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ALGINATE/κ-CARRAGEENAN MICROSPHERES AND THEIR APPLICATION FOR PROTEIN DRUGS CONTROLLED RELEASE

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Abstract. pH-Responsive microspheres were prepared and their feasibility as potential carriers for oral delivery of protein drugs was evaluated. The microspheres were prepared from the ionotropically-crosslinked mixture of sodium alginate and κ-carrageenan. The morphology and size of the microspheres were investigated. A model protein drug α-amylase was entrapped and *in vitro* drug release profiles were established. The preliminary investigation of the microspheres showed a consistent swelling pattern, high entrapment efficiency and sustained release profiles of the enzyme. All the results indicated that the alginate/κ-carrageenan microspheres could be potentially useful in drug delivery systems.

Keywords: pH-responsive microspheres, sodium alginate, κ-carrageenan, α-amylase.

1. Introduction

Proteins and peptides are excellent therapeutic agents with very specific and strong biological functions. However, delivery of these drugs is limited because of parenteral administration and necessity of frequent injections due to their short half-life in human blood [1]. Preparation of biodegradable microspheres containing proteins and peptides has received much attention in recent years [2, 3]. Despite the rapid development of encapsulation technologies in past few decades, peptides and proteins encapsulation is one of the most complex tasks in the field of the controlled drug delivery systems. Peptides are highly sensitive compounds and may be denatured during the encapsulation process. This may cause unpredictable side effects such as toxicity and immunogenicity [2]. Therefore, it is difficult to achieve the preservation of their functionality. Typically, peptides and proteins are entrapped into biodegradable microspheres by double emulsion [4], phase separation [5], and spray drying techniques [6].

pH-Sensitive microspheres have to meet the following requirements [7]:

- The size to a maximum of 1000 μm;
- Biocompatibility;
- Uniformity index no more than 2;
- Sphericity;
- High encapsulation efficiency;
- The swelling degree should significantly vary with pH changing;
- Drug release degree has to be no more than 20 % in acidic media, and maximum in neutral and alkaline media;
- Gradual drug release;
- The biodegradation of the microspheres will not lead to the formation of toxic compounds.

The essential class of biopolymers that meets these requirements is polysaccharides such as agarose, sodium alginate, dextran, chitosan, *etc*. Many shells for the microspheres are based on sodium alginate, due to its ability to gel in the presence of divalent cations. Sodium alginate is combined with other natural and synthetic polymers such as chitosan [8], guar gum [9], sodium hyaluronate [10], tamarind seed polysaccharide [11], poly(vinyl alcohol) [12], polyethylene glycol [13].

In this study, we have developed smart delivery system of α-amylase triggered by an external pH change. Microspheres with different behavior in acidic and neutral environments provide a prolonged release of protein and target delivery of the gastrolabile drugs to the intestine. In our system, enzyme is incorporated within pH-sensitive microspheres of sodium alginate and κ-carrageenan at different polymer ratio.

Sodium alginate is a water-soluble salt of alginic acid. It is a natural polysaccharide extracted from brown seaweed, and consists of 1→4 linked D-mannuronic and L-glucuronic acids [14].

Carrageenans are obtained by extraction with water or aqueous alkali from red seaweed. They are composed of repeating disaccharide units and classified into three types, κ, ι and λ, according to the number of sulfonic groups per repeat unit of the disaccharide. κ-Carrageenan consists of an alternating linear chain of (1→3)-*β*-D-galactose-4SO_3-(1→4)-3,6-anhydro-α-D-galactose [15].

pH-Responsive swelling and degradation, release behavior of the microspheres, the loading efficiency of α-amylase in the microspheres according to the polymer composition and its concentration, protein amount, and protective ability of microspheres for enzyme were investigated.

2. Experimental

2.1. Materials

Sodium alginate and κ-carrageenan were purchased from Fluka (Japan) and used without further purification. α-Amylase from porcine pancreas and Tween 80 were purchased from Sigma Aldrich (USA). Other reagents were all of analytical grade and were used as received.

2.2. Preparation of Alginate/ κ-Carrageenan Films

Alginate/κ-carrageenan mixtures with mass ratio 1:1, 1:3 and 3:1 were dissolved into deionized water to prepare 2 wt % solutions. The mixtures were stirred at room temperature for 24 h to obtain the homogeneous solution. After degassing, the solutions were cast on a plastic plate and dried in the air at 309 K for 40 h. The obtained films were kept in a 0.3 M $CaCl_2$ aqueous solution for 30 min. After crosslinking, the films were washed with the deionized water.

2.3. Swelling and Degradation Experiments

The kinetics of films swelling and degradation was studied by the gravimetric method. The degree of swelling/ degradation A was calculated from the following equation:

$$A = \frac{W_t - W_0}{W_0}$$

where W_t was the film weight at time t, W_0 was the weight of the dry film.

2.4. Molecular Weight between Crosslinks

Molecular weight between the crosslinks M_c was calculated from the equilibrium swelling data using Flory-Rehner equation [16]:

$$\frac{1}{M_c} = \frac{1}{M_n} - \frac{v/v_1\left[\ln(1-v_{2s}) + v_{2s} + c v_{2s}^2\right]}{v_{2r}\left[(v_{2s}/v_{2r})^{1/3} - 1/2(v_{2s}/v_{2r})\right]}$$

where M_n is the number average molecular weight of the alginate polymer before crosslinking (450,000); v is the specific volume of alginate (1.142 cm^3/g); v_1 is the molar volume of the water (18.1 cm^3/mol); v_{2r} is the volume fraction of the crosslinked polymer at the relaxed state; v_{2s} is the volume fraction of the crosslinked polymer in hydrogel at equilibrium swelling, and χ was the Flory polymer-solvent interaction parameter for the polymer.

The crosslinking density v_e of the films was calculated as follows [17]:

$$v_e = \frac{r_p}{M_c}$$

where ρ_p is a sodium alginate density.

2.5. Preparation of Alginate/ κ-Carrageenan Microspheres

The alginate/κ-carrageenan microspheres were prepared by an emulsification method using calcium chloride as a crosslinking agent. α-Amylase was added to the mixture solution of alginate and κ-carrageenan. Then the homogeneous solution was poured into 150 ml of vegetable oil, which contained 2.2 g of nonionic surfactant Tween-80. The mixture was stirred for 30 min at 1200 rpm to obtain a stable emulsion. Then 30 ml of 0.3 M aqueous solution of calcium chloride was added for crosslinking. The emulsion was stirred for another 30 min. After this, the stirring plate was switched off and the emulsion was left for 2 h to harden the microspheres. Following this, the microspheres were filtered and washed with ethanol three times in the ultrafiltration cell Amicon 8200 (Milipore, USA) under the applied pressure of 0.3 MPa.

2.6. Determination of Microspheres Size

The optical microscope (Kruiser MBL 2000, Germany) was used to characterize the shape and size of alginate and alginate/κ-carrageenan microspheres. A small amount of dry microspheres was suspended in the deionized water. The suspension was ultrasonicated for 5 s. After that the diameter of at least 300 microspheres were measured using a calibrated ocular micrometer. Uniformity index (UI) was determined as [18]:

$$UI = D_w / D_n$$

where D_w and D_n are weight average diameter and number average diameter, respectively.

2.7. Encapsulation Efficiency Measurements

The encapsulation efficiency was measured by dissolving the sample of the microspheres in PBS buffer at pH 6.8. Drug concentration measurements were done using UV spectroscope UV-1200 (LAB InTech, China) at a wavelength 215 nm. Encapsulation efficiency (EE) was calculated from the following equation [19]:

$$EE = \frac{W_{enc}}{W_{\text{int}}} \cdot 100\%$$

where W_{enc} is an enzyme amount in microspheres, and W_{int} is an initial enzyme amount in the reaction mixture.

2.8. Release Studies

To study the protein release from the microspheres, 100 mg of the sample was placed in a plastic centrifuge tube and 10 ml of the medium solution was added. 0.1 M HCl was used to simulate the pH value of the stomach (pH = 1.8) while the PBS solution (pH = 6.8) was used to simulate the intestine pH value. Each sample was then left in the water bath at 310 K under constant stirring [20]. Drug concentration was measured as in the previous experiment. All the samples were measured in triplicate.

2.9. Determination of a-Amylase Catalytic Activity

α-Amylase activity was determined by the degree of starch conversion [21]. 5 ml of 0.1 % starch solution was added to 1 ml of enzyme solution of desired concentration in PBS (pH = 6.8). The sample was selected at regular time intervals; the solution was then analyzed to determine a starch concentration. The degree of starch conversion α was calculated as follows:

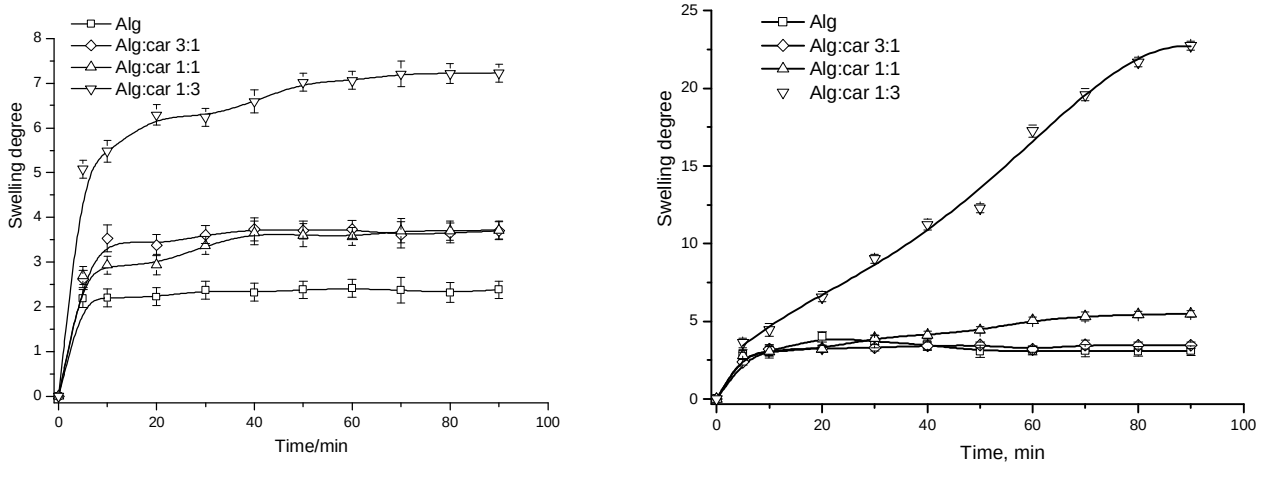
$$a = \frac{C_0 - C_t}{C_0} \cdot 100$$

where C_0 and C_t are the initial ($t = 0$) and measured at t time starch concentrations.

3. Results and Discussion

3.1. Swelling and Degradation Kinetics

Swelling kinetics of alginate-carrageenan films is similar with swelling of alginate matrix (Fig. 1). The carrageenan addition at 50 % (alg:car 1:1) insignificantly increases the films swelling degree in acidic and near neutral media. But at alginate/κ-carrageenan ratio of 1:3 the swelling degree is rapidly increasing, which can be explained by high molecular weight between crosslinks (11608 g/mol) and is in accordance with the low crosslinking density (0.08 mol/dm^3) (Table 1).



a) b)

Fig. 1. Swelling kinetics of alginate and alginate-κ-carrageenan films at pH 1.8 (a) and pH 6.8 (b)

Table 1

Film properties

Film	M_c, g/mol	Crosslinking density, mol/dm^3	pH sensitivity (β)
Alginate	698	1.26	1.31
Alg:car 3:1	1425	0.61	0.94
Alg:car 1:1	6787	0.13	1.47
Alg:car 1:3	11608	0.08	3.15

The high swelling degree of alginate-κ-carrageenan samples can be accounted for the ionization of sulfo-groups in the polymer matrix. Due to acid group dissociation and country-ions influx, the ion concentration in the inner polymer network is higher than the one in the environment. This causes an osmotic pressure difference, resulting in the solvent penetration into the film.

pH-Sensitivity β of prepared films can be evaluated by proportion of swelling ratio at pH 6.8/1.8. At an alginate-carrageenan ratio of 3:1 the loss of pH sensitivity is evidenced ($\beta = 0.94$). However, further increasing κ-carrageenan content (1:1, 1:3) led to pH sensitivity growth compared to the alginate films ($\beta = 1.47$, 3.15 and 1.31, respectively). The lower swelling degree in acidic medium is explained by the dominance of polymer-polymer interactions over polymer-solvent ones due to formation of hydrogen bonds between the non-dissociated carboxyl groups in alginate [18].

As can be seen from Table 1, molecular weight between crosslinks grows and crosslinking density decreases with increasing κ-carrageen content in polymer mixture, promoting increase in films swelling degree. This trend can be explained by the formation of interpenetrating nets between the alginate and carrageenan molecular chains, which minimizes the probability of two carboxyl groups location nearby for interactions with Ca^{2+}.

Fig. 2 shows dependence of weight loss for polymeric films on residence time in phosphate buffer. In the initial stages of degradation, the alginate film and alginate-carrageenan film with high content of alginate (alg:car 3:1) leaching of low-polymeric fraction is observable. Further weight loss is caused by Ca-alginate bonds destruction. For alginate-carrageenan ratios of 1:1 and 1:3, leaching of low-polymeric fraction can also be seen. But after that the films weight remains constant, possibly due to carrageen chains structuring under the influence of potassium cautions in the PBS buffer.

3.2. Microspheres Characteristics

Analysis of obtained microspheres with the optical microscopy indicated that the wet microspheres had the smooth surface and the spherical shape, as shown in Fig. 3, whereas the dried ones were irregular in shape.

Table 2 demonstrates the minimal, maximum and average diameters of microspheres in dependence of alginate-κ-carrageenan ratios. So, increase in the mixture carrageenan content resulted in the microspheres size grow. With the sulfated polysaccharide administration average diameter of spheres increases by 4–7 times depending on the amount of carrageenan. This phenomenon may be caused by the increasing of the solutions viscosity, that prevents dispersion of the polymer mixture during the process of microcapsulation. The obtained microspheres had a wide size distribution, which is evident from the values of the uniformity index (UI) [18].

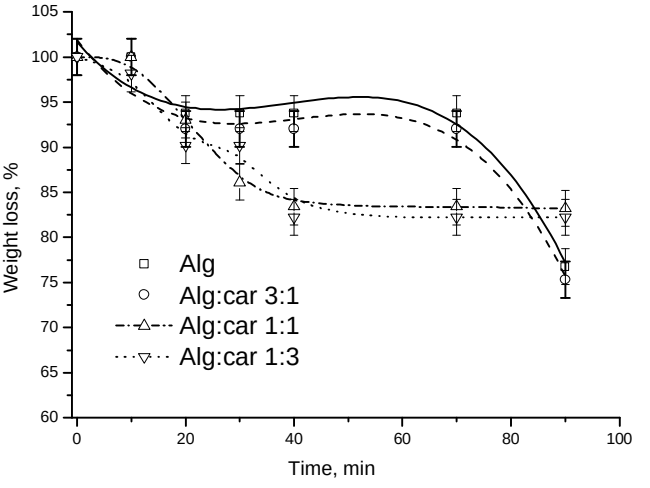


Fig. 2. Degradation kinetics of alginate and alginate/κ-carrageenan films at pH 6.8

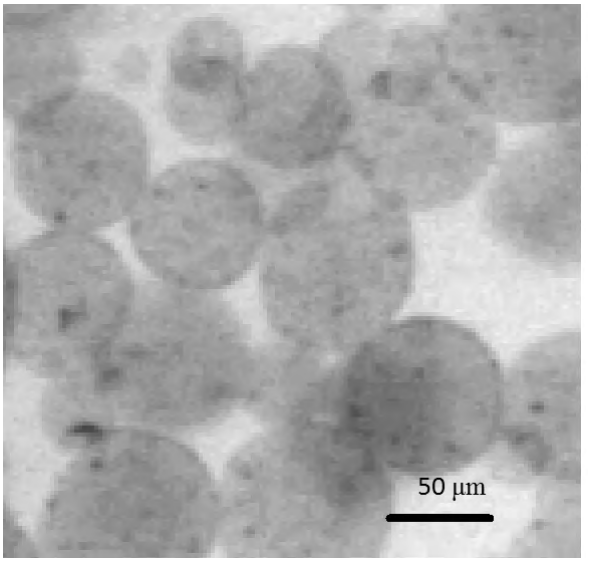


Fig. 3. Photograph of the wet alginate/κ-carrageenan (1:1) microspheres

3.3. Effect of Alginate-κ-Carrageenan Ratio on Release Kinetics of α-Amylase

Microspheres from the alginate-carrageenan mixture at various ratios 1:3, 1:1, and 3:1 were obtained. The initial amount of enzyme was 10 wt %. The effect of alginate-carrageen ratio on the enzyme release kinetics is shown in Fig. 4.

Table 2

Microspheres characteristics

Polymer mixture	Minimal diameter, μm	Mean diameter, μm	Maximal diameter, μm	UI
Alginate	5.2	11.5±5.84	35.4	2.18
Alg:car 3:1	6.7	42.4±22.39	150.0	2.21
Alg:car 1:1	14.2	51.7±32.04	168.3	2.08
Alg:car 1:3	22.5	75.3±41.96	216.7	2.01

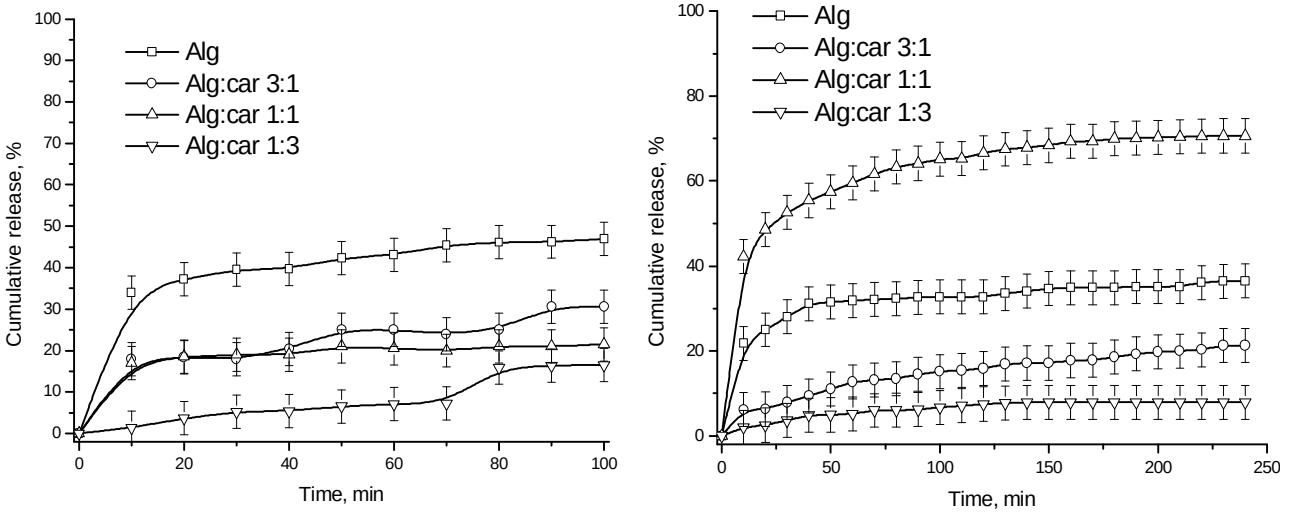


Fig. 4. α-Amylase release kinetics from alginate and alginate-κ-carrageenan microspheres at pH 1.8 (a) and pH 6.8 (b)

Table 3

Microspheres properties

Polymer mixture	EE, %	Cumulative release (pH 1.8+pH 6.8), %
Alginate	60.8	83.4
Alg:car 3:1	98.0	51.9
Alg:car 1:1	100.0	92.2
Alg:car 1:3	94.5	24.4

The release degree of α-amylase in the acid environment is directly correlated with the κ-carrageenan content in the polymer mixture (Fig. 4a). Alginate microspheres are characterized by the greatest degree of enzyme release at pH 1.8, that do not match the requirements for drug delivery systems to the intestine.

For other microspheres the loss of protein is approximately 20 %, which fully match the acceptable level of drug release. Enzyme release degree increases at pH 6.8. This is typical for alginate-based matrices, which erode in a phosphate buffer due to a calcium elution [22]. But in neutral environment there is no strong correlation between the carrageenan content and release degree. For microspheres with polysaccharides ratio 3:1, pH-insensitivity was confirmed because the enzyme release rate was the same in the acidic and neutral media (20 %). Maximum and minimum protein releases were observed for 1:1 and 1:3 ratios, respectively. This fact can be explained by two parallel processes flowing: the structuring of κ-carrageenan in the solution with K^+ and alginate-Ca^{2+}-coordination bonds destruction in the phosphate buffer, which leads to changing the protein release mechanism from microspheres.

As can be seen in Fig. 4b, a prolonged release was observed for microspheres from alginate/κ-carrageenan in ratios 1:1 and 3:1. This fact allows choosing necessary polymer composite for needed drug amount, depending on its therapeutic window.

Increasing κ-carrageenan amount in microspheres provides nearly 100 % α-amylase encapsulation efficiency

(Table 3) and reduces drug release in the acidic medium because of the strong electrostatic interactions between positively charged protein and negatively charged sulfo groups of κ-carrageenan.

Considering the enzyme encapsulation efficiency of 100 % and the total release degree of 92 %, the 1:1 ratio of alginate/κ-carrageenan was chosen to maximize the therapeutic effect.

3.4. Effect of Enzyme Amount in Microspheres on Release Kinetics of α-Amylase

In microspheres with alginate/κ-carrageenan ratio 1:1, 10, 25 and 50 wt % amount of α-amylase was added.

It was determined that increase in microspheres protein content reduced polymer mixture capacity toward enzyme due to lack of available active sites for the protein binding. Thus, as can be seen in Fig. 5, the maximum efficiency of encapsulation was achieved by the addition of 10 wt % α-amylase. The total amount of the enzyme does not depend on the initial protein content and is equal to 10 wt % of the polymer.

Fig. 5. The encapsulation efficiency of α-amylase at different initial protein content in the polymer matrix

Fig. 6 shows release kinetics of α-amylase from alginate/κ-carrageenan microspheres in acidic medium. It can be seen, that initial enzyme contents practically have no effect on the degree of its release from microspheres. Changing of kinetics profile for microspheres with high initial protein content may indicate a pore formation in polymer structure.

Fig. 7 shows release kinetics of α-amylase from alginate/κ-carrageenan microspheres in neutral medium. Reduction in the protein release with increasing its initial content was observed. It can be assumed that increase in protein amount changes the mechanism of its release from microspheres. This effect may be due to different protein-polymer interactions at different enzyme concentrations. So, at low concentrations, electrostatic interactions may dominate, and for high ones strength of donor-acceptor and hydrophobic-hydrophobic interactions may increase. Also, as mentioned earlier, this effect may be caused by the pore formation, which allows the protein to diffuse out the polymer matrix freely and release more sustainable.

3.5. Effect of Polymer Mixture Concentration on Release Kinetics of α-Amylase

Influence of polymer mixture concentration on release kinetics of protein was investigated. For this purpose, the concentration of alginate-κ-carrageenan (1:1) mixture was varied from 2 to 1 %. Fig. 8 illustrates enzyme release kinetics from obtained microspheres at acidic (a) and neutral (b) media. When concentration of polymer mixture decreases, encapsulation efficiency remains close to 10 wt % of the polymer. However, this changing negatively affects the protein release in acidic medium (Fig. 8a) because of protein loss approximately 40 %. View of kinetic curves of α-amylase release in the neutral medium for polymer concentrations 1.5 and 2.0 % is almost the same (Fig. 8b). But two-stage kinetics observed for enzyme release from microspheres fabricated from 1.0 % polymer solution.

Firstly, a rapid non-linear release occurred. In the interval of 90–150 min this was followed by a well-defined linear section (y = 0.9114x – 36.257) of the kinetic curve which corresponds to the zero-order kinetics (R^2 = 0.9983). For microcapsules obtained from other solutions, the second stage of release was not observed. This effect can be explained by more rapid degradation of the polymer matrix fabricated from low-viscous solutions.

3.6. α-Amylase Catalytic Activity after Encapsulation

Activity of released α-amylase was investigated by the degree of starch conversion and compared with those of native enzyme. As native α-amylase is inactivated in the acidic medium, experiments with it were carried out only in the PBS buffer, while the microspheres with enzyme firstly were immersed in the solution with pH 1.8 for 2 h.

The degrees of starch conversion after 30 min were 62.9 and 98.0 % for released and native α-amylase, respectively (Fig. 9). Consequently, enzyme activity remained 64.2 % compared with the native α-amylase.

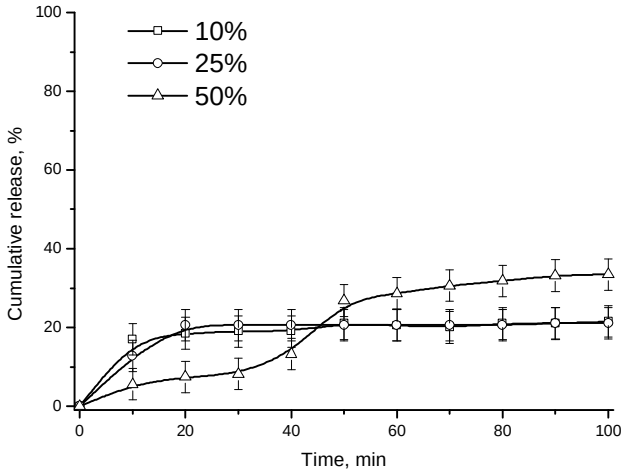


Fig. 6. α-Amylase release kinetics from alginate/κ-carrageenan (1:1) microspheres at different initial protein content at pH 1.8

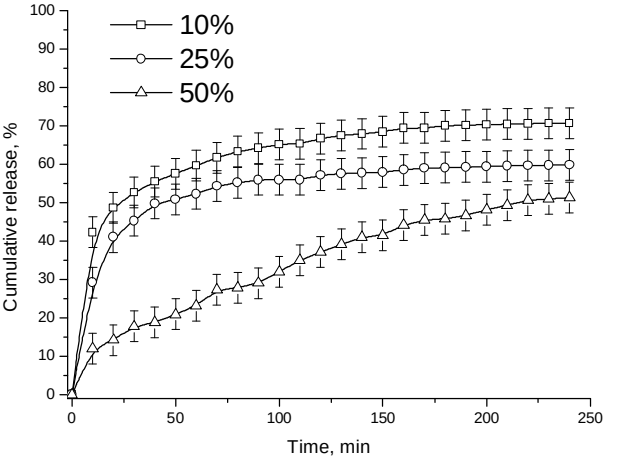
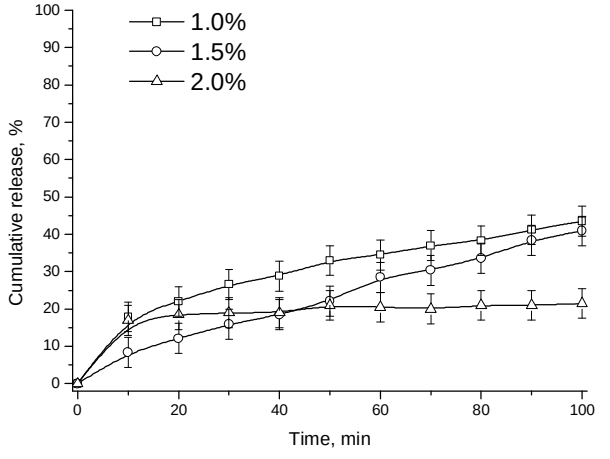
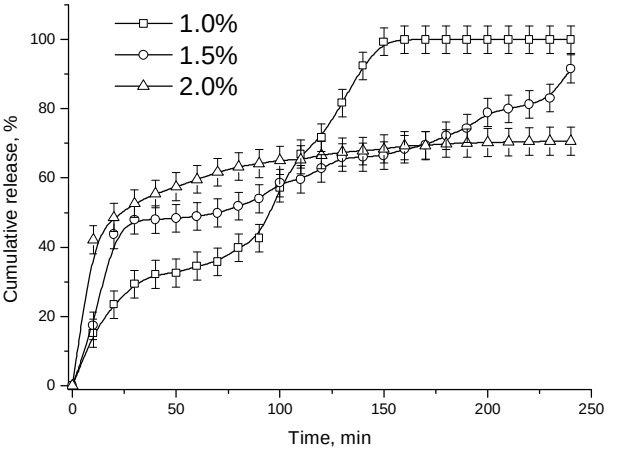


Fig. 7. α-Amylase release kinetics from alginate/κ-carrageenan (1:1) microspheres at different initial protein content at pH 6.8



a)



b)

Fig. 8. α-Amylase release kinetics from alginate-carrageenan (1:1) microspheres at different polymer mixture concentration at pH 1.8 (a) and pH 6.8 (b)

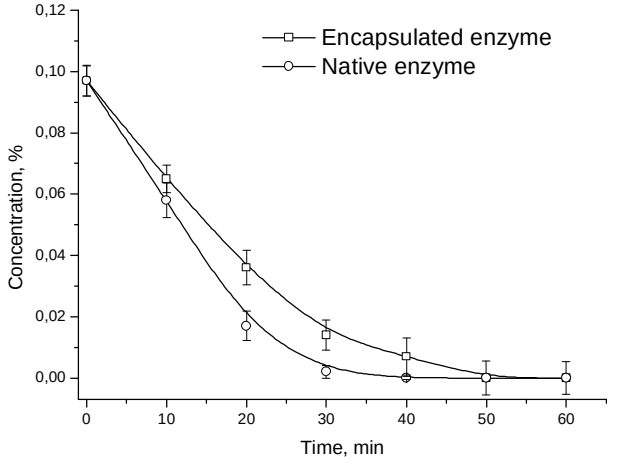


Fig. 9. Kinetics of starch conversion by α-amylase released from alginate/κ-carrageenan (1:1) microspheres at pH 6.8

Thus, the process of enzyme microencapsulation in alginate/κ-carrageenan microspheres achieved not only high levels of protein release at pH 6.8, but also protected activity of α-amylase after its exposure to aggressive stomach environment.

4. Conclusions

A new pH-responsive drug delivery system based on polymer mixture of sodium alginate and κ-carrageenan was developed. Release of α-amylase from such microspheres at acidic pH was significantly reduced compared to alginate/Ca^{2+} microspheres; in addition, these systems were able to effectively protect the drug from the acidic environment. Consequently, the burst effect of the drug was reduced and the drug efficiency was improved.

The proposed alginate/κ-carrageenan microspheres could be considered as a potent candidate for a protein delivery matrix to the intestine *via* the oral route.

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МІКРОСФЕРИ НА ОСНОВІ НАТРІЙ АЛЬГІНАТУ/κ-КАРАГІНАНУ ТА ЇХ ВИКОРИСТАННЯ ДЛЯ КОНТРОЛЬОВАНОГО ВИВІЛЬНЕННЯ ЛІКІВ БІЛКОВОЇ ПРИРОДИ

Анотація. *Одержано pH-чутливі мікросфери та показана можливість їхнього застосування як потенційних носіїв для пероральної доставки ліків білкової природи. Мікросфери були одержані методом йонотропного гелеутворення із суміші натрій альгінату та κ-карагінану. Досліджені морфологія та розмір мікросфер. Модельний лікарський засіб α-амілаза був закапсульований та in vitro досліджена кінетика його вивільнення. Попередні дослідження показали поступовий характер набрякання полімерної матриці, високу ефективність капсулювання ліків та пролонговане вивільнення ферменту. Одержані результати показують, що мікросфери на основі натрій альгінату та κ-карагінану можуть бути успішними системами доставки лікарських засобів.*

Ключові слова: *pH-чутливі мікросфери, натрій альгінат, κ-карагінан, α-амілаза.*