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Ability of chitosan/carrageenan complex to encapsulate bovine serum albumin (BSA) for potential use in protein delivery

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Abstract

The purpose of this study was to examine the capability of chitosan/carrageenan complex formed by electrostatic interaction as a carrier system for protein delivery. Chitosan/carrageenan complex was formulated at charge ratios (+/-) of 1,3,5,7 while bovine serum albumin (BSA) was the model protein. Microencapsulation of BSA was carried out by mixing BSA with chitosan solution followed by -, -, or -carrageenan then agitated at 200 rpm for 24 hours at 4 °C. The resulting complex was then evaluated by different physical methods. Results showed that the polyelectrolyte complex with -carrageenan had the highest encapsulation efficiency at 74 % with the order of >> and was stable in pH 1.2 to 7.3. The complex of chitosan/carrageenan was more efficient in the encapsulation of BSA than using chitosan or carrageenan alone. Controlled release of BSA from the complex was observed when treated with enzymes and different physiological solutions. Among the complexes prepared, chitosan/-carrageenan complex was the most stable releasing the BSA at the lowest rate. SDS-PAGE analysis of the complex showed no degradation of BSA when exposed in pH 1.2 solution 37 °C for 1 hour while gradual degradation was observed in pepsin solution (1 mg/mL). Zeta potential ranged from -40 mV to -50 mV with z-average size from 334 nm to 782 nm. Atomic Force Microscopy (AFM) images of chitosan/carrageenan complex loaded with BSA showed compact structure with almost uniform circular structure.

INTRODUCTION

At present, researches being done on protein drugs are directed towards developing effective oral formulations and increasing the oral absorption of intact protein through the use of formulations that protect the macromolecule and/or enhance it's uptake into the intestinal mucosa [1]. In order to effect the delivery of peptides and proteins via the gastrointestinal tract, a carrier system should overcome significant enzymatic and diffusion barriers. The most widely used carrier is the cationic polysaccharide chitosan. It is biodegradable, biocompatible, bioadhesive and has permeation enhancing properties [2]. Previous study revealed that drug release from polyion complexes affords more sustained effect than the preformed complexes [3]. Polymer blends can combine attributes of different polymers to give a superior quality for the dosage form [4]. Since carrageenan reacts with chitosan forming a polyelectrolyte complex [5] the objective of this study is to evaluate the possibility of using polyelectrolyte complexes from chitosan and different types of carrageenan (, ,) produced from Philippine *Eucheuma* species as carrier system for protein delivery. The model protein used in this study is bovine serum albumin (BSA). Carrageenan is an anionic polymer extracted from marine red algae and has also been used in many published studies as a carrier system for drug delivery [6,7,8,9,10,]. It shows potential use for new drug delivery system providing more control over the release rate of drugs. Previous studies showed that polyelectrolyte complex (PEC) in the form of beads and microspheres that are formed by cationic polymer(s) and anionic polymer(s) could enhance the controlled or prolonged release of a drug. Examples of PEC for controlling drug release include alginate/chitosan [5], chitosan-cellulose multicore microparticles [6], chitosan-coated pectin [7], Chitosan/poly(acrylic acid) complexes [8], poly(vinyl alcohol)/sodium alginate blend beads [9], poly(methacrylic acid-g-ethylene glycol) particles [10]. The potential use of carrageenan for oral protein delivery will indirectly boost the carrageenan industry in the Philippines since all of the carrageenan samples used in this study were produced in the said country.

METHODOLOGY

Materials:

Chitosan (average MW = 45 kDa, with degree of acetylation of 75.4%) was obtained fromYSK YaizuSuisankagaku Industry Co, Ltd., Japan. -carrageenan (Bengel KK-100, Lot No. XO300-2, average MW = 510 kDa), -carrageenan (Benvisco SI-100, Lot No.M1400-1, average MW= 560 kDa) and -carrageenan (Benvisco SL-100, Lot No. S2703-2, average MW = 750 kDa) were obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Bovine serum albumin (BSA) was purchased from Sigma Aldrich Japan K.K while dextran sodium sulphate (average MW = 5 kDa) was purchased from Wako Japan. All other chemicals and reagents used were of analytical grade.

Preparation of Solution:

Chitosan solution was prepared at 1mg/mL in MES buffer solution at pH 6.0. Carrageenan (1mg/mL), Dextran sodium sulphate (1 mg/mL) and BSA (4 mg/mL) solutions were prepared in MilliQ water.

Preparation of Complex:

BSA solution was added to chitosan solution, mixed thoroughly and allowed to stabilize at room temperature for 15 minutes. Carrageenan solution was then slowly added to the mixture, mixed again followed by another 15 minutes stabilization at room temperature. Final concentration of BSA in

the complex was 1mg/mL. Thereafter the final mixture was incubated for 24 hours at 4 ^{0}C with shaking (200 rpm). The volumes used to prepare the complex at various charge ratios (+/-) and type of carrageenan are shown in Table 1.

Encapsulation Efficiency (E.E.):

After incubation of the complex, the solution was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C. The resulting supernatant was then assayed for its protein content following the manufacture's instruction (Bio-Rad Laboratories, Japan). All experiments were performed in triplicate. Encapsulation efficiency was determined according to the following equation:

$$\text{E.E. (100\%)} = \frac{\text{Initial concentration of Protein - Protein Reading from Assay}}{\text{Initial concentration of Protein}} \times 100$$

Relative Stability at Different Physiological Solutions:

After centrifugation of the solution, the supernatant was removed and the resulting precipitate was subjected to various simulated physiological solutions: a. 35 mMNaCl solution adjusted to pH 1.2; b. 50 mM Acetic acid adjusted to pH 4.0; c. 200 mMNaCl in MES buffer adjusted to pH 6.0; d. 50 mM Potassium phosphate buffer adjusted to pH 6.8; e. MilliQ H_2O; f. PBS solution at pH 7.3 The relative stability was calculated according to the following equation:

$$\text{Relative Stability (\%)} = \frac{\text{Turbidity at 500 nm of the complex in physiological solution}}{\text{Turbidity at 500 nm of 0 mMNaCl, MES buffer solution, pH 6.0}} \times 100$$

Atomic force microscopy (AFM) measurement:

After incubation for 24 hours at 4 °C with shaking (200 rpm), 10 uL of the solution was diluted with MES buffer (pH 6.0) to make 100 uL then applied 20 uL drop deposition onto the surface of a mica disk as previously described [11]. Following adsorption for 2 minutes at room temperature, excess fluid was removed by absorption with filter paper. Then 50 L of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging. A commercial atomic force microscope (SPA-300 system of Seiko Instruments, Inc. Japan) was used for imaging using a Si_3N_4 tip on the cantilever (100 m x 400 nm, SN-AF01-A, Olympus Optical Co.) with tapping mode and non-contact recording under ambient condition.

Measurement of zeta potential and particle size:

Surface charges of the complexes were evaluated using a Coulter DELSA 440SX Zeta Potential Analyzer. The complex after incubation for 24 hours at 4 °C with shaking (200 rpm) was diluted with MES buffer (pH 6.0) to make a final volume of 1.5 mL. Size measurement was determined by using a High Performance Particle Sizer (Malvern Instrument, UK) diluting 3 uL of the complex with MES buffer (pH 6.0) to make a final volume of 1.2 mL.

Cumulative release of BSA from the complex after treatment with different physiological solutions:

The precipitate formed after centrifugation was treated with 1 mL each of the different physiological solutions at 37 ^{0}C with mild agitation (60 rpm) for 10, 30, 60, 120, 180 minutes. After each time interval, the treated samples were assayed for its protein content as previously described. Cumulative release of BSA from the complex was determined according to the following equation:

$$\text{Cumulative release} = \frac{\text{\% Concentration of the protein in the complex–Protein Reading from assay x 100}}{\text{Concentration of the protein in the complex}}$$

Cumulative release of BSA from the complex after treatment with enzymes (chitosanase & papain, 1 mg/mL, pepsin & trypsin, 0.1 mg/mL):

The same procedure was followed as previously described. Release of BSA from the complex was determined by protein assay of the supernatant collected after each time interval of 1, 3, 5, 7, 9 hours.

SDS PAGE analysis:

Release and degradation of BSA from the complex after exposure to pepsin (1 mg/mL) and pH 1.2 was monitored by SDS-PAGE electrophoresis using Bioradredy gels J (161J341V) in running buffer (1 x Tris-Glycine, 0.1% SDS) with constant current mode, 20 mA. About 5 uL of sample was applied in each well. A pre-stained protein marker was also used. Staining solution used was Coomasie Brilliant Blue.

RESULTS

Encapsulation Efficiency:

In this study, different charge ratios (+/-) of 1,3,5,7 were prepared to determine the efficiency of the polyelectrolyte complex to encapsulate BSA. As shown in Figure 1A, the complex of chitosan/-carrageenan had the highest encapsulation efficiency of 74% followed by chitosan/-carrageenan. The least efficient was the -type. Chitosan/dextran sodium sulphate complex showed an encapsulation efficiency of 68%. Encapsulation efficiency of each of the polymers was also investigated. - carrageenan exhibited the highest efficiency (Fig. 1B) followed by -carrageenan and -carrageenan. Chitosan only showed an efficiency of about 10%. Since charge ratios of 3 & 5 exhibited high encapsulation efficiency, chitosan/carrageenan complexes prepared at these ratios were furthered investigated and studied according to its morphology, stability and physical properties.

Morphology of the Complex:

As observed in Figures 2J, 2L, 2M, 2N, AFM images of the polymers (carrageenan, chitosan) and BSA (Figure 2K) showed morphologies that are fibrous in shape. Complexation of chitosan with carrageenan through electrostatic interaction formed a good complex with BSA (Figures 2A-2I). AFM images of chitosan/carrageenan complex loaded with BSA (Figures 2A-2I) showed compact structure with almost uniform circular structure confirming a complex formation. The complexes of chitosan/-carrageenan (Figure 2A) and chitosan/dextran sodium sulphate (Figs. 2D & 2H) showed a good complex formation with BSA as compared to -, -carrageenan and chitosan (Figs 2B, 2C, 2F, 2G, 2I) where some BSA could be seen looping out of the complex. These findings conformed to the results of the encapsulation efficiency (Figure 1) of the polyion complex. As observed in the AFM images, complexes with encapsulation efficiency of < 85% showed some unbound BSA in the background.

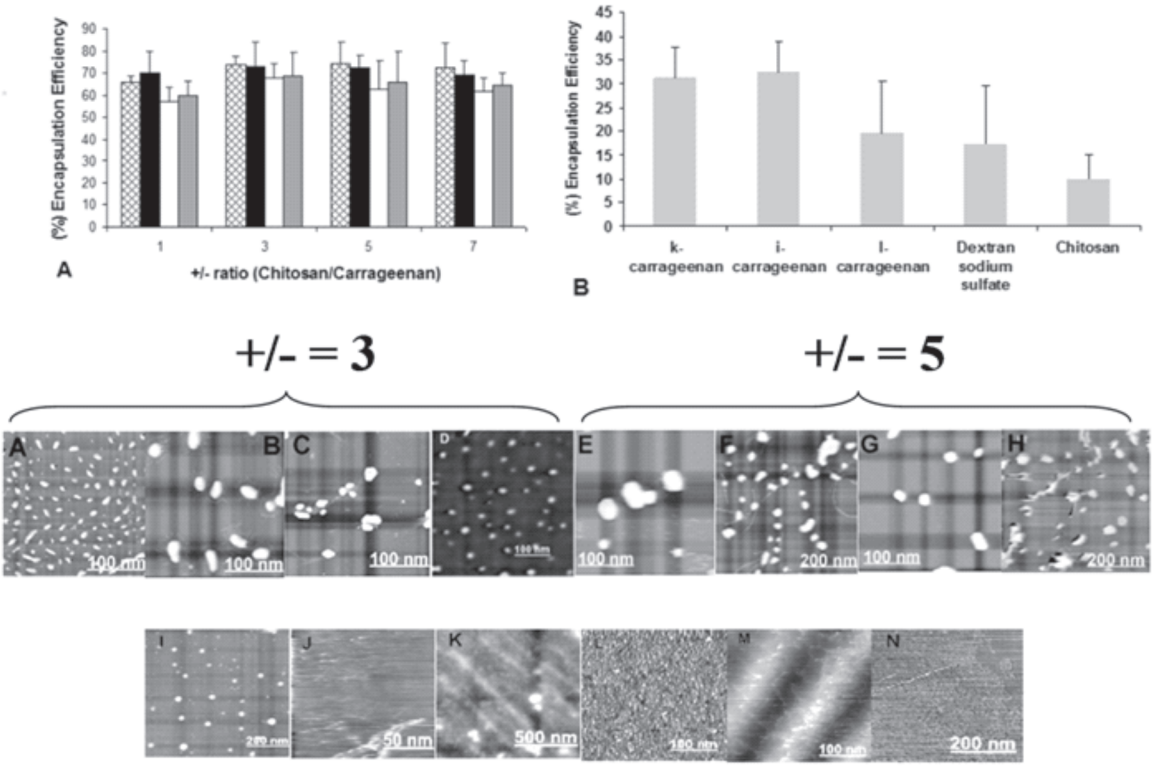

Figure 1. Efficiency of chitosan/carrageenan complex and single polymers to encapsulate BSA (1mg/mL) at different charge (+/-) ratios and AFM images of BSA encapsulated with the complex. ⬚ : chitosan/-carrageenan; ■ :chitosan/-carrageenan; □ : chitosan/-carrageenan; ▩ : chitosan/dextran sodium sulphate. The values represent the mean ± SD, n = 4. BSA indicates bovine serum albumin. AFM images: A & E: chitosan/-carrageenan; B & F: chitosan/ carrageenan; C & G: chitosan/carrageenan; D & H: chitosan/dextran sodium sulphate; I: chitosan (with BSA); J: -carrageenan; K: BSA; L: carrageenan; M: - carrageenan; N: chitosan;

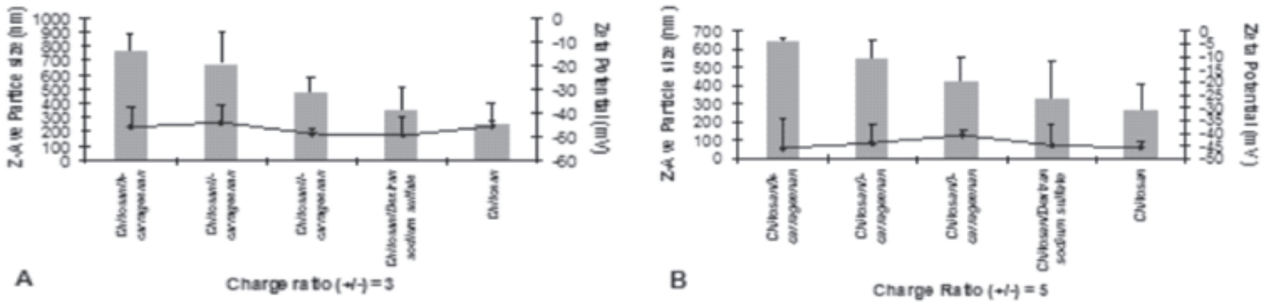

Figure 2 Zeta potential and particle size of the chitosan/carrageenan complex with BSA (1 mg/mL). The values represent the mean ± SD, n = 4. BSA indicates bovine serum albumin

Zeta Potential and Particle Size:

Negative zeta potential (Figure 3) was observed in all of the complexes prepared. There was no significance change in zeta potential in both charge ratios. Charge ratio (+/-) = 3 displayed negative zeta potential at > - 45 while charge ratio (+/-) = 5 exhibited negative zeta potential of < - 46. High values of zeta potential suggested high stability of the complex. Particle size of chitosan/carrageenan complexes with BSA were higher in values than chitosan/BSA alone. Increase of particle size was expected in charge ratio (+/-) = 3 as compared to charge ratio (+/-) = 5 since the former contained more carrageenan than the latter. The high values of particle size showed that entrapment of BSA and complexation of chitosan with carrageenan were achieved.

In Vitro Release Studies:

The release profile of BSA from the chitosan/carrageenan complex were evaluated in 0.1 mg/mL solution of chitosanase, pepsin and trypsin and different physiological solutions. In Figures 4-6 controlled release of BSA was observed in which the lowest rate was exhibited by chitosan/-carrageenan complex. The electrostatic interaction of the amino group of chitosan with the sulphate group of -carrageenan was more stable than the interaction between chitosan to - & -carrageenan. The complex of chitosan and dextran sodium sulphate also showed slow release rate of BSA next to chitosan/-carrageenan complex. Chitosan showed the highest release rate of BSA at about > 60% after one hour incubation in chitosanase (Figure 4) and pepsin (Figure 5).

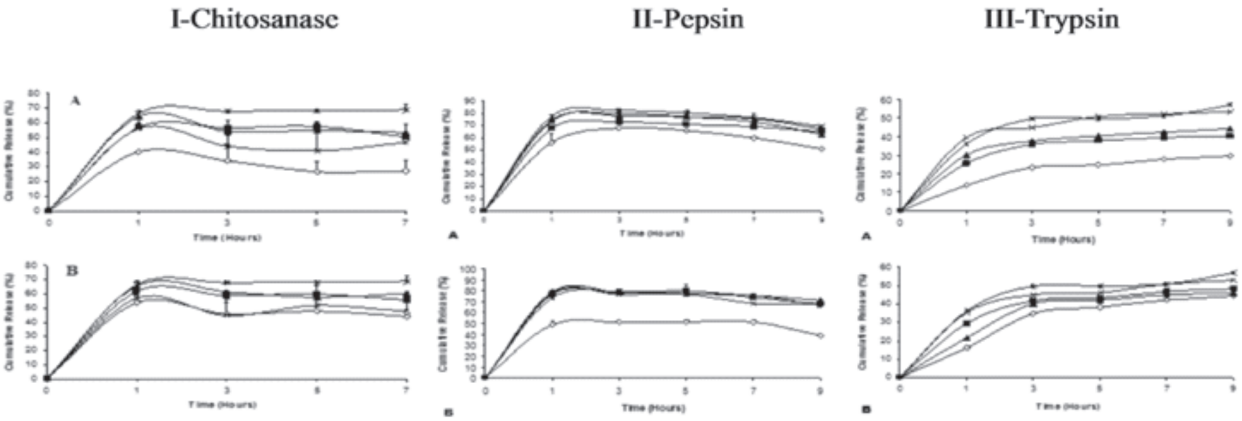

Figure 3 Cumulative release of BSA from chitosan/carrageenan complex prepared at various charge ratios in different enzyme solutions (0.1 mg/mL). A: (+/-) = 3; B: (+/-) = 5; ◊ = chitosan/-carrageenan; ■ = chitosan/-carrageenan; ▲= chitosan/-carrageenan; = chitosan/dextran sodium sulphate. Incubation temperature: 37 0C. pH: 6.0. The values represent the mean ± SD, n = 3. BSA indicates bovine serum albumin.

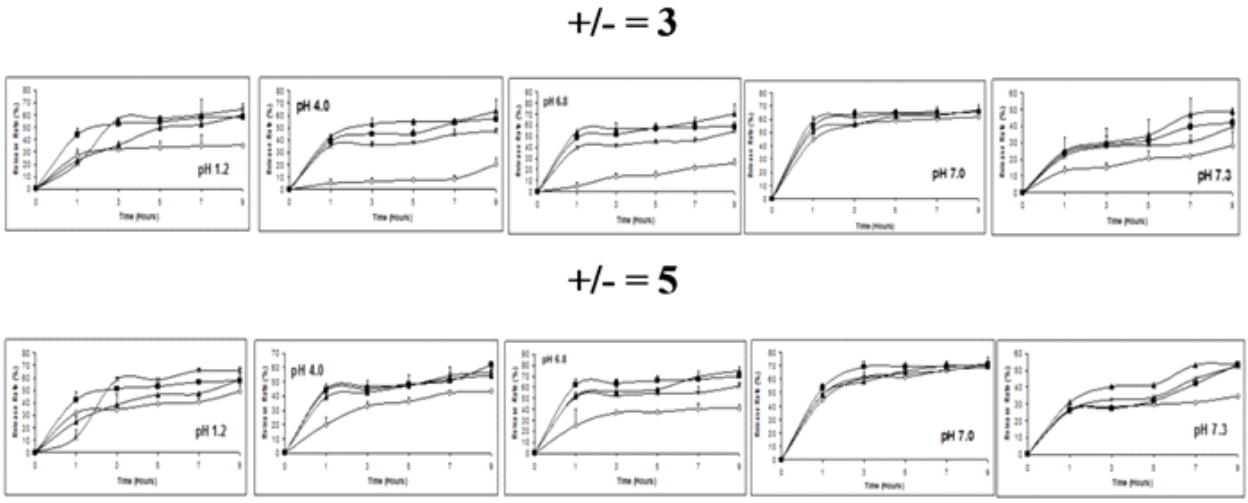

Figure 4. Cumulative release of BSA from chitosan/carrageenan complex prepared at various charge ratios in various physiological solutions. ◊ = chitosan/-carrageenan; ■ = chitosan/-carrageenan; ▲= chitosan/-carrageenan; = chitosan/dextran sodium sulphate. Incubation temperature: 37 0C. The values represent the mean± SD, n = 3. BSA indicates bovine serum albumin.

Using different physiological solutions (Figures 7-8), the chitosan/-carrageenan still showed the lowest release rate of BSA. Even at low pH of 1.2, the chitosan/-carrageenan complex was the most stable among the complexes releasing the BSA in a much controlled rate. At this condition (below the isoelectric point of BSA), the BSA was positively charged and was bound more to carrageenan making the release rate more slower. Complexation of chitosan with carrageenan controlled the release of BSA at a given time and temperature.

Relative Stability at Different Physiological Solutions:

The complexes were relatively stable in different physiological solutions at various pH (Figure 9). Among the complexes prepared, chitosan/dextran sodium sulfate has the highest turbidity reading as compared to chitosan/carrageenan complex. The chitosan/-carrageenan complex was the most stable as there was no significant change observed in the turbidity reading at various pH. Turbidity reading increases at pH 6.0 in chitosan/-carrageenan, chitosan/-carrageenan and chitosan/dextran sodium sulfate complexes.

Stability of BSA in pepsin solution and pH 1.2:

In protein delivery, the protein must be protected by the complex formation during its application. The stability and integrity of BSA in the complex were evaluated by gel elecrophoresis. The use of pepsin solution and pH 1.2 was employed to investigate the integrity of BSA. Figure 10 shows the SDS-PAGE analysis of release fractions of BSA in chitosan, carrageenan and dextran sodium sulfate (Figure 10A) and BSA in chitosan/carrageenan complexes (Figure 10B), free BSA and treated free BSA that were used as a control. The study showed that the treated free BSA (Figure 10A & B, Lane 3) was degraded completely as compared to the BSA released from the polymers and complexes. In Figure 10A, the BSA released from chitosan (Lane 8), carrageenan (Lanes 4-6) and dextran sodium sulfate (Lane 7) was degraded in a slower rate based on the formation of more than one band as compared to treated free BSA in which only one band (Lane 3) was observed. The BSA fractions released from chitosan/carrageenan complex showed also degradation but in a much slower rate. In Figure 10B, most of the

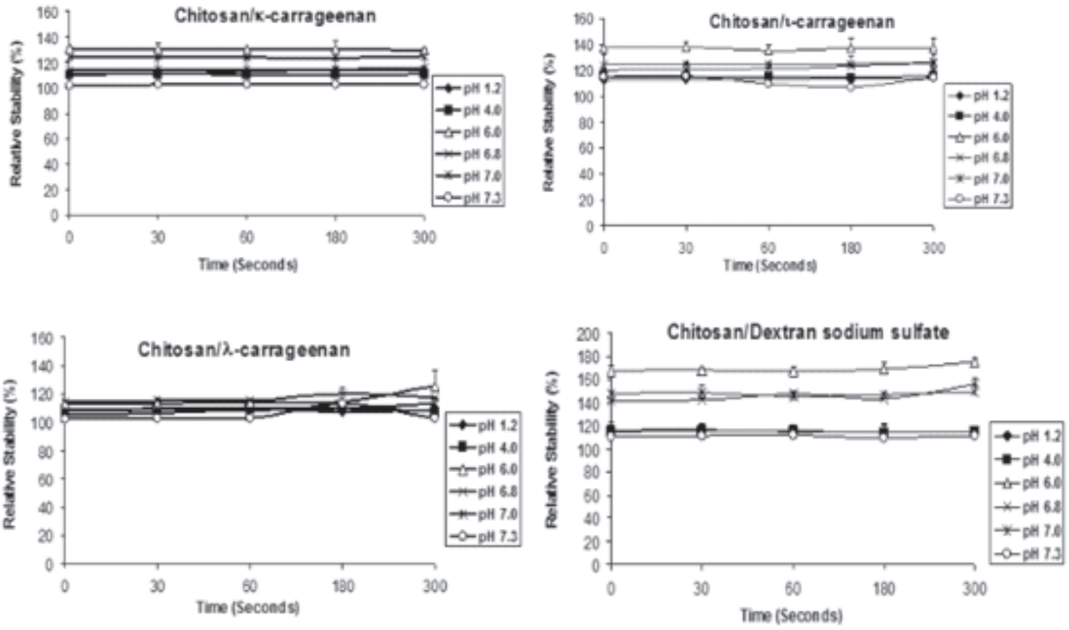

Figure 5. Relative stability of encapsulated BSA in chitosan/carrageenan complex (+/- ratio = 3) using different physiological solutions. pH 1.2: 35 mMNaCl; pH 4.0: 50 mM acetic acid; pH 6.0: 200 mMNaCl in MES buffer; pH 6.8: 50 mM potassium phosphate buffer; pH 7.0: H20; pH 7.3: PBS (0.01 g/mL). Turbidity of the encapsulated BSA with the different physiological solutions was measured at 500 nm against the control (0 mm NaCl solution). BSA indicates bovine serum albumin; PBS, phosphate buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid.

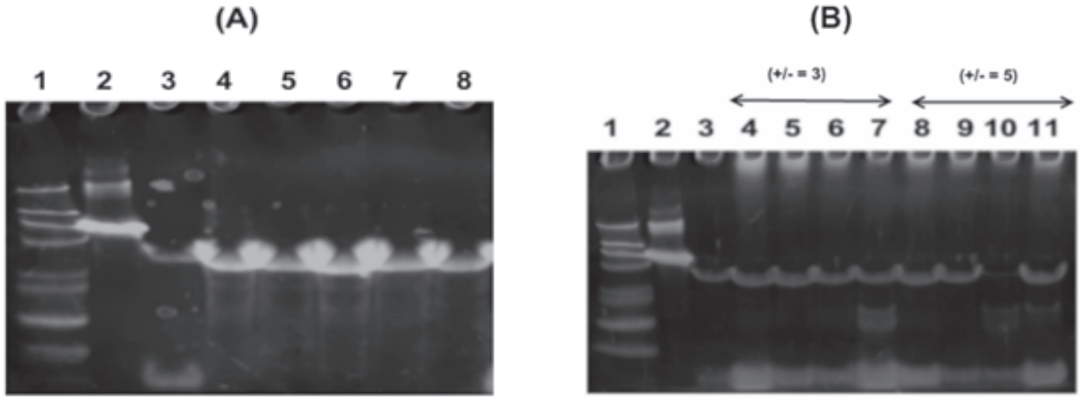

Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis for stability of BSA released from the complex treated with pepsin (1 mg/mL) and incubated at 37 0C for one hour. A. BSA released in carrageenan, dextran sodium sulphate and chitosan. Lane 1: Protein marker; Lane 2: Free BSA; Lane 3: Treated BSA; Lane 4: -carrageenan; Lane 5: -carrageenan; Lane 6: -carrageenan; Lane 7: Dextran sodium sulphate; Lane 8: Chitosan; B. BSA released in chitosan/carrageenan complex. Lane 1: Protein marker; Lane 2: Free BSA; Lane 3: Treated BSA; Lanes 4 & 8: chitosan/-carrageenan; Lanes 5 & 9; chitosan/-carrageenan; Lanes 6 & 10; chitosan/-carrageenan; Lanes 7 & 11; chitosan/dextran sodium sulphate; BSA indicates bovine serum albumin.

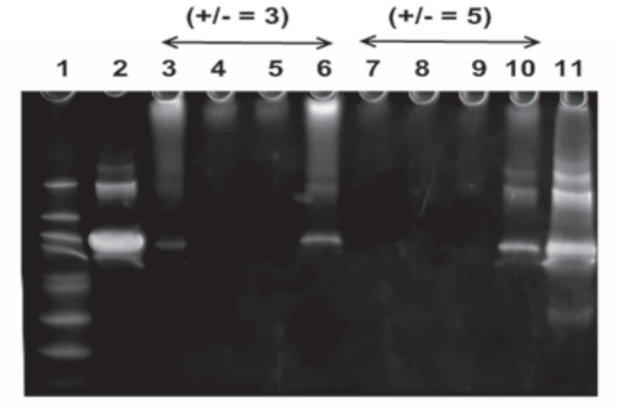

Figure 11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis for stability of BSA released from chitosan/carrageenan complex in pH 1.2 incubated at 37 0C for one hour. Lane 1: Protein marker; Lane 2: Free BSA; Lanes 3 & 7: chitosan/-carrageenan; Lanes 4 & 8; chitosan/-carrageenan; Lanes 5 & 9; chitosan/-carrageenan; Lanes 6 & 10; chitosan/dextran sodium sulphate; Lane 11: Treated BSA; BSA indicates bovine serum albumin.

BSA were still in the well as observed in the intensity of the bands showing resistance to release and degradation. Most of the lanes showed more than one band showing the slower rate of BSA degradation in the polyion complex than using one polymer in the encapsulation process. The integrity of BSA in pH 1.2 was also investigated by gel electrophoresis. In Figure 11, BSA fractions released from the complexes were not degraded as compared to the treated free BSA (Figure 11, Lane 11). The BSA was protected from being degraded and hydrolyzed. The use of chitosan/carrageenan complex prevented degradation of BSA in acidic solution.

DISCUSSION

The formation of the micro particles of chitosan/carrageenan complex was due to its opposite charges between the positively charged amine groups (NH_3) in chitosan and the negatively charged sulphate groups (SO4) in carrageenan. Chitosan/dextran sodium sulphate was used as the control in this study since previous study showed the high efficiency of this complex to entrap BSA and Rhodamine at an efficiency of 98% [12]. The low efficiency of chitosan to encapsulate BSA might be attributed to the condition used in this study wherein no cross linker was used as compared to the previous study which make used of tripolyphosphate (TPP) as cross linker [13]. Here, we were able to show that polyion complex (chitosan/carrageenan) was more efficient than using just one polymer without the use of a crosslinking agent. The electrostatic interaction of the sulphate group of carrageenan and the amine group of chitosan make a better entrapment of BSA than chitosan alone. Charge ratios (+/-) of 3 & 5 were the most efficient as compared to 1 & 7 ratios. The difference of encapsulation efficiency among the three types of carrageenan could be due to its chemical structure and molecular weight. - carrageenan had the highest molecular weight of 750 kDa and being the most anionic due to its three sulphate groups of the alternating 1,3-linked β-D-galactopyranosyl and 1,4-linked - D-galactopyranosyl sugar units while -carrageenan has two sulphate groups attached to its alternating sugar [14]. The isoelectric point of BSA is 4.8 [12] and the pH used in this study for complex formation was pH 6.0 which is above the isoelectric point of BSA. At this condition, the BSA would be predominantly negatively charged thus would ionically interact with chitosan competing with the more anionic carrageenan. The swelling mechanism of chitosan/carrageenan complex showed that in an acidic solution, no swelling was observed [15]. This is due to the presence of electrostatic bonds between chitosan and carrageenan. The sulfate groups of carrageenan will remain negatively charged and interact with the positively charged amino groups of chitosan hence, the cumulative release rate of BSA was not too excessive. In alkaline solution, the amino groups of chitosan will be neutralized, and the sulfate groups of carrageenan will remain negatively charged therefore, the electrostatic linkage between the two polymers disappears [15]. The electrostatic repulsion between sulfonate groups will contribute to the swelling mechanism [15]; thus, higher release rate of BSA was observed. In alkaline solution, BSA is negatively charged; thus, there is no electrostatic interaction with chitosan. BSA, which has a pI of 4.7 [16], is anionic at physiological pH of 7.3. Among the prepared complexes, chitosan/-carrageenan complex showed the lowest release rate of BSA. This behavior might be due to its chemical structure. Since - carrageenan contains only one sulfate group attached to its alternating sugar units, less electrostatic repulsion is expected with fewer ionic binding sites. It also undergoes a helix-coil transition, creating additional cross-links and forming a more rigid system [17]. - Carrageenan had the highest release rate of BSA among the three types. This type contains three sulfate groups of the alternating sugar units, creating more electrostatic repulsion and ionic binding sites. This type does not gel and does not undergo a helixcoil transition, which precludes additional cross-links [17].

CONCLUSION

This study has demonstrated that chitosan/carrageenan complex at charge ratios of 3 & 5 can allow entrapment of BSA and control its release. Electrostatic interaction is the main mechanism involved in the incorporation of protein and its release from the complex. It has found importance in the application of chitosan/carrageenan complex for protein delivery. The ease of preparation and the ability of protecting protein integrity, make chitosan/carrageenan complex a promising drug delivery system for oral administration of peptides and proteins.

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REFERENCES

1. Semalty A, Semalty M, Singh R, Saraf SK, Saraf S. Properties and formulation of oral drug delivery systems of protein and peptides. Indian J Pharm Sci 2007;69:741-747.

2. Paul W, Sharma CP. Chitosan, a drug carrier for the 21st century: a review. STP Pharma Sciences 2000;10:5-22.

3. Meshali MM, Gabr KE. Effect of Interpolymer Complex Formation of Chitosan with Pectin or Acacia on Release Behaviour of Chlorpromazine HCL Int J Pharm 1993;89:177-181.

4. Lee JW, Park JH, Robinson JR. Bioadhesive-based dosage forms: the next generation. J Pharm Sci 2000;89:850-866.

5. Sakayama T, Chu C, Fujii T, Yano T, Preparation of a polyelectrolyte complex gel from chitosan and k-carrageenan and its pH-sensitive swelling, J. Appl. Polym. Sci. 1993;50:2021 2025.

6. Makino K, Idenuma R, Ohshima H. A model for erosion kinetics of a hydrogel matrix. Colloids and Surfaces B: Biointerfaces 1996;8:93-100.

7. Makino K, Idenuma R, Ohshima H. Design of a rate- and time-programming drug release device using a hydrogel: pulsatile drug release from kappa-carrageenan hydrogel device by surface erosion of the hydrogel. Colloids and Surfaces B: Biointerfaces 2001;20: 355-359.

8. Garcia AM, Ghaly ES, Preliminary spherical agglomerates of water soluble drug using natural polymer and cross-linking technique. J Control Release 1996;40:179-186.

9. Sipahigil O, B. Dortunc B. Preparation and in vitro evaluation of verapamil HCl and ibuprofen containing carrageenan beads. Int J Pharm 2001; 228: 119-128.

10. Tomida H, Nakamura C, Kiryu S. A novel method for the preparation of controlled-release theophylline capsules coated with a polyelectrolyte complex of k-carrageenan and chitosan. Chem Pharm Bull 1994;42:979-981.

11. Balnois E, Wilkinson KJ.Sample preparation for the observation of environmental biopolymers by atomic force microscopy. Colloids and Surface A: Physicochemical and Engineering Aspects 2002: 207: 229-242.

12. Chen Y, Mohanraj VJ, Wang F, Benson HA.Designing chitosan-dextran sulfate nanoparticles using charge ratios. AAPS PharmSciTech 2007: 8(4): E1-E9.

13. Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier-Systematic examination of fabrication conditions for efficient loading and release. Colloids and Surfaces B: Biointerfaces 2007:59(1):24-34.

14. Guisely KB, Stanley NF, Whitehouse PA.Carrageenan. In: R.L. Davidson, (Ed). Handbook of water-soluble gums and resins, McGraw-Hill Book Co., New York, 1980.p.5-1.

15. Sakiyama T, Chu CH, Fujii T, Yano T. Preparation of a polyelectrolyte complex gel from chitosan and k-carrageenan and its pH-sensitive swelling. J Appl Polym Sci. 1993: 50:20212025.

16. Ge S, Kojio K, Takahara A, Kajiyama T . Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: influence of the phase separation on protein adsorption patterns. *J Biomater Sci Polym Ed.* 1998:9(2):13150.

17. Shumilina EV, Shchipunov YA, Chitosan-carrageenan Gels. Colloid *J.* 2002:64:372-378.