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Preparation of chitosan–alginate complex beads and their use for maltogenase immobilization

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Polyelectrolyte complex formation from chitosan of various molecular weight and degree of deacetylation and sodium alginate of various molecular weight and content of β-D-manuronic and α-L-guluronic acids as a function of their molar ratios and pH has been studied. Interpolymer complex formation between chitosan and alginate was studied by turbidity measurements. Chitosan–alginate complex beads were prepared from chitosan–alginate complexes by using a mixture of $CaCl_2$ and GA as a cross-linker. The structure of chitosan–alginate complexes and complex beads was characterized by FT-IR spectra. The stability of chitosan–alginate complex beads was investigated by storing them at room temperature for 1 to 7 days in a citrate buffer, pH 2.5 or 6.5, and distilled water. A possible application of complex beads for immobilization of enzymes was studied. A high efficiency of maltogenase immobilized onto chitosan–alginate complex beads was achieved.

Key words: chitosan, sodium alginate, polyelectrolyte complex, beads, maltogenase

INTRODUCTION

Polyelectrolyte complexes (PEC) may be formed when oppositely charged polyelectrolytes are mixed and interact via electrostatic interactions. Recently, PEC from natural polysaccharides such as chitosan (Chs) and sodium alginate (NaAlg) [1–7], carboxymethylcelullose [1–4, 8], carrageenans [1–3] dextran [1, 2] etc. attracted considerable attention due to their biocompatibility, biodegradability, low toxicity, rich resources and potential applications in various biotechnological processes, dressings, controlled-release drugs or protein delivery systems, immobilization of drugs, enzymes, cells and proteins [2]. Chs-based PEC are able to form fibers, membranes, films or microcapsules from solutions [9]. Chs is a unique cationic, linear, "ecologically-friendly" polysaccharide. Chs is β-(1 → 4)-2-amino-2-deoxy-D-glucose prepared by deacetylation of chitin acetamide groups. It is positively charged in acidic to neutral solutions and has limited solubility in the neutral or a higher pH region [1]. NaAlg is a water-soluble anionic polysaccharide derived from seaweed, consisting of unbranched binary copolymer of (1 → 4) linked residues of β-D-mannuronic (M) and α-L-guluronic (G) acid (Scheme).

The ratio of M and G blocks in NaAlg influenced the physical properties of biopolymers. These monomers are epimers resulting in a different orientation of the polymer chain; however, only the G units are oriented in a manner that renders the carboxylate moieties accessible for ionic cross-linking. Addition of calcium ions to an aqueous solution of NaAlg results in the formation of a three-dimensional calcium alginate hydrogel as the divalent calcium cations' cross-link adjacent biopolymer chains [9]. Microcapsules, beads or membranes prepared from Chs / Alg complexes without a cross-linker [10–15], Chs / Alg complexes with $CaCl_2$ or $BaCl_2$ [16–22] and Chs / Alg complexes with glutaraldehyde [23] were used for encapsulation of drugs (rifamicin, *L*-dopa, sodium diclofenac, paracetamol etc.) [10, 11, 16–18], immobilization of proteins [19], antibodies [23] and enzymes such as α-amylase [12], β-galactosidase [20, 21], tanase [22], invertase [13], catalase [14], dextransucrase [15]. However, only divalent calcium or barium cations were used for preparation of beads and microcapsules for the immobilization of enzymes [12–15, 20–22].

In the present work, the optimal conditions of the formation of Chs / Alg complexes from commercial Chs and NaAlg of various molecular weight at different pH values and molar ratios were determined. Preparation of beads of these complexes by using a mixture of $CaCl_2$ and glutaraldehyde as a cross-linker and their use as polymeric carriers for maltogenase immobilization are presented.

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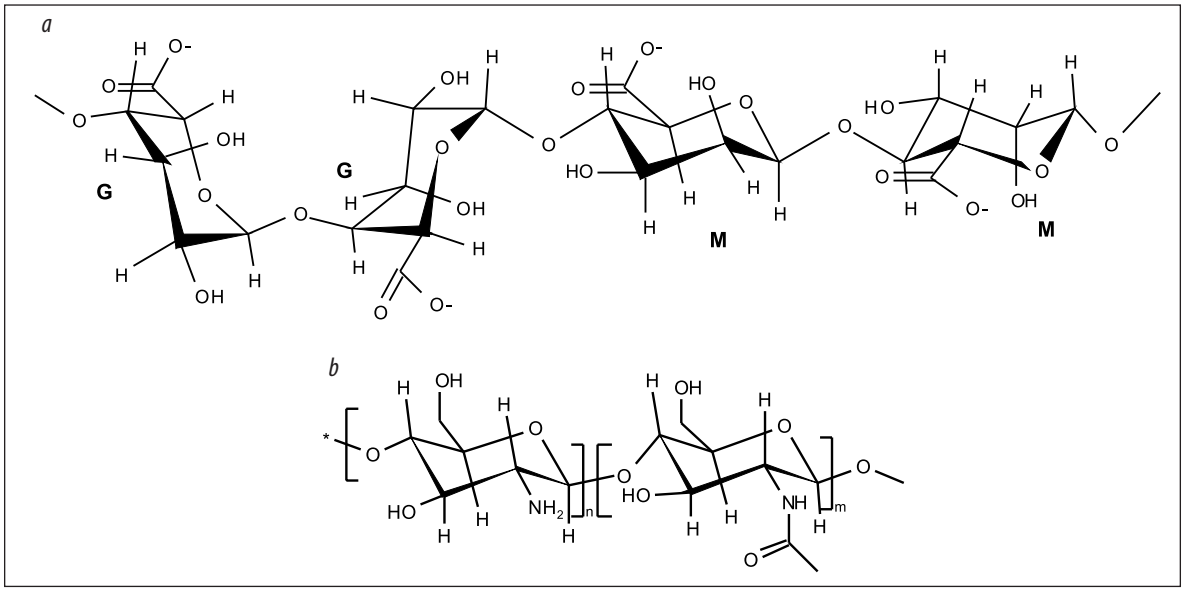

Scheme. Structure of alginate (*a*) and chitosan (*b*)

MATERIALS AND METHODS

Materials

Chs(I) (M_w 400 000, deacetylation degree (DD) 72% (Fluka)); Chs(II) (M_w 70 000, DD 77% (Fluka)); NaAlg(I) (M_n 440 000, viscosity of 2% solution in water at 25 °C, 3500 cps, M/G 1,6 (Sigma)); NaAlg(II) (M_n 230 000, viscosity of 2% solution in water at 25 °C, 250 cps, M/G 1,5 (Sigma)); NaAlg(III) (M_n 60 000, viscosity of 2% solution in water at 25 °C, 200 cps, M/G 1,0 (Reachim)); acetic acid and sodium hydroxide (POCh); glutaraldehyde (GA) (25% aqueous solution (Aldrich). All reagents were used as received.

Enzymes

Maltogenase (MG) (Novozymes, Denmark) was from *Bacillus stearothermophilus* (glucan 1.4-α-maltohydrolase, E.C.3.2.1.133) recombinant exo-acting maltogenic amylase, which removes maltose units (through 1.4-α-D-glucosidic linkages) from the non-reducing chain ends in maltooligosaccharides, such as starch and amylopectin.

α-Amylase was from *Bacillus subtilis* (Biosintezė, Lithuania).

Complexation of Chs and NaAlg and preparation of Chs / Alg beads

1% Chs(I) or Chs(II) solution in 2% acetic acid (initial pH 3.3 or corrected to pH 2.5 or 5.5) was mixed with 1% NaAlg(I–III) aqueous solution (NaAlg(I, II), initial pH 6.1 and NaAlg(III), initial pH 6.3 or corrected to pH 2.5 or 5.5) in various molar ratios. The corrected pH of Chs or NaAlg solution was adjusted by using 0.5 M HCI or NaOH aqueous solution. Complex beads were prepared by dropping 20 cm^3 of formed Chs / Alg complexes in the form of gel through a syringe into 40 cm^3 of 0.05 M $CaCl_2$ solution containing 0.012 M of GA. The mixture was stirred for 3 h at 50 °C, then the beads were filtered off, rinsed with distilled water and weighed. The yield of beads was defined as the bead quantity in percentage from the quantity of gel used for the preparation of beads.

Stability of Chs / Alg beads

The stability of beads was assayed in 0.1 M citrate buffer, pH 2.5 or 6.5, and distilled water after 1–7 days. The percentage stability, which depends on swelling or shrinkage of Chs / Alg beads, was calculated by the formula:

$$\text{Stability} = \frac{W_e}{W_0} \cdot 100\%,$$

where W_e is the weight of Chs / Alg beads at equilibrium swelling or shrinkage, and W_0 is the initial weight of Chs / Alg beads.

Characterization of Chs / Alg complexes and beads

Formation of Chs / Alg complexes was estimated by turbidity. Solutions of Chs(I, II) and NaAlg(I–III) were used as prepared, or their pH had been adjusted to 2.5 or 5.5 before complexation. The maximal value of turbidity by direct titration of 0.1% NaAlg(I–III) solution in distilled water with 0.1% Chs(I, II) solution in 2% acetic acid or conversely corresponds to formation of complexes. Turbidity t (%) was measured with a nefelometer (LMF-69).

The structure of Chs / Alg complexes and beads was characterized by using a FT-IR spectrophotometer (Perkin–Elmer 1000).

The content of nitrogen (%) in Chs and Chs / Alg complex beads was estimated by the Kjeldahl method [24]. Samples of complexes and beads for FT-IR and nitrogen analysis were freeze-dried using a lyophilizer (Christ ALPHA2-4LSC).

The morphology of beads was characterized employing an optical microscope (LEITZ).

Preparation of liquefied starch solution

100 cm^3 of a 5% potato starch solution in 0.1 M citrate buffer, pH 5.0 was heated to boiling and mixed by mechanical stirring until a viscous colloidal starch solution was obtained. The colloid was cooled and incubated at 40 °C. After that, 0.5 cm^3 (20 International Units (IU)) of α-amylase from *Bacillus subtilis* solution was added. The mixture was vigorously stirred for 10 min at 40 °C, and the enzyme was inactivated by heating the solution for 20 min in a bath of boiling water; as a result, a liquefied starch solution was obtained. The dextrose equivalent (a measure of the total amount of reducing sugars in a solution) of the prepared liquefied starch was 2–4%.

Immobilization of maltogenase via covalent binding on Chs / Alg beads

Immobilization of maltogenase onto Chs / Alg complex beads prepared from complexes using as a crosslinker mixture of $CaCl_2$ and GA was carried out by attaching the amino group of the enzyme to the aldehyde group of GA yielding Schiff bases [25]. The mixture of 0.25 cm^3 (500 IU) of MG, 5 cm^3 of 0.1 M citrate buffer, pH 6.5 and 3.5 g of wet Chs / Alg complex beads was stirred at 40 °C for 30 min and left overnight at 4 °C. The next day, the immobilized enzyme was thoroughly washed with buffer. The efficiency of immobilization (EI) was defined as the activity of immobilized MG in percentage from the activity of the native enzyme used for immobilization. The yield of immobilization (YI) was defined as the protein content in the immobilized enzyme as a percentage from the content of protein in the native enzyme used for immobilization.

Determination of maltogenase enzymatic activity

The activity of native and immobilized MG in the native enzyme solution or MG remaining in the solution after immobilization was assayed by the Somogyi–Nelson method [26]. The activity unit of native or immobilized MG was defined as the amount of enzyme which under standard conditions (at 40 °C, pH 5.0) produced 1 μmol of reduced sugars per minute.

Determination of protein content

The content of protein in native and immobilized MG was assayed by the bicinchinonic acid method (BCA kit, Interchim, France).

RESULTS AND DISCUSSION

Preparation and characterization of Chs / Alg complexes and beads

The complexation of Chs and NaAlg was followed by ionic interaction between protonated amino groups ($-NH_3^+$) of Chs and carboxylate groups ($-COO^-$) of NaAlg. The reaction between Chs and NaAlg is fast and gives insoluble coacervates upon mixing, but not all amino and carboxyl groups react due to steric hindrance and conformation restriction. It is known that the pH of Chs and NaAlg solutions has a strong influence on dissociation of the functional groups of polyelectrolytes and the conformation of chains. It is also known that the degree of dissociation of amino groups of Chs and carboxyl groups of NaAlg at pH 4.8–5.5 is about 70–80%. Each polysaccharide has a rigid, linear conformation; the complexation is almost full, and the membranes formed from Chs / Alg complexes are dense. The complexation of polysaccharides at pH 2.5 or pH ≥ 5.5 is suppressed and not full because of the formation of some kinds of loops and tails from NaAlg at pH 2.5 or from Chs at pH ≥ 5.5. Formation of loops makes membranes from Chs / Alg complexes less dense [2, 7].

Determination of the molar ratios of constituents in PEC by turbidity measurements was described elsewhere [4, 27–29]. Formation of complexes of poly(ethylene imine) and carboxymethylcellulose, or heparin, or sulphomethylcellulose, and determination of molar ratios in PEC from turbidity measurements were studied in detail [27]. This method was applied for determining the molar ratios of Chs and NaAlg in Chs / Alg complexes in our work. Turbidity experiments were carried out by keeping the NaAlg concentration constant and varying the Chs concentration in small steps, or conversely. The observed increase of turbidity could be due to intensified Alg–Chs interactions. The maximal value of turbidity corresponds to maximal complex strength. The molar ratios of the monomer units of Chs and Alg in Chs / Alg complexes were evaluated with respect to DD of Chs. The molar ratios of Chs and NaAlg in the complexes were found to depend on the molecular weight of Chs and NaAlg, DD of Chs, the ratios of M / G blocks in NaAlg and the pH of complexation solutions (Table 1).

The molar ratios of Chs and Alg in complexes were 1.00 : 1.08–1.16 and 1.00 : 1.21–1.32 when the complexes were formed from Chs(I) or Chs(II) with NaAlg(I–III) at the initial pH, respectively. These complexes were nearly stoichiometric in the case of Chs(I). The content of Alg in complexes was increased when Chs with a lower molecular weight and higher a DD and NaAlg with a higher content of G units were used for complexation. The stoichiometry of PEC of weak polyacids and weak polybases is known to be pH dependent due to the change of their dissociation degree with pH [3]. The complexation of Chs and NaAlg at corrected pH 5.5 is stoichiometric, and the molar ratios are as follows: Chs(I) : Alg(I–III) = 1.00 : 1.00–1.03 and Chs(II) : Alg(I–III) = 1.00 : 1.00–1.01. The influence of molecular weight of Chs and NaAlg, DD of Chs and M/G in NaAlg on the composition of complexes formed at pH 5.5 was negligible. The complexation of Chs and NaAlg at the corrected pH 2.5 was nonstoichiometric, and the molar ratios were as follows: Chs(I) : Alg(I–III) = 1.00 : 2.09–2.57 and Chs(II) : Alg(I–III) = 1.00 : 1.82–2.98. The content of Alg(I–III) in the

Table 1. **Molar ratios of Chs and NaAlg in complexes, estimated from turbidity measurements**

Initial solutions				Molar ratios of Chs and Alg in complexes[6]	
Chs	pH of Chs	NaAlg	pH of NaAlg	Chs:	Alg
Chs(I)[1]	3.3	NaAlg(I)[3]	6.1	1.00	1.13
Chs(I)	2.5	NaAlg(I)	2.5	1.00	2.09
Chs(I)	5.5	NaAlg(I)	5.5	1.00	1.02
Chs(I)	3.3	NaAlg(II)[4]	6.1	1.00	1.08
Chs(I)	2.5	NaAlg(II)	2.5	1.00	2.30
Chs(I)	5.5	NaAlg(II)	5.5	1.00	1.00
Chs(I)	3.3	NaAlg(III)[5]	6.3	1.00	1.16
Chs(I)	2.5	NaAlg(III)	2.5	1.00	2.57
Chs(I)	5.5	NaAlg(III)	5.5	1.00	1.03
Chs(II)[2]	3.3	NaAlg(I)[3]	6.1	1.00	1.23
Chs(II)	2.5	NaAlg(I)	2.5	1.00	1.82
Chs(II)	5.5	NaAlg(I)	5.5	1.00	1.01
Chs(II)	3.3	NaAlg(II)[4]	6.1	1.00	1.21
Chs(II)	2.5	NaAlg(II)	2.5	1.00	1.90
Chs(II)	5.5	NaAlg(II)	5.5	1.00	1.00
Chs(II)	3.3	NaAlg(III)[5]	6.3	1.00	1.32
Chs(II)	2.5	NaAlg(III)	2.5	1.00	2.98
Chs(II)	5.5	NaAlg(III)	5.5	1.00	1.00

[1] Chs(I), M_w 400 000, DD 71%; [2] Chs(II), M_w 70 000, DD 77%; [3] NaAlg(I), M_n 440 000, M/G 1.6; [4] NaAlg(II), M_n 230 000, M/G 1.5; [5] NaAlg(III), M_n 60 000, M/G 1.0;
[6] Initial molar ratio was estimated from turbidity measurements.

Table 2. **Stability of Chs(I)/Alg (I–III) complex beads**

No.	Type of NaAlg	Complexation of Chs(I) and NaAlg(I–III)		Yield of beads, %	Stability of beads, %			
		Initial molar ratio*	pH of initial solutions		Assay (buffer pH or H_2O)	After 1 day	After 3 days	After 7 days
1.	NaAlg (I)	1.00 : 1.13	3.3 : 6.1	19	2.5	95	97	103
					6.5	150	160	15
					H_2O	90	92	97
2.	NaAlg (I)	1.00 : 2.09	2.5 : 2.5	20	2.5	120	110	100
					6.5	65	60	54
					H_2O	96	100	100
3.	NaAlg (I)	1.00 : 1.02	5.5 : 5.5	35	2.5	100	100	103
					6.5	50	41	24
					H_2O	100	102	108
4.	NaAlg (II)	1.00 : 1.08	3.3 : 6.1	30	2.5	96	93	90
					6.5	20	15	–**
					H_2O	85	84	90
5.	NaAlg (II)	1.00 : 2.30	2.5 : 2.5	23	2.5	120	120	120
					6.5	55	45	30
					H_2O	100	105	106
6.	NaAlg (II)	1.00 : 1.00	5.5 : 5.5	30	2.5	100	92	90
					6.5	110	–**	–
					H_2O	90	90	94
7.	NaAlg (III)	1.00 : 1.16	3.3 : 6.3	30	2.5	106	93	80
					6.5	20	–**	–
					H_2O	96	96	96
8.	NaAlg (III)	1.00 : 2.57	2.5 : 2.5	15	2.5	110	90	90
					6.5	70	67	50
					H_2O	100	100	102
9.	NaAlg (III)	1.00 : 1.03	5.5 : 5.5	25	2.5	95	91	90
					6.5	65	20	–**
					H_2O	86	85	85

* Initial molar ratio was estimated from turbidity measurements.
** Decomposition of beads.

complexes was increased because of some loops and tails formed from alginic acid. The influence of the molecular weight of Chs and NaAlg as well as DD of Chs and the content of G units in NaAlg was similar to those in the case when complexes were formed from solutions with the initial pH.

The spheres and beads are defined as spherical particles, their size varying from 50 nm to 2 mm [1]. Chs / Alg beads were prepared from the complexes that had been obtained from Chz(I, II) and NaAlg(I–III) in the molar ratios estimated from turbidity measurements (Table 1) with a $CaCl_2$ and GA mixture used as a cross-linker. Usually, $CaCl_2$ is used as a cross-linker for NaAlg, and GA is used as a cross-linker for Chs. The beads prepared from Chs / Alg complexes using only $CaCl_2$ or GA were unstable, and their yields were lower compared with beads obtained using a $CaCl_2$ and GA mixture. For example, the yields of Chs / Alg complex beads were 30 and 32% when they had been prepared from complexes obtained from solutions at the initial pH, with the molar ratios of Chs(I) : Alg(III) = 1.00 : 1.16 or Chs(II) : Alg(III) = 1.00 : 1.32 using a $CaCl_2$ and GA mixture, but the yields were 22 and 25% when using only $CaCl_2$, or 12 and 13% when using only GA. The best results were achieved when a cross-linker mixture of $CaCl_2$ and GA for the preparation of Chs / Alg beads was used (yield 15–40%) (Tables 2 and 3).

The yield of Chs / Alg beads prepared from Chs(I, II) and NaAlg(I–III) complexes at pH 5.5 was higher than the yield of beads prepared at pH 2.5 or the initial pH. We supposed that the complexation at pH 5.5 was stoichiometric or nearly stoichiometric (Tables 2 and 3).

A proof of the structure of complex beads by the Kjeldahl nitrogen analysis [24] was performed on the freeze-dried Chs(I) and Chs / Alg beads prepared from Chs(I) and NaAlg(I) complexes, using a cross-linker mixture of $CaCl_2$ and GA, when the molar ratio of Chs(I) and Alg(I) was 1.00 : 1.13 at the initial pH, 1.00 : 2.09 at the corrected pH 2.5 and 1.00 : 1.02 at the corrected pH 5.5. The content of nitrogen was found to be 7.3% in Chs and 2.2%, 1.5% and 2.6% in the complex beads, respectively. These results showed the presence of Chs in the complex beads.

The FT-IR data analysis of Chs / Alg complexes had been used to the prove the complexation and structure of complexes in [7, 9, 30, 31]. Analogous results were observed in our case. The complexes and beads of Chs / Alg were characterized by FT-IR data. In Chs(I) spectrum, there are absorption

Table 3. **Stability of Chs(II) / Alg (I–III) complex beads**

No.	Type of NaAlg	Complexation of Chs(II) and NaAlg(I–III)		Yield of beads, %	Stability of beads, %			
		Initial molar ratio	pH of initial solutions		Assay (buffer pH or H_2O)	After 1 day	After 3 days	After 7 days
1.	NaAlg (I)	1.00 : 1.23	3.3 : 6.1	20	2.5	114	115	115
					6.5	180	–**	–
					H_2O	80	94	99
2.	NaAlg (I)	1.00 : 1.82	2.5 : 2.5	20	2.5	91	103	10
					6.5	44	–**	–
					H_2O	89	89	88
3.	NaAlg (I)	1.00 : 1.01	5.5 : 5.5	23	2.5	102	104	104
					6.5	110	–**	–
					H_2O	98	90	90
4.	NaAlg (II)	1.00 : 1.21	3.3 : 6.1	25	2.5	106	100	98
					6.5	57	20	–**
					H_2O	82	84	90
5.	NaAlg (II)	1.00 : 1.90	2.5 : 2.5	21	2.5	100	112	122
					6.5	65	–**	–
					H_2O	100	100	105
6.	NaAlg (II)	1.00 : 1.00	5.5 : 5.5	30	2.5	100	95	95
					6.5	110	–**	–
					H_2O	90	90	94
7.	NaAlg (III)	1.00 : 1.32	3.3 : 6.3	15	2.5	115	90	80
					6.5	24	–**	–
					H_2O	122	120	118
8.	NaAlg (III)	1.00 : 2.98	2.5 : 2.5	15	2.5	100	95	95
					6.5	65	–**	–
					H_2O	100	100	100
9.	NaAlg (III)	1.00 : 1.00	5.5 : 5.5	25	2.5	97	95	92
					6.5	65	20	–**
					H_2O	96	90	85

* Initial molar ratio was estimated from turbidity measurements.

** Decomposition of beads.

bands at 1 634 cm^{-1} (Amide I), 1 560 cm^{-1} (NH_2), 1 550 cm^{-1} (Amide II), 1 412 cm^{-1} (CH_2), 1 153–1 031 cm^{-1}(saccharide structure). In NaAlg(I) spectrum, there are broad absorption bands at 1 616 cm^{-1} (asymmetric COONa), 1 419 cm^{-1} (symmetric COONa), 1 257 cm^{-1} (skeletal vibration) and 1 152–1 031 cm^{-1} (saccharide structure). In the spectrum of Chs / Alg complex beads prepared from Chs(I) and NaAlg(I) complexes using a cross-linker mixture of $CaCl_2$ and GA, there was an evident sharpening of the band at 1 616 cm^{-1} due to the COO^- groups in the alginate and an almost complete disappearance of the Chs amino band at 1 560 cm^{-1}. There were new absorption bands at 1 530 cm^{-1} (NH_3^+), 1 710 cm^{-1} (COO^-) (by ionic interaction between protonated amino groups of Chs and carboxylate groups of NaAlg). In the spectra of complexes prepared from Chs(I) and NaAlg(I) solutions at the initial pH or at pH 2.5 corrected to or 5.5, there were absorption bands at 1 535–1 540 cm^{-1} (NH_3^+) and 1 710–1 706 cm^{-1} (COO^-) formed by ionic interaction, and almost a complete disappearance of the Chs amino band at 1 560 cm^{-1}. Besides, in the spectra of complexes prepared at pH 2.5, there were absorption bands at 1 735 cm^{-1}(COOH) caused by Alg loops.

The Chs / Alg complex beads were investigated with an optical microscope. The size of the beads was in the range of 0.2–2 mm.

The stability of Chs / Alg complex beads

The stability of Chs / Alg beads was strongly depended on the conditions of bead formation. It is known that PEC of Chs / Alg are highly hydrophilic and can produce highly swelling systems in aqueous solutions. The degree of swelling is dependent on the content of Alg in beads and the pH of aqueous solutions. The stability of Chs / Alg complex beads was investigated by storaging them at room temperature for 1–7 days in 0.1 M citrate buffer, pH 2.5 or 6.5, and distilled water. Usually, immobilization of enzymes and capsulation of drugs for oral applications are carried out in a citrate buffer pH 6.5 and 2.5, respectively. Thus, it is important to keep beads stable in those buffer solutions [32, 33]. The influence of the molecular weight of Chs and NaAlg, their molar ratios and the pH of the initial solutions on the stability of Chs / Alg beads was investigated (Tables 2 and 3). Chs / Alg complex beads prepared from complexes obtained at pH 5.5 were stable during storage for 7 days in 0.1 M citrate buffer, pH 2.5 in all cases. Chs / Alg beads prepared from Chs and NaAlg solutions at the initial pH or from solutions at the corrected pH 2.5 were stable or swelled little during storage in 0.1 M citrate buffer, pH 2.5. In all cases, the complex beads were more stable in 0.1 M citrate buffer, pH 2.5 and distilled water, than in 0.1 M citrate buffer, pH 6.5. A high concentration of ($-COO^-$) or protonated ($-NH_3^+$) groups in the interface of beads from loops and strong electrostatic interactions between these groups restrain the swelling of beads. The degree of swelling of Chs / Alg complex beads increased during storage in 0.1 M citrate buffer, pH 6.5, for 7 days (Tables 2 and 3). In some cases, a contraction or decomposition of beads was observed in this buffer after 1 or 3 days, possibly because smaller Na^+ ions from the citrate buffer could replace Ca^{2+} ions from Alg which is a constituent part of beads and could cause their decomposition [33]. These results can be attributed to the combined effects of diffusion and complex dissociation during swelling.

Maltogenase immobilization on Chs / Alg complex beads

Enzyme immobilization offers several advantages: improvement of enzyme stability, easy product separation and a control of the release of an enzyme, increase the surface area, etc. [2, 23]. The possible application of Chs / Alg complex beads for enzyme immobilization was studied. MG was chosen as a modeling enzyme. This enzyme was immobilized on Chs / Alg complex beads by covalent binding. Chs / Alg complex beads were prepared using a mixture of $CaCl_2$ and GA as a cross-

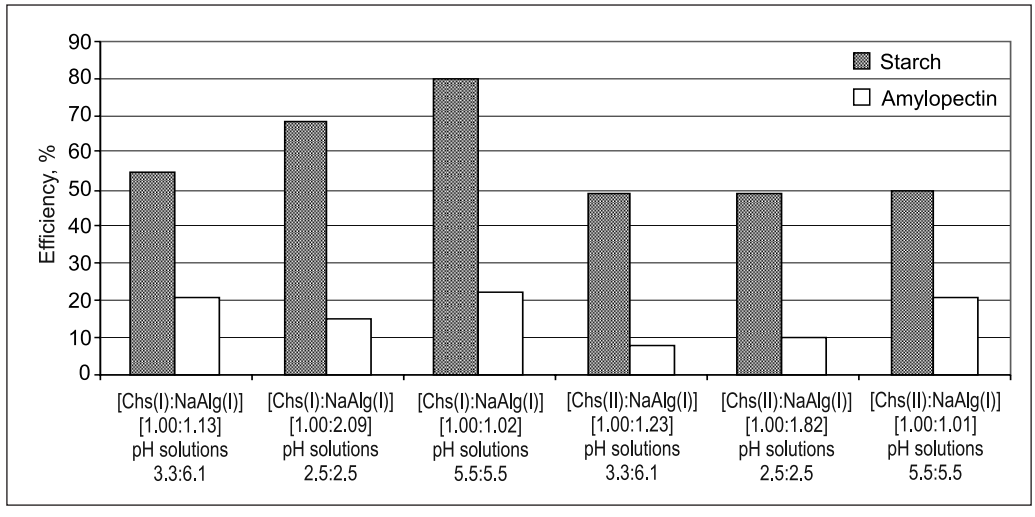

Figure. Efficiency of maltogenase immobilization on Chs(I, II) / Alg(I) complex beads prepared from complexes obtained under different initial conditions

linker. GA had been extensively used as an enzyme immobilizing agent for many years [25]. Herein, during preparation of Chs / Alg complex beads, free aldehyde groups from GA remain in complex beads which could be used for the attachment of enzyme. It is usually accepted that reactions between the aldehyde group of GA and the amino group of enzymes take place, yielding Schiff bases [25, 34].

MG is industrially used in the production of high-maltose syrups via saccharification of starch or its constituents amylopectin and amylose. A partially hydrolyzed starch solution and amylopectin were used as a substrate for determining the activity of native and immobilized enzymes.

There are two parameters, YI and EI, which are extremely important in evaluating the immobilization of enzymes. YI is defined as a protein amount bound to the carrier, and it could be determined indirectly by subtracting the amount of protein found in the washed solvent from the amount of protein used for immobilization. However, one of the most important parameters used to describe the immobilization process is EI. EI was defined as the total units of enzyme activity obtained in the immobilized preparation and divided by the activity of the immobilized enzyme molecules present in the free state, i. e. the activity of immobilized MG in % from the activity of a native enzyme used for immobilization. EI is a more important parameter than YI, because it evaluates the residual activity of the enzyme after immobilization, even in cases when the activity of the enzyme was reduced after immobilization. In our case, YI correlated with EI when liquefied starch was used as a substrate for the determination of MG activity, and it was 54–79%, that indicating there had been no loss of enzyme activity after immobilization. A high efficiency of MG immobilization was achieved when partially hydrolyzed starch solution as a substrate was used (Figure).

The efficiency of MG immobilization was lower using amylopectin as a substrate. This difference is caused by a high molecular weight of amylopectin as well as branching. The efficiency of immobilization depended on the conditions of bead preparation. The best results of immobilization were achieved when Chs(I) / Alg(I) complex beads had been prepared from complexes obtained at pH 5.5 in the molar ratio 1.00 : 1.02. The efficiency of immobilization in this case was about 80%.

CONCLUSIONS

Intermolecular complexation between Chs and NaAlg depends on the molecular weight and deacetylation degree of Chs, the molecular weight and β-D-mannuronic and α-L-guluronic acid values of NaAlg, and the initial pH of solutions. Nonstoichiometric PEC with excess of Alg were obtained from Chs and NaAlg solutions at the initial pH or at the pH corrected to 2.5. The amount of Alg increased when Chs of a lower molecular weight and a higher DD and NaAlg with a higher content of G units were used for complex formation. The complexation of Chs and NaAlg at pH 5.5 was stoichiometric. Chs / NaAlg beads were prepared from these complexes obtained with various molar ratios at the initial pH or at the pH corrected to 2.5 or 5.5 by using a mixture of $CaCI_2$ and GA as a cross-linker. The structure of the complexes and beads was characterized by FT–IR spectra, and ionic interactions between protonated amino groups of Chs and carboxylate groups of Alg were confirmed. The size of beads ranged within 0.2–2 mm. The beads were most stable in 0.1 M citrate buffer, pH 2.5, and distilled water. The synthesized Chs / Alg beads were used as carriers for maltogenase immobilization. The highest efficiency of immobilization (80%) was achieved using as a carrier Chs(I) / Alg(I) complex beads prepared from complexes obtained at pH 5.5. Chs / Alg complex beads could be used as polymeric carriers.

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References

1. M. N. V. Ravi Kumar, R. A. A. Muzzarelli, C. Muzzarelli, H. Sashiva, A. J. Domb, *Chem. Rev*., **104**, 6017 (2004).
2. M. A. Krayukhina, N. A. Samoilova, I. A. Yamskov, *Russ. Chem. Rev*., **77**, 799 (2008).
3. M. Rinaudo, *Polym. Int*., **57**, 397 (2008).
4. C. Peniche, W. Arguelles-Monal, *Macromol. Symp*., **168**, 103 (2001).
5. K. Y. Lee, W. H. Park, W. S. Ha, *J. Appl. Polym. Sci*., **63**, 425 (1997).
6. H. V. Saether, H. K. Holme, G. Maurstad, O. Smidsrod, B. T. Stokke, *Carbohydrat. Polym*., **74**, 813 (2008).
7. F. A. Simsek-Ege, G. M. Bond, J. Stringer, *J. Appl. Polymer Sci*., **88**, 346 (2003).
8. M. Gomez-Burgaz, B. Garcia-Ochoa, S. Torrado-Santiago, *Int. J. Pharmaceut*., **359**, 135 (2008).
9. G. Lawrie, I. Keen et al., *Biomacromolecules*, **8**, 2533 (2007).
10. J. Filipovic, D. Maysinger, B. Zorc, I. Jasenjak, *Int. J. Pharmaceut*., **116**, 39 (1995).
11. H. L. Lai, A. A. Khalil, D. Q. M. Craig, *Int. J. Pharmaceut*., **251**, 175 (2003).
12. M. G. Sankalia, R. C. Mashru, J. M. Sankalia, V. B. Sutariya, *Eur. J. Pharmaceut. Biopharmaceut*., **65**, 215 (2007).
13. L. Gomez, H. L. Ramirez, M. L. Villalonga, J. Hernandez, R. Villalonga, *Enzym. Microbial Technol*., **38**, 22 (2006).
14. S. A. Cetinus, H. N. Oztop, D. Saraydin, *Enzym. Microbial Technol*., **41**, 447 (2007).
15. C. Kubik, B. Sikora, S. Bielecki, *Enzym. Microbial Technol*., **34**, 555 (2004).

16. M. L. Gonzalez-Rodriguez, M. A. Holgado, C. Sanchez-Lafuente, A. M. Rabasco, *Int. J. Pharmaceut*., **175**, 225 (2002).
17. R. Pandey, G. K. Khuller, *J. Antimicrob. Chemother.*, **53**, 653 (2004).
18. O. Diez-Sales, M. Dolz, M. J. Hernandez, A. Casanovas, M. Herraez, *J. Pharmaceut. Sci*., **96**, 1653 (2007).
19. G. W. Vandenberg, C. Drolet, S. L. Scott, J. Noue, *J. Controll. Rel.*, **77**, 297 (2001).
20. E. Tagieddin, M. Armiji, *Biomaterial*., **25**, 1937 (2004).
21. E. Udrenaite, I. Masevič, S. Budriene, A. Zubriene, G. Dienys, *Proceedings of Baltic Polymer Symposium*, Jurmala, 191 (2003).
22. X. Yu, Y. Li, D. Wu, *J. Chem. Technol. Biotechnol*., **79**, 475 (2004).
23. M. Albarghouthi, D. A. Fara, M. M. Saleem, T. El-Thaher, K. Matalka, A. Badwan, *Int. J. Pharmaceut*., **206**, 23 (2006).
24. R. Makuška (ed.), *Synthesis and Characterization of Polymers* (in Lithuanian), VU leidykla, Vilnius (2006).
25. A. Zubrienė, S. Budrienė, N. Gorochovceva, T. Romaškevič, E. Matulionis, G. Dienys, *Chemija*, **14(4)**, 226 (2003)].
26. M. Samogyi-Nelson, *J. Biol. Chem*., **195**, 19 (1952).
27. E. A. Bekturov, L. A. Bimendina, *Interpolimernyje kompleksy* (in Russian), 109, Nauka, Alma-Ata (1977).
28. O. Stoilova, N. Koseva, N. Manolova, I. Rashkov, *Polym. Bull*., **43**, 67 (1999).
29. B. Philipp, H. Dautzenberg, K. J. Lonow, J. Kotz, W. Dawydoff, *Prog. Polym. Sci*., **14**, 91 (1989).
30. L. Wang, E. Khor, L.-Y. Lim, *J. Pharmaceut. Sci.*, **90**, 1134 (2001).
31. F. O. M. S. Abreu, C. Bianchini, M. M. C. Forte, T. B. L. Kist, *Carbohydrat. Polym*., **74**, 283 (2008).
32. T. Romaskevič, S. Budrienė, A. Liubertienė, I. Gerasimčik, A. Zubriene, G. Dienys, *Chemija*, **18**(**3**), 33 (2007).
33. Y. Dai, P. Li, J. Zhang, A. Wang, Q. Wei, *J. Biomed. Mater. Res.*, **86**(**2**), 493 (2008).
34. S. S. W. Park, Y. I. Kim, K. H. Chung, S. I. Hong, *React. Funct. Polym*., **51**, 79 (2002).

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DALELIŲ IŠ CHITOZANO–ALGINATO KOMPLEKSŲ SINTEZĖ IR JŲ PANAUDOJIMAS MALTOGENAZEI IMOBILIZUOTI

S a n t r a u k a

Tirta skirtingų molekulinių masių bei deacetilinimo laipsnio chitozano ir skirtingų molekulinių masių bei iš skirtingo kiekio gulurono ir manurono rūgščių liekanų sudaryto natrio alginato kompleksų susidarymo priklausomumas nuo pH. Iš drumstumo kreivių maksimumų, gautų titruojant alginatą chitozanu arba atvirkščiai, nustatytas kompleksų susidarymas. Chitozano ir alginato kompleksų stecheometriškumas priklauso nuo pH. Chitozano–alginato kompleksai praturtinti natrio alginatu, esant pradiniams tirpalų pH bei pakoreguotiems iki pH 2,5, o esant pakoreguotiems iki pH 5,5 – stecheometriniai. Iš chitozano–alginato kompleksų, susiuvimui naudojant $CaCl_2$ ir glutaro aldehido mišinį, susintetintos chitozano–alginato dalelės. Kompleksų ir dalelių struktūra patvirtinta IR spektrų duomenimis. Tirtas chitozano–alginato dalelių stabilumas 0,1 M citratiniame buferyje, esant pH 2,5 arba 6,5, bei distiliuotame vandenyje. Chitozano–alginato dalelės stabilios distiliuotame vandenyje, o citratiniame buferyje, kurio pH 2,5, yra stabilesnės negu buferyje, kurio pH 6,5. Chitozano–alginato daleles galima naudoti kaip polimerinius nešiklius maltogenazei imobilizuoti. Maltogenazės imobilizavimo efektyvumas ant chitozano–alginato dalelių siekia 80 %.