl)amino]phenyl]butyric acid [6], styrene derivatives [7], chitosans [8], and poly(lactic-co-glycolic) acid [9] have been explored. Biopolymers like alginates have also been extensively studied [10,11]. Major benefits to these systems include efficiency in protein delivery, control of the amount protein released, and enhanced protein stability. Despite these benefits, there remains a need for delivery vehicles that provide improved protection of carried proteins against rapid acid or proteolytic degradation while also maximizing protein loading. Furthermore, the opportunity to incorporate polymers as delivery vehicles that provide more than just a passive delivery role would be an attractive feature leading towards more functional or multifunctional drug delivery devices. This could lead to combined selective biological activation and drug release, with appropriate synergistic responses to the delivered therapeutics. Finally, such systems may also find utility in a broader range of applications, including food and personal care products.

The aim of the present study was to examine emulsan/alginate beads as carriers for proteins in terms of functional features of the adsorbed proteins and controlled or enzymatic triggered release of the adsorbed proteins. Enzymatic cleavage of azo-BSA, used as pre-protein model and preadsorbed in the beads was studied using *Candida rugosa* lipase and subtilisin protease. This 'triggered' approach to protein release offers additional control over function in these emulsan-based delivery systems. Finally, the stability of enzymes adsorbed to the microspheres was determined. The results suggest that emulsan/alginate beads offer new options to the field of controlled release of labile proteins due to their protein binding capacity, the ability to trigger release, and the biological activation features of the polymer used as the delivery vehicle.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and solvents were of analytical grade, and other reagents and microbiological media were of highest available grade (Aldrich Milwaukee, WI; Difco, Franklin Lakes, NJ). Low viscosity alginate (A 2158), azo-BSA (bovine serum albumin covalently linked to sulfanilic acid, A 2382), *C. rugosa* lipase, L 8525), and subtilisin (from *Bacillus licheniformis*, P4860) were purchased from Sigma (St. Louis, MO).

2.2. Bacterial cultures and emulsan purification

Emulsan synthesis by *A. venetianus* strain RAG-1 (ATCC 31012) was in saline medium supplemented with ethanol, and purified according to our previously reported techniques [4].

2.3. Synthesis and characterization of beads

Two grams of alginate sodium salt was dissolved in 100 ml of distilled water. Alginate solution was pumped at 1.0 ml/min into an aqueous solution containing 25 mM $CaCl_2$ with or without 3.00 mg/ml emulsan under continuous stirring. Fresh microspheres were incubated in the calcium solutions for 72 h in a shaker at 1.0 Hz at room temperature. The microspheres were then filtered out of calcium solution on Whatman #1 filter paper (Whatman, Clifton, NJ). Filtered microspheres were kept in 70% ethanol at 5 °C until use. Carbohydrate analysis by phenol-sulfuric assay was used to determine the composition of beads. Thirteen alginate and emulsan/alginate beads were placed in test tubes containing 1.0 ml of distilled water and 3.2 ml of concentrated sulfuric acid was added; the tubes were kept at room

temperature for 1 min and then cooled in a water bath. Fifty microliter of 90% phenol was added to the vials, and spectrophotometric readings were taken at 480 nm after 30 min incubation [11]. Differences between emulsan/alginate and alginate microsphere carbohydrate quantities were considered as the amount of emulsan incorporated into the beads. This assay was also used to determine the stability of the microspheres by analyzing carbohydrate leached from the emulsan/alginate and the alginate beads. The leaching was performed in distilled water and pH 7.7 buffer solutions at 37 °C for 1 h and the liquid phase analyzed with the sulfuric acid–phenol assay above.

2.4. Adsorption and release of proteins

To study adsorption of proteins, 100 mg of alginate or emulsan/alginate beads were placed in 1.5 ml tubes and filled with 1.0 ml of experimental solutions. Experimental solutions contained azo-BSA (1.0 mg/ml), or subtilisin (5000.000 U/ml, in 25 mM Tris–HCl buffer, pH=7.7), or lipase (5000 U/ml, 50 mM MES buffer pH=6.0) in each case. Adsorption experiments were carried out incubating the tubes at 37 °C for 60 min, and then centrifuged at 10,000 ×*g* at room temperature for 2 min. Release experiments were performed using vials containing azo-BSA adsorbed by the microspheres incubated with subtilisin or lipase for 30 min at 37 °C. Aliquots of 0.50 ml of supernatants before and after incubations were filtered through a 100 kDa ultrafiltration device (Microcon, Millipore, Billerica, MA), and the filtered products were analyzed for sulfanilic acid and/or BSA release. Spectrophotometric quantification of adsorbed azo-BSA was performed by dissolving the beads for 1 h in the presence of 10 mM EDTA and 100 mM phosphate buffer (pH=7.0) at 37 °C. Sulfanilic acid quantification was performed spectrophotometrically at 334 nm using a calibration curve after precipitating azo-BSA and BSA with 5% trifluoroacetic acid at 0 °C for 15 min, and centrifuging the supernatant at 10,000 ×*g* for 5 min. Determination of BSA was carried out using Coomassie Brilliant Blue dye binding assay and Bovine Serum Albumin (BSA, Fraction V) as a standard [12]. Release of adsorbed enzyme from the beads was studied by incubating the beads containing the adsorbed lipase in the presence of hydrogen disodium phosphate 100 mM–sodium citrate 9.0 mM buffer (pH=7.45) at 37 °C until total bead dissolution (1 h). Aliquots of the viscous alginate and emulsan–alginate solutions containing lipase were assayed with *p*-nitrophenyl acetate as described above.

2.5. Light microscopy (LM)

LM was performed with an Axiovert S-100 inverted microscope (Carl Zeiss, Jena, Germany) using fresh alginate and emulsan/alginate beads previously incubated with azo-BSA as described above.

2.6. Enzyme assays

C. rugosa lipase was assayed by incubating the sample in the presence of 150 μM *p*-nitrophenylacetate, 50 mM MES buffer (pH=6.0) at 37 °C for 30 min. One lipase unit was defined as the amount of enzyme able to produce 1 μmol of *p*-nitrophenol per minute at 400 nm in a 1 cm light path cuvette. To assess lipase stability adsorbed to the microspheres, lipase-bound microspheres were incubated at 37 °C for 40 min and lipase activity was monitored with *p*-nitrophenylacetate conversion to *p*-nitrophenol. Subtilisin Carlsberg from *B. licheniformis*, protease, activity was determined by incubating a 1 ml reaction vial containing 400 μg/ml azo-BSA/50 mM Tris–HCl buffer (pH=7.7) solution at 37 °C for 10 min. Free dye in the supernatant was determined at 334 nm using an appropriate standard. Alternatively, protease activity was assayed in the presence of 10 μg/ml casein as previously reported [13]. The reaction was stopped by adding 50 μl of 5% tricholoroacetic acid, and then centrifuged (2 min at 10,000 ×*g*). Analysis of supernatant proteins was quantified using Coomassie Brilliant Blue assay as described above. Controls without protease, and with protease previously inhibited with 1.0 mM diisopropyl fluorophosphate (DFP), were included.

2.7. SDS–PAGE

Samples were run in 10% Novex 1 mm Tricine gels at 110 mV for 2 h (Invitrogen, CA). Molecular weight of samples was estimated using protein standards covering 6515 to 212,000 kDa (New England Biolabs). Gels were stained by silver (SilverXpress, Invitrogen, Ca).

2.8. Sample analysis

Experiments were run in triplicates to quintuplicates ($n=3$ to 5). Data in the graphs represent the average ± standard deviation.

3. Results

Alginate and emulsan/alginate beads exhibited an average weight of 220 ± 20 μg of polymeric material per bead, with diameter of 400 ± 80 μm. The emulsan content in the beads, determined by the sulfuric acid–phenol assay, was 24.0 ± 4.3 μg per bead, thus the emulsan/alginate beads consisted on average of about 10.9% emulsan. Analysis of carbohydrates leaching from the emulsan/alginate and alginate beads suggested that the structures were stable and no evidence for polymer solubilization was found after sugar analysis over 3 h. The difference in azo-BSA content adsorbed by the beads was initially visualized by light microscopy (Fig. 1). Spectrophotometric quantification of azo-BSA adsorbed in the microspheres confirmed visual differences, 0.637 ± 0.004 μg/mg ($n=5$) and 0.170 ± 0.007 μg/mg ($n=5$) of azo-BSA adsorbed per emulsan/alginate and alginate bead, respectively. The azo-BSA adsorption ratio between emulsan/alginate to alginate microspheres was 3.7 indicating significantly higher capacity of the beads for the protein due to the presence of the emulsan.

C. rugosa lipase was used to examine the 'triggered' release of physically bound protein by the cleavage of the emulsan-based fatty acid esters (Fig. 2). Beads with adsorbed azo-BSA were treated with lipase for 20 min at 37 °C. Due to the potential for non-specific protease-like activity in the lipase preparations [14], the release of sulfanilic acid (in the absence of emulsan or alginate) from the azo-BSA was estimated by precipitation with 5% trifluoroacetic acid at 0 °C and found to be negligible (data not shown). In addition, in the absence of the lipase the release of azo-BSA and sulfanilic acid from both types of beads was negligible. The release of azo-BSA from the alginate beads was not significantly increased in the presence of the lipase. In contrast, the release of azo-BSA from the emulsan/alginate beads by the lipase was nearly complete in 10 min (Fig. 3).

To study the adsorption of prodrugs that could subsequently be activated and/or released by specific enzymes, the beads were loaded with azo-BSA followed by treatment with subtilisin. Subtilisin is a serine protease in the same group as trypsin and chymotrypsin and present in significant amounts in the mammalian gastro-intestinal tract. Sulfanilic acid release from azo-BSA adsorbed to the beads via enzymatic hydrolysis was characterized. Reactions were run with and without (control) subtilisin, and with subtilisin previously inhibited with DFP (control) as an irreversible protease inhibitor (Fig. 4). The rate of sulfanilic acid release from the emulsan/alginate and alginate beads without protease or with the DFP-inhibited subtilisin was negligible. Upon treatment with subtilisin, kinetic analysis of dye release in the linear range showed reaction rate constants of

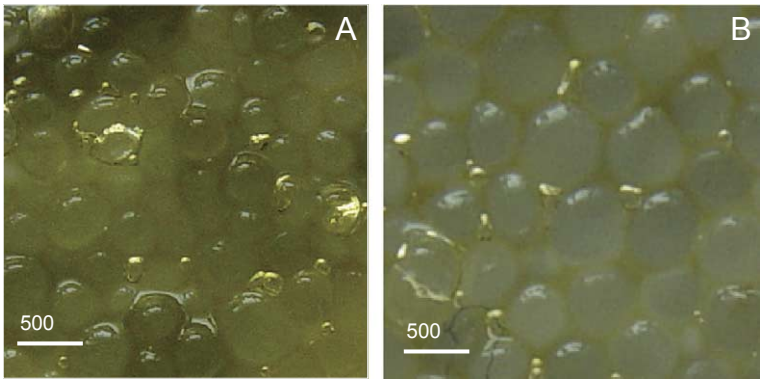

Fig. 1. Emulsan/alginate (A), and alginate (B) beads containing adsorbed azo-BSA.

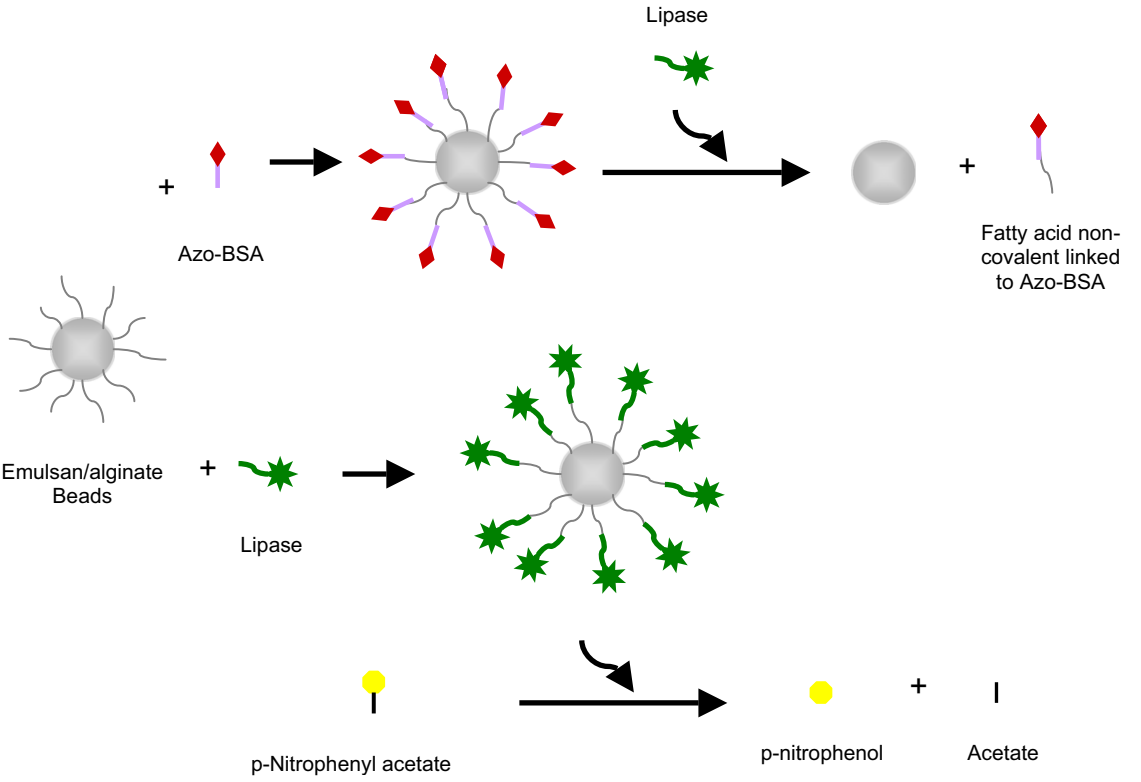

Fig. 2. Scheme of assays performed on emulsan/alginate beads with lipase.

11.34×10^{-3} min^{-1} and 4.02×10^{-3} min^{-1} for the emulsan/alginate and alginate beads, respectively, with a release ratio of 3.33 (Fig. 5). The adsorption and dye release ratios for the two types of beads suggest that no major changes in subtilisin activity were induced by the emulsan in the beads.

Subtilisin activity was monitored during the incubation of the beads containing azo-BSA by SDS–PAGE. Analysis of azo-BSA bead supernatants containing subtilisin by SDS–PAGE showed the presence of a strong band close to 66.0 kDa, indicating the release of free BSA (M_W=66.4–66.7 kDa, [15]) from both types of beads. SDS–PAGE analysis of bead supernatants showed a similar profile after 10, 30, or 50 min, suggesting no changes in subtilisin activity in the presence of both types of beads. Also, autolysis, a common feature with proteases and particularly in subtilisin, was not in evidence (Fig. 6).

Subtilisin adsorption by the two types of beads was characterized in order to assess the potential for delivery of a biologically active protein. For these experiments, soluble azo-BSA was used to quantify protease activity adsorbed to the microspheres.

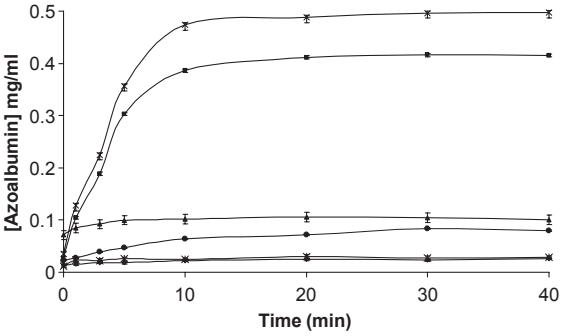

Fig. 3. Release of azo-BSA and sulfanilic acid adsorbed by beads using *C. rugosa* lipase. Symbols: × and , ■ sulfanilic acid and azo-BSA release from emulsan/alginate and alginate, respectively; ▲ and ●, sulfanilic acid release from emulsan/alginate and alginate beads, respectively; ✳ and ◆, sulfanilic acid and azo-BSA release from emulsan/alginate and alginate beads, respectively, incubated without lipase.

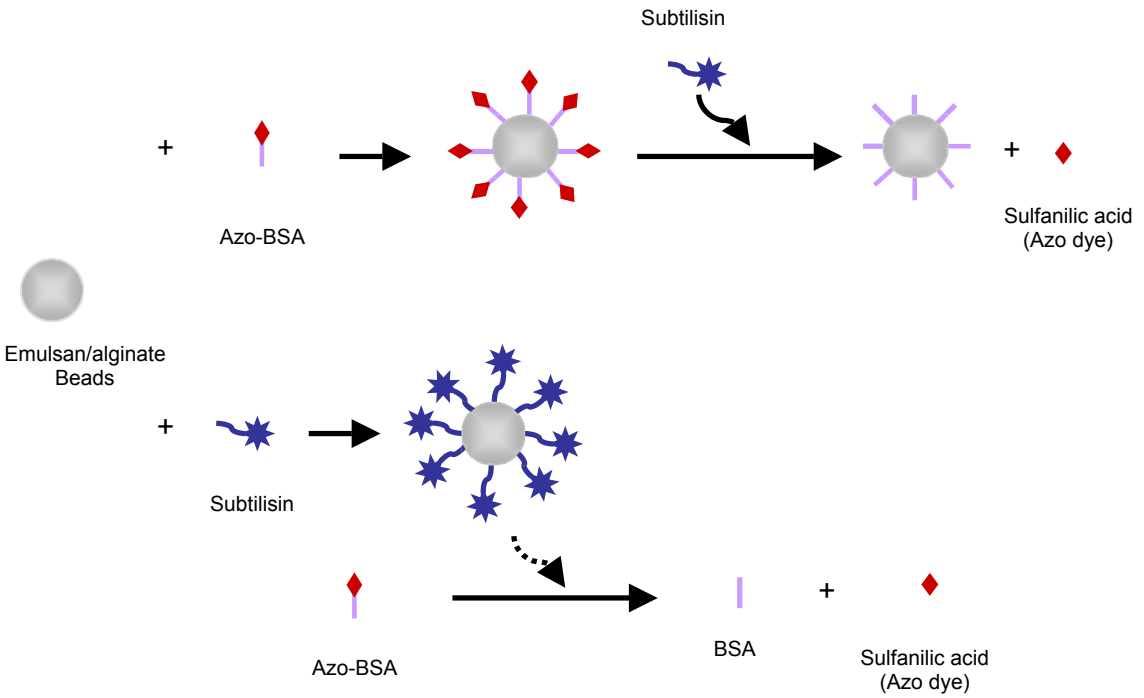

Fig. 4. Scheme of assays performed with the beads using subtilisin.

However, it was necessary to first address the potential problem of azo-BSA adsorption which might reduce the availability of azo-BSA for subtilisin reactions, thereby leading to an underestimate of subtilisin activity. After protease adsorption, a blocking step utilizing BSA (without azo dye) was performed. BSA may act as a competing substrate for the active site of subtilisin, and could therefore reduce the observed cleavage of azo-BSA. The strong pH-dependence of subtilisin activity was utilized to block with BSA at pH 6.0 where subtilisin Carlsberg activity is reversibly reduced to almost zero [16]. This

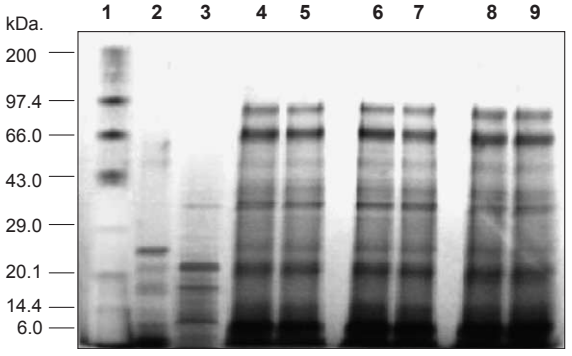

Fig. 5. SDS–PAGE—Lanes: 1, molecular weight markers; 2, subtilisin inhibited with DFP; 3, casein, at zero time; 4, 6 and 8, alginate bead supernatant incubated with subtilisin at 10, 30 and 50 min, respectively; 5, 7 and 9, emulsan/alginate bead supernatant incubated at 10, 30 and 50 min.

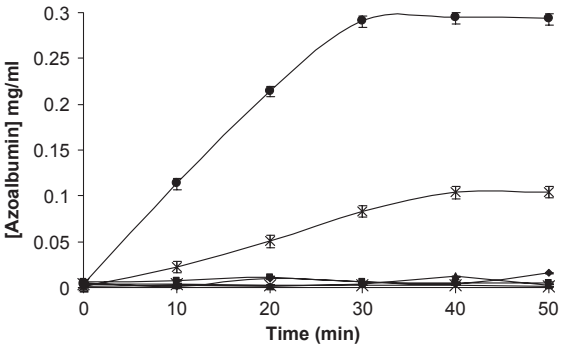

Fig. 6. Cleavage of sulfanilic acid from azo-BSA adsorbed on the beads. Symbols: ♦, emulsan/alginate; ■, alginate; ▲ and ×, emulsan/alginate and alginate beads incubated with subtilisin previously inhibited with DFP, respectively; ● and ✳, emulsan/alginate and alginate beads incubated with subtilisin, respectively.

shift in pH also provided an increase in BSA adsorption by the beads as was previously described [2]. The blocking step was followed by detection of subtilisin activity under standard conditions (pH=7.7). In both bead types, enzymatic activity reached a plateau after 5 min of incubation and no differences in subtilisin activity were detected in the two types of beads at 37 °C for 20 min (Fig. 7). Therefore, during the adsorption process of subtilisin into the beads, enzymatic activity was not inactivated, and consequently it is possible to infer that the protein retained biological activity.

The hydrolysis rate of soluble azo-BSA by subtilisin adsorbed to each type of bead exhibited similar values, 8.645×10^{-6} and 8.997×10^{-6} mM azo-BSA per nM of subtilisin per min at 37 °C for the emulsan/alginate and alginate beads, respectively. These rates were significantly lower than that found for soluble subtilisin which exhibited a rate of azo-BSA hydrolysis of 3.967×10^{-2} mM azo-BSA per nM of subtilisin per minute under the same experimental conditions, and can be explained by the rigidification of the enzyme structure induced by immobilization on the bead surface. Of particular interest, however, the activity of subtilisin adsorbed in either bead preparation remained constant over 4 h of incubation (data not shown), while the half-life of soluble subtilisin was less than 30 min [16]. The stability of the lipase adsorbed in the beads was examined. *C. rugosa* lipase was first adsorbed to beads, followed by a blocking step with BSA. Release

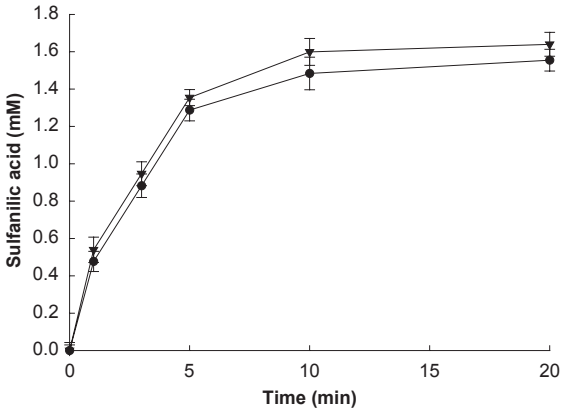

Fig. 7. Activity of subtilisin adsorbed on emulsan/alginate (●) and alginate (▼) beads, respectively.

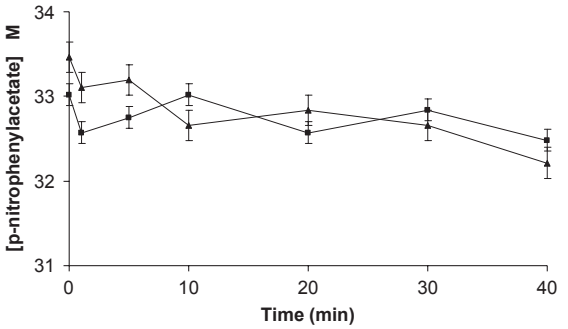

Fig. 8. Stability of lipase adsorbed on emulsan/alginate (●) and alginate (▼) beads, respectively.

of *p*-nitrophenol by the adsorbed lipase showed similar and stable activities over 36 h with both types of beads (Fig. 8).

4. Discussion

In previous work, the higher protein adsorption capacity of emulsan/alginate beads when compared to alginate beads was attributed to the presence of fatty acid esters on emulsan [2]. The present studies utilizing azo-BSA adsorption confirmed our previous findings, indicating high protein adsorption by beads containing emulsan when compared to those consisting of only alginate, and this adsorbed protein could be released via treatment with a lipase that would hydrolyze these fatty acid side chains. The present work also demonstrated that pre-proteins, like pro-hormones and pro-enzymes, adsorbed on emulsan/alginate beads (in this study in the form of azo-BSA) can be cleaved, meaning activated, under physiological conditions by common enzymes. Thus, in both cases, triggered release of the bound 'drug' could be demonstrated, either by clipping off the fatty acid side chains from the emulsan in the beads, or by enzymatically hydrolyzing the conjugated form of the drug physically bound to the beads to facilitate release from the beads.

Lipase was utilized to induce release of physically bound protein from emulsan microspheres. Lipase treatment of the emulsan/alginate beads containing adsorbed azo-BSA showed release of dye coupled BSA, and little conversion of azo-BSA into sulfanilic acid and BSA (Fig. 3). This specific release profile

induced by lipase can likely be attributed to the release of fatty acids from the emulsan, therefore decreasing the binding capacity of the emulsan/alginate system. This strategy may be useful in instances wherein 'triggered' approaches to release are desirable, such as small peptides and proteins which are susceptible to degradation under physiological conditions mediated by proteases [18].

Protease autolysis is a significant drawback in the use of serine proteases. In particular, subtilisin Carlsberg, one of the most active enzymes of this family, has a half-life of approximately 25 min in aqueous medium at 30 °C and the autolysis rate increases about 10-fold at 37 °C [17]. Reaction rates of subtilisin adsorbed on the two types of beads were not significantly different, indicating that the biological activity of subtilisin is unaffected by adsorption in the beads. Soluble subtilisin showed $\sim 4 \times 10^3$ higher rates of activity compared to adsorbed subtilisin in the emulsan/alginate and alginate beads. A decrease of enzyme activity is a common result of enzyme immobilization processes, due to enzyme structure rigidification and steric hindrance, which drastically reduces the rate of molecular transfer in the catalytic triad center of the enzyme [18]. The significant decrease in subtilisin activity when adsorbed in the beads is partially compensated by the more than 8-fold increase in enzyme stability when compared to the soluble enzyme.

The decrease in lipase activity adsorbed to both types of beads was low after 40 min, on the order of 10^{-4} μmol/min. In the case of the alginate beads, lipase inactivation was practically negligible, but the reduction of lipase activity was 3.75 times higher in the emulsan/alginate system, which could be due to inhibition by free fatty acids released from emulsan, a competitor with *p*-nitrophenyl acetate substrate for the active site of the enzyme rather than biocatalyst inactivation by experimental conditions (e.g. temperature, pH). In addition, experiments with adsorbed enzymes such as subtilisin and lipase showed that protein structure was not drastically changed by the adsorption procedure, suggesting the tertiary and quaternary protein structures are maintained by the proteins.

Lipase release from the beads was studied by dissolving the beads and measuring enzyme activity in solution (Table 1). The results confirmed that the adsorption process in emulsan/alginate beads is a reversible process and that the reduced enzymatic activity of lipase when bound to the beads should be attributed to steric hindrance. Also, optical microscopy controls of both types of beads did not reveal any aggregates of the enzymes. Moreover, similar values of enzymatic activity were recovered from the dissolved alginate beads, and a slight decrease of enzymatic activity was detected in emulsan/alginate beads compared to the controls, probably because of the presence of free fatty acids as a product of emulsan partial hydrolysis. Lipase enhanced activity released from alginate microspheres can be interpreted in terms of the increase of the hydrophobic environment compared to the aqueous media due to alginate dissolution in the aqueous media [19]. Retention of biological activity is an important issue in pharmaceutical delivery in which protein–protein (e.g. aggregation) and protein–matrix interactions of are important reasons for protein unfolding and consequently lack of biological activity.

Table 1
Activity of *C. rugosa* lipase released from alginate and emulsan/alginate beads

Lipase	Enzymatic activity (%)
Control (aqueous media)	100.0 ± 2.1
Released form alginate beads	109.8 ± 4.7
Released form emulsan/alginate beads	89.5 ± 6.5

In conclusion, proteins bound to emulsan/alginate beads can be specifically released (triggered release) by treatment with lipase, which cleaves fatty esters from the emulsan structure thereby releasing the bound protein, or release by selective hydrolysis of the bound prodrug. The emulsan/alginate bead preparations allowed lipase and subtilisin to maintain activity while adsorbed, albeit at a lower level than in solution, and extended the half-life of the physically bound enzyme. These results further establish the versatility and utility of emulsan/alginate beads for protein binding and delivery functions.

Acknowledgments

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