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Preparation of chitosan nanoparticles for encapsulation and release of protein

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Abstract−Chitosan nanoparticles were prepared by ionic cross-linking with tripolyphosphate (TPP). The major effect on encapsulation and release of protein in chitosan-TPP nanoparticles was investigated in order to control the loading and release efficiency. A set of the same molecular weight (MW) proteins with different pI and a set of the same pI proteins with different MW were studied. The influence of protein concentration, pH of solution, and the activity of released protein were examined. It was found that the encapsulation efficiency (EE) of a set of the different MW protein decreased with increasing of MW of protein and protein concentration. The protein with having pI higher than pH of solution was attracted to the positively charged chitosan, resulting in increasing of EE. The release of protein from the nanoparticles showed that the protein release decreased with increasing of chitosan concentration, high MW protein, low pH, and less swelling of the particle. The released protein in chitosan-TPP matrix was still active in the buffer solution.

Key words: Chitosan, Nanoparticles, Tripolyphosphate, Controlled Release, Protein

INTRODUCTION

Chitosan is a biodegradable and bioadhesive polysaccharide which is a copolymer of glucosamine and N-acetyl-D-glucosamine linked by β(1,4) glycosidic bonds. It has been widely used in pharmaceutical and medical areas to deliver therapeutic peptide, protein, oligonucleotide, and genes because it has biocompatibility and low toxicity. Chitosan nanoparticles were prepared by various methods such as chemical cross-linking [1,2], ionic cross-linking [3], emulsion droplet coalescence [4] reverse micellar [5], and self assembly chemical modification [6]. The disadvantages of some methods are undesirable effects of cross-linking agent, more solvent and high energy consumption for chemical cross-linking, and reverse micellar, respectively. To overcome the disadvantages, the ionic cross-linking with tripolyphosphate (TPP), which is nontoxic, and multivalent anions has been investigated by many researchers [7-11]. The chitosan matrix is formed by ionic interaction between positively charged amino groups of chitosan and negatively charged TPP. The charge density of TPP, which is dependent on pH of solution, could control the interaction. Moreover, the nanoparticles can be prepared under mild conditions, and have homogeneous cross-linked structure, and adjustable size [8].

In the present work, the major effect on encapsulation and release of protein in chitosan-TPP nanoparticles was investigated in order to control the loading and release efficiency. A set of the same molecular weight (MW) protein with different pI and a set of the same pI protein with different MW were examined on the encapsulation efficiency and release rate. The influence of protein concentration and pH of solution was also investigated. Finally, the activity of released protein was studied.

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MATERIALS AND METHODS

Chitosan from a shrimp shell was obtained from Sea Fresh Co. Ltd. (Surathani, Thailand). Acetic acid was AR grade and obtained from Merck. Bovine serum albumin (BSA) (pI 10.2; MW 14.5 kDa), Cytochrome C (pI 10.2; MW 14.5 kDa), Ribonuclease A (pI 10.2; MW 14.5 kDa), Fibrinogen (pI 10.2; MW 14.5 kDa), α-Lactalbumin (pI 10.2; MW 14.5 kDa) and Transferrin (pI 10.2; MW 14.5 kDa) were obtained from Sigma. Tripolyphosphate was analytical grade from Merck. All other chemicals were analytical grade purchased from Sigma.

1. CS Nanoparticles Preparation

Chitosan solution concentration of 0.75 mg/ml was prepared by dissolving 11.25 mg of chitosan with deacetylation degree of 90 in 1.125 mg/ml of acetic acid. Then, 6 ml of tripolyphosphate (TPP) solution concentration of 1 mg/ml was added to 15 ml of chitosan solution. An opalescent suspension was formed spontaneously by stirring at room temperature for 20 min. Finally, the chitosan nanoparticles were separated by centrifugation at speed of 20,000 g and temperature of 4 °C for 30 min.

2. Encapsulation

Tested protein was dissolved in chitosan solution as described above. 6 ml of 1 mg/ml Tripolyphosphate was slowly added into the solution under magnetic stirring for 20 min. Then, the chitosan nanoparticles were separated by centrifugation at speed of 20,000 g and temperature of 4 °C for 30 min. Finally, the protein concentration in supernatant was measured by UV spectrophotometry. The encapsulation efficiency (EE) was then calculated from Eq. (1).

$$EE=(A-B)/A\times 100 \quad (1)$$

Where A is total amount of protein (mg/*l*); B is free amount of protein (mg/*l*).

3. Protein Release from Nanoparticles *In Vitro*

The protein-loaded chitosan nanoparticles after separation from suspension were re-suspended into 6 ml of 0.2 mol/*l* phosphate buffer saline (PBS) of pH 7.4. Then, they were incubated at 37 °C under shaking at 100 rpm. The samples were taken after 1 to 7 days, consecutively. The interval samples were centrifuged at a speed of 20,000 g and temperature of 4 °C for 30 min. Finally, the protein concentration in supernatant was measured by UV spectrophotometry. The diffusion coefficient (D) of the released protein was calculated from Eq. (2) [12]

$$m_t/m_o = (6/r)\sqrt{Dt/\pi} \quad (2)$$

Where: m_t is the released protein during the time of t (mg/ml); m_0 is the encapsulated protein in chitosan nanoparticles (mg/ml); r is radius of chitosan nanoparticles (m); D is diffusion coefficient (m^2/s); and t is sampling time (s). The slope of the plot m_t/m_o vs $\sqrt{t}$ was first determined. Then, the radius of particle measured from experiment was put into the Eq. (2).

4. Protein Activity

After encapsulation, the activity of protein should be remaining. In this study, ribonuclease A was selected as a model protein for activity study presented as initial velocity which was described by Crook et al. [13]. The substrate, 0.25 Cytidine 2′,3′-cyclic monophosphate (CP), was mixed with 2.5 ml Tris buffer. Then, 0.01 ml of 8 mg/ml ribonuclease A solution was mixed with the substrate. The hydrolysis rate was measured by UV-spectrophotometer at 288 nm. The optical density was recorded every 1 min for 2 h which the reaction was completed. Finally, the hydrolysis rate was determined from the plot of log (A−x) against time. The initial velocity of hydrolysis (V, $DE_{288\,nm}$/min) was calculated from Eq. (3):

$$V = -2.303A\, d \log (A-x)/dt \quad (3)$$

Where, A is the change in extinction at complete reaction time.
x is the change in extinction at time t.

RESULTS AND DISCUSSION

1. Encapsulation

1-1. Effect of MW

The effect of MW on encapsulation efficiency was examined with a set of protein which has similar pI but different MW (listed in Table 1), as shown in Table 2. The set of proteins was selected because the influence of electrostatic interaction of the proteins on EE should be the same. It was found that the encapsulation efficiency increased with decreasing of MW. At the pH 5 of solution, which is close to pI of protein, the net charge on the protein surface should be close to zero. Hence, the encapsulation process should depend on the size and diffusion rate of protein. The lower EE might be attributed to lower moving rate of the larger protein molecule. Jensen et al. [14] examined the loading of four different molecular weight proteins (vasopressin, MW 1,084 Da; aprotinin, MW 6,512 Da; lysozyme, MW 14,400 Da; and bovine serum albumin, MW 67,000 Da) in the chondroitin 4 sulfate hydrogel. They reported that the loading efficiency increased with decreasing molecule size of solute. Moreover, the effect of protein concentration of this set of proteins on the EE is also presented in Table 2. It was found that increasing the protein concentration reduced the EE. The same trend was also reported by Xu and Du [3].

1-2. Effect of pI

The effect of pI on encapsulation efficiency is shown in Table 3 for a set of similar MW but different pI protein (listed in Table 1). The set of proteins was selected because the influence of molecular size of the proteins on EE should be the same. It was observed that encapsulation efficiency of the proteins with pI<pH was higher

Table 1. Two sets of tested protein

A set of proteins with the same pI , different MW			A set of proteins with the same MW , different pI		
Protein	pI	MW (kDa)	Protein	pI	MW (kDa)
α-Lactalbumin	5.1	17.4	α-Lactalbumin	5.1	17.4
BSA	4.6	55	Cytochrome c	10.6	12.3
Transferrin	5.8	76-81	Ribonuclease A	7.8	13.7
Fibrinogen	5.8	340			

Table 2. Effect of MW and protein concentration on EE

Protein	Concentration (mg/ml)	EE (%)
α-Lactalbumin	0.5	82.64
	1.0	78.23
	1.5	75.39
	2.0	73.81
BSA	0.5	63.66
	1.0	59.46
	1.5	55.71
	2.0	52.23
Transferrin	0.5	51.47
	1.0	48.21
	1.5	45.79
	2.0	42.05
Fibrinogen	0.5	38.55
	1.0	32.25
	1.5	28.14
	2.0	27.95

Table 3. Effect of pI and protein concentration on EE

Protein	Concentration (mg/ml)	EE (%)
α-Lactalbumin	0.5	82.64
	1.0	78.23
	1.5	75.39
	2.0	73.81
Cytochrome c	0.5	39.72
	1.0	36.68
	1.5	33.52
	2.0	30.10
Ribonuclease A	0.5	29.81
	1.0	25.14
	1.5	23.06
	2.0	21.65

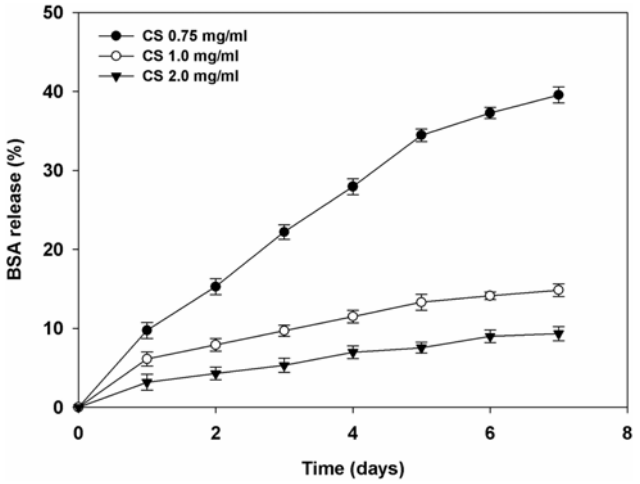

Fig. 1. Effect of chitosan concentration on protein release.

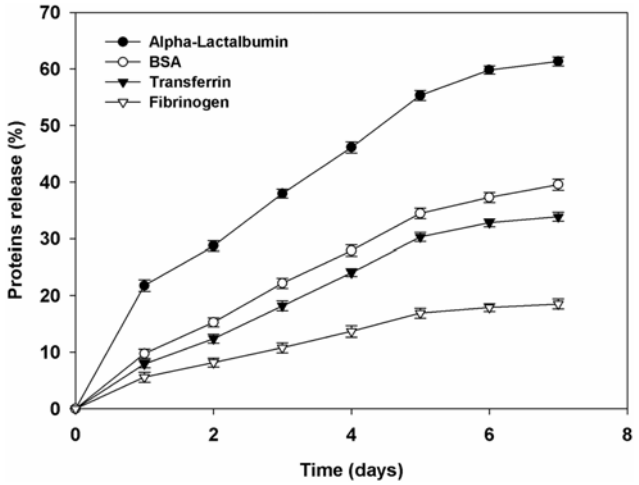

Fig. 2. Effect of protein MW on protein release.

than the proteins with pI>pH. The efficiency of the proteins with pI close to pH was minimum. When considering the charge on the protein surface, the net charge of α-Lactalbumin is negatively charged, whereas the net charge of Cytochrome c and Ribonuclease A is positively charged. Therefore, the net negatively charged protein should be attracted with positively charged chitosan, causing high encapsulation. On the other hand, the net positive charge of proteins should be repulsed with positive charge of chitosan but attracted with negative charge of TPP, resulting in less encapsulation, although the size of the proteins was the same. The effect of protein concentration of this set of proteins on the EE is also in Table 3. It was found that increasing of protein concentration also reduced the EE.

2. *In vitro* Release

2-1. Effect of Chitosan Concentration

Fig. 1 shows the effect of chitosan concentration on BSA release behavior. The initial mean particle size of the chitosan concentration of 0.75 mg/ml, 1 mg/ml, and 2 mg/ml was 58 nm, 157 nm, and 227 nm, respectively. The released protein increased sharply at the beginning period. It reached to plateau after 7 days. It was observed that 0.75 mg/ml chitosan concentration provided the highest yield of 40%. Moreover, the diffusion coefficient of low chitosan concentration was higher than that of high chitosan concentration, as shown in Table 4. As the concentration of chitosan increased, the viscosity of chitosan solution increased. The high viscosity of the solution should form a strong wall of particles, resulting in lower swelling ability which reduces the release of protein. The same effect of chitosan concentration on protein release was reported by Lim et al. [15], Ko et al. [16], and Gan and Wang [9].

2-2. Effect of MW

Fig. 2 shows the effect of MW on protein release behavior. The initial mean size diameter of chitosan nanoparticles encapsulated with α-Lactalbumin, BSA, Transferrin, and Fibrinogen was 31 nm, 58 nm, 42 nm, and 40 nm, respectively. It was found that the protein release of low MW protein (α-Lactalbumin) was higher than that of high MW protein (Fibrinogen). The proteins were rapidly released at the initial period. Then, the proteins were released at lower release rate and reached a plateau. The diffusion coefficient also shows the same trend, as shown in Table 5. This might be explained as that the small size of protein should be diffusing through the chitosan matrix faster than large sized proteins. The same trend was also reported by Mohanraj [17].

Table 4. Effect of chitosan concentration on diffusion coefficient

Chitosan concentration (mg/ml)	D (m^2/s)
0.75	2.32×10^{-23}
1.0	2.07×10^{-23}
2.0	1.78×10^{-23}

Table 5. Effect of protein MW on diffusion coefficient

Protein	D (m^2/s)
α-Lactalbumin	3.96×10^{-23}
BSA	2.32×10^{-23}
Transferrin	1.88×10^{-23}
Fibrinogen	5.22×10^{-24}

2-3. Effect of pI

The effect of pI protein on protein release and diffusion coefficient is shown in Fig. 3 and Table 6, respectively. The mean size

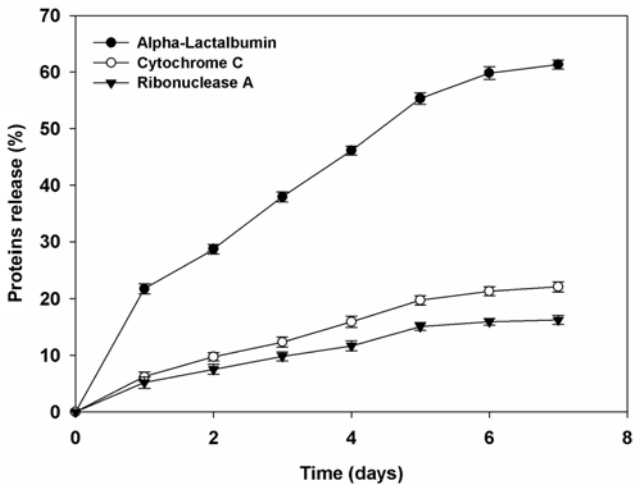

Fig. 3. Effect of pI of protein on protein release.

Table 6. Effect of protein pI on diffusion coefficient

Protein	D (m^2/s)
α-Lactalbumin	3.96×10^{-23}
Cytochrome c	6.35×10^{-24}
Ribonuclease A	3.12×10^{-24}

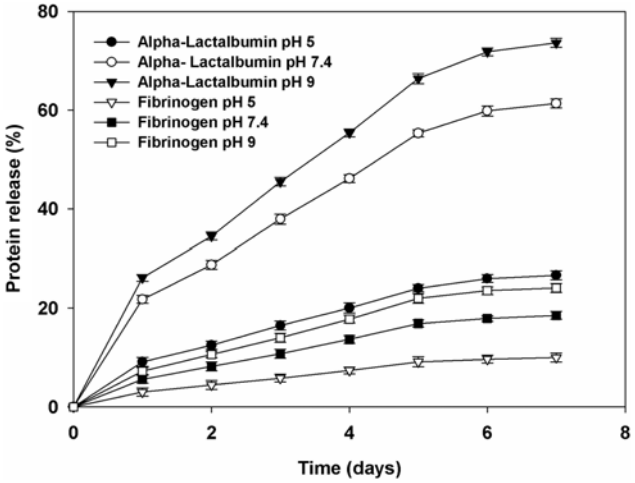

Fig. 4. Effect of pH of buffer solution on protein release.

diameter of chitosan nanoparticles encapsulated with α-Lactalbumin, Cytochrome C, and Ribonuclease A was 31 nm, 43 nm, and 44 nm, respectively. It was found that the protein release was high with the protein which has the same charge as the charge of chitosan. This might be explained as that the lower crosslink of α-Lactalbumin and chitosan resulted in more swelling ability and easier to release [16]. For cytochrome C and ribonuclease A, the higher crosslink of the proteins and TPP resulted in less swelling and difficulty of releasing. When considering the diffusion rate, at the same size of the proteins, the diffusion rate of α-Lactalbumin increased to ten times higher than that of cytochrome C and ribonuclease A. Moreover, the release of ribonuclease A was the lowest because the pI of protein was close to the pH of phosphate buffer solution, causing less solubility of the protein.

2-4. Effect of pH Solution

Fig. 4 shows the effect of pH on protein release of α-Lactalbumin and fibrinogen. For both proteins, the release rate was highest at pH 9, whereas it was lowest at pH 5. At pH 5, the pI of both proteins was close to this pH, resulting in low solubility of them. However, at high pH, more erosion and swelling of nanoparticle could be occurring, resulting in high release rate. Ko et al. [16] reported that open porous and low density structure was observed at high pH. This structure was more degradable than high density structure.

3. Enzyme Activity

Ribonuclease A was selected to investigate the enzyme activity after releasing from the nanoparticle. To our best knowledge, the activity of released protein has rarely been examined. Fig. 5 shows the hydrolysis rate of CP by normal ribonuclease A and released ribonuclease A. It was found that the hydrolysis rate of normal ribonuclease A was 0.16 compared with the rate of 0.12 from released ribonuclease A. The result indicated that the released ribonuclease

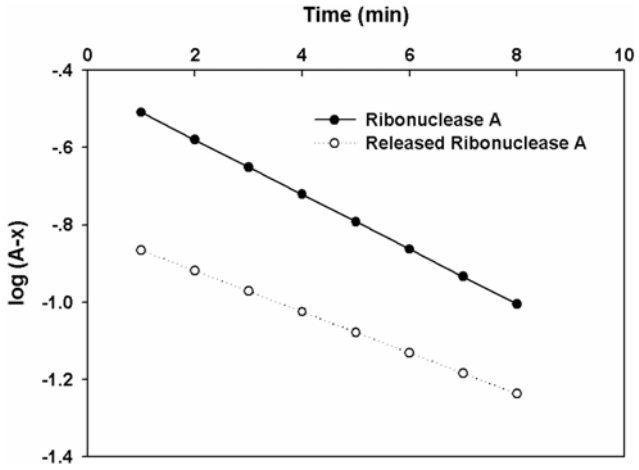

Fig. 5. Hydrolysis rate of cytidine 2′,3′-cyclic monophosphate (CP) from Ribonuclease A.

A was still active. Colona et al. [18] reported the same trend with the encapsulation of prolidase in TPP-chitosan nanoparticles. The result indicated the ability of chitosan to stabilize proteins during the preparation and release process.

CONCLUSION

The EE of a set of different MW proteins demonstrated that the efficiency decreased with increasing of MW of proteins and protein concentration. For the effect of pI of proteins on EE, the protein having pI higher than pH of solution was attracted to positively charged chitosan, resulting in higher EE. The release of protein from chitosan nanoparticle showed that the protein release decreased with increasing of chitosan concentration, high MW protein, low pH, and less swelling of the particle. Finally, the released protein in chitosan-TPP matrix was still active in the buffer solution.

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