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Synthesis of chitosan-*graft*-poly(ethylene glycol) methyl ether methacrylate copolymer and its application for immobilization of maltogenase

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Grafting of poly(ethylene glycol) methyl ether methacrylate macromonomer (methoxy poly(ethylene glycol) methacrylate) onto chitosan backbone was studied under homogeneous conditions in a 1% acetic acid solution. The grafting was initiated by cerium ammonium nitrate. Graft copolymers were characterized by FT-IR spectra and chemical analytical methods. The mechanism of grafting reaction and molar compositions of copolymers were determined. Microspheres from the grafted chitosan were applied for the immobilization of maltogenase. High efficiency and stability of maltogenase immobilized onto the grafted chitosan was achieved.

Key words: copolymers, synthesys, chytosan, grafting, maltogenase, immobilization

INTRODUCTION

Chitosan (Chs) is a water-soluble biocompatible and biodegradable polymer. Chemical modification of Chs is important for the production of biofunctional materials with a wide practical application in many areas. Among various methods, graft copolymerization is most attractive because it is a useful technique for modifying the chemical and physical properties of natural polymers. Grafting of Chs is a common way to improve Chs properties, such as increasing chelating [1] or complexation properties [2], bacteriostatic effect [3] or enhancing the adsorption properties [4]. Several methods of grafting onto Chs are known, such as grafting initiated by free radicals, grafting using radiation, enzymatic grafting [5], graft copolymerization via polycondensation, graft copolymerization via oxidative coupling, cyclic monomer graft copolymerization (ring-opening) [6], etc. Graft copolymerization onto Chs using free radical initiation has attracted the attention of many scientists in the last two decades [5]. A number of initiators, such as ammonium persulphate, potassium persulphate, thiocarbonation-potasium bromate, potassium diperiodatocuprate (III), 2, 2`- azobisisobutyronitrile, ferrous ammonium sulphate and ceric ammonium nitrate (CAN) have been developed to initiate grafting copolymerization. CAN redox initiator has been used to produce free radical sites on many kinds of polymers. Chs molecules not only took part in the graft copolymerization, but were also used as a surfactant, providing the stability of the dispersed particles [5]. Poly(vinyl acetate) was grafted onto Chs at 60 °C using CAN. It was noticed that the incorporation of poly(vinyl acetate) into the Chs chains increased the toughness and decreased the water absorption of Chs. [7]. CAN also induced *N,N*-Dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium [8], *N*-isopropylacrylamide [9], acrylonitrile [10], 2-hydroxy ethyl methacrylate [11], acrylamide [12], acrylic acid [13], methacrylic acid [13], methyl acrylate, methyl methacrylate, 4-vinylpyridine [14], poly(ethylene glycol) diacrylate [15] graft copolymerization onto Chs backbone, however, no macromonomers were grafted onto Chs.

The aim of this study was to synthesize and characterize Chs-*g*-poly(ethylene glycol)methyl ether methacrylate (PEGMEMA) (methoxy poly(ethylene glycol)methacrylate) copolymers using CAN and to apply the use of microspheres form Chs graft copolymers for the immobilization of maltogenase.

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MATERIALS AND METHODS

Materials

The following materials were used in this research: chitosan (M_w 70000, 400000, 2000000, Fluka), cerium ammonium nitrate (Aldrich), PEGMEMA (M_w 300, 474, 1100, 2080, Aldrich), acetic acid (POCh), sodium hydroxide (POCh), glutaraldehyde (GA) (25% aqueous solution, Aldrich). All reagents were used as received.

Synthesis of graft copolymers

The synthesis of Chs-*g*-PEGMEMA copolymers was carried out in a three-neck flask with a nitrogen inlet, under homogeneous conditions at 40–70 °C for 1–6 hours. PEGMEMA (0.06–0.6 M) was added to 0.06 M of Chs in an acetic acid (1%) solution. After that, a quantity of CAN proportional to the quantity of PEGMEMA (0.001–0.03 M in 0.5 ml 0.01 M HNO_3 solution), which was used as initiator, was added to the reaction mixture. After grafting reaction, the microspheres from graft Chs were prepared using 0.5 M sodium hydroxide solution. The reaction mixture was stirred for 10 minutes, then filtered and washed with distilled water.

Activation and crosslinking of Chs-g-PEGMEMA copolymers

The microspheres from graft Chs copolymers were prepared by the coacervation method according to the previous description, with an exception that before filtering, the reaction mixture was treated with GA (0.5 ml, 25% aqueous solution) and stirred for 30 minutes at 50 °C. After that, the precipitated copolymers were filtered and washed with distilled water.

Determination of the content of functional groups and nitrogen in copolymers

The amount of hydroxyl- and amino- or iminogroups, as well as the amount of nitrogen was determined according to [16].

FT-IR analysis

The structure of the copolymers was characterized using a FT-IR spectrometer (Perkin-Elmer 1000).

Enzyme

Maltogenase (MG) (Novozymes, Denmark) from *Bacillus Stearothermophilus* (glucan 1,4-α-maltohydrolase, E.C.3.2.1.133) recombinant exo-acting maltogenic amylase, removes maltose units (through 1,4-α-D-glucosidic linkages) from the non-reducing chain ends in maltooligosaccharides and polysaccharides, such as amylase and amylopectin.

Preparation of liquefied starch solution

100 ml^3 of a 5% potato starch suspension in 0.1 M citrate buffer (pH 5.0) was mixed with 0.5 ml^3 (20 International Units (IU)) of α-amylase from *Bacillus Subtilis* solution. The mixture was stirred for 10 minutes at 40 °C and after that the enzyme was inactivated by heating the solution for 20 minutes in a bath of boiling water. Dextrose equivalent (a measure of the total amount of reducing sugars in a solution) of the prepared starch was 2–4%.

Determination of enzymatic activity of maltogenase

The activity of native and immobilized MG in the native enzyme solution or remaining in the solution after immobilization was assayed by the Somogyi-Nelson method [17]. The activity unit of native or immobilized MG was defined as an amount of enzyme which under standard conditions (at 40 °C, pH 5.0) produced 1 μmol of reduced sugars per minute.

Immobilization of maltogenase via covalent binding

The immobilization of MG was carried out in 0.1 M citrate buffer (pH 6.5). The mixture of 0.25 cm^3 (500 IU) of MG, 5 ml^3 of buffer and 3.5 g of wet carrier activated with GA Chs-*g*-PEGMEMA (immediately after the synthesis) was stirred at 40 °C for 30 minutes and was left overnight at 4 °C. On the next day, the immobilized enzyme was thoroughly washed with buffer. The efficiency of immobilization was defined as the activity of the immobilized MG in percent values from the activity of the native enzyme used for the immobilization. The yield of immobilization was defined as the protein quantity of the immobilized enzyme in percent values from the quantity of the protein of the native enzyme used for the immobilization.

RESULTS AND DISCUSSION

Synthesis and characterization of Chs-g-PEGMEMA

PEGMEMA of various molecular weights (M_n 300, 475, 1100, 2080) was grafted onto Chs backbone of cerium ammonium nitrate under homogeneous conditions in acetic acid solution.

The Chs and Chs graft copolymers were characterized by FT-IR spectra. From the Chs spectrum distinctive absorption bands at 1654 (Amide I), 1587 (-NH_2 binding) and 1380 (Amide III) can be found. The absorption bands at 1155 cm^{-1} (anti-symmetric stretching of the C–O–C bridge), 1075 and 1033 cm^{-1} (skeletal vibration involving the C–O stretching) are characteristic of its saccharide structure [8]. The absorbance at 2879 cm^{-1} (υ_s CH_2), was stronger in the copolymer. The new band at

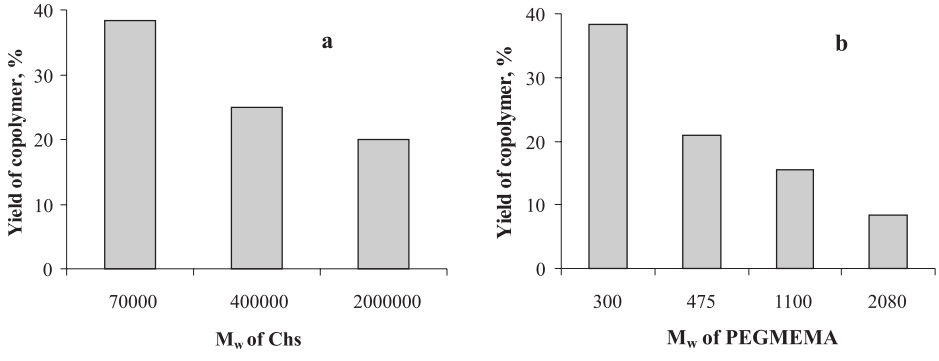


Fig. 1. Yield of Chs-*g*-PEGMEMA copolymer as a function of molecular weight of Chs (a), and PEGMEMA (b), (120 min, 50 °C, [Chs]:[PEGMEMEA] = 1:3)

1719 cm^{-1}, which corresponds to C = O of the ester group stretching vibration of PEGMEMA, was observed in the grafted Chs spectrum. This is a typical absorption of PEGMEMA, which confirms that PEGMEMA has been successfully grafted onto the Chs backbone. The absorbtion due to vinyl unsaturation at 1640 is absent. [15].

Chs and PEGMEMA of various molecular weights were used for grafting (Fig. 1). The best results were achieved in the case when Chs of molecular weight 70000 was used (Fig. 1a). The yield of the graft copolymers decreased with an increasing molecular weight of PEGMEMA (Fig. 1b). It is supposed, that the addition of PEGMEMA with a higher molecular weight was complicated by steric hydrance. Another reason of the decrease in the yield of copolymers could be their solubility in water, as a result, incomplete separation could be observed.

The influence of the initial [Chs]:[PEGMEMA] molar ratio and the concentration of the initiator on copolymerization yield was investigated (Fig. 2). The highest yield of copolymers was in the case when the molar ratio of [Chs]:[PEGMEMA] = 1:3. Increasing the quantity of PEGMEMA in initial substances [Chs]:[PEGMEMA] from 1:3 up to 1:10 resulted in a decrease in the yield of copolymers (Fig. 2a). The yield of copolymers was increased with an increasing CAN concentration used for grafting, and no crosslinking effect in copolymers was observed. It is supposed, that at higher CAN concentration more active centres could have composed, and as a result, more PEGMEMA grafts could have formed (Fig. 2b).

The influence of the grafting time and temperature on the yield of copolymers was also studied. No grafting time effect on the yield of copolymers was observed (Fig. 3a). It was noticed, that the increase in temperature from 40 °C to 50 °C, increased the yield of copolymers from 16.2 to 38.3%. The further temperature increase up to 70 °C influenced the decrease in the yield of copolymers.

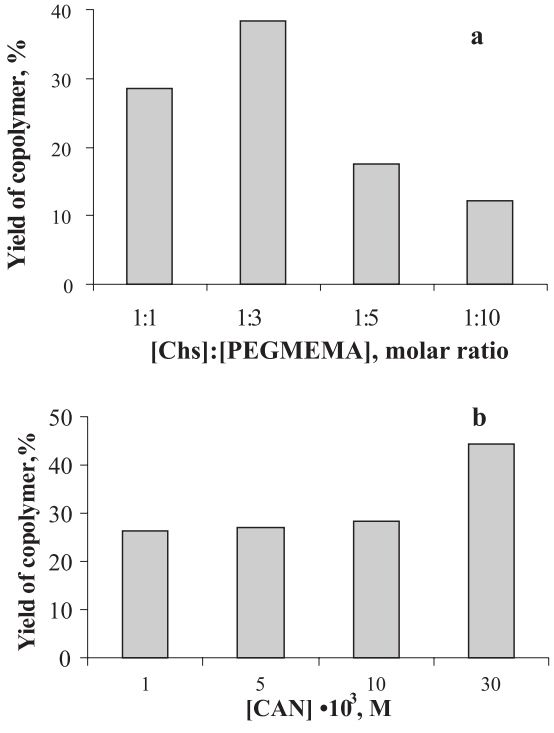


Fig. 2. Yield of copolymers as a function of [Chs]:[PEGMEMA] molar ratio (a, 50 °C, 120 min), and concentration of initiator (b, [Chs]:[PEGMEMA] = 1:1, 120 min, 50 °C).

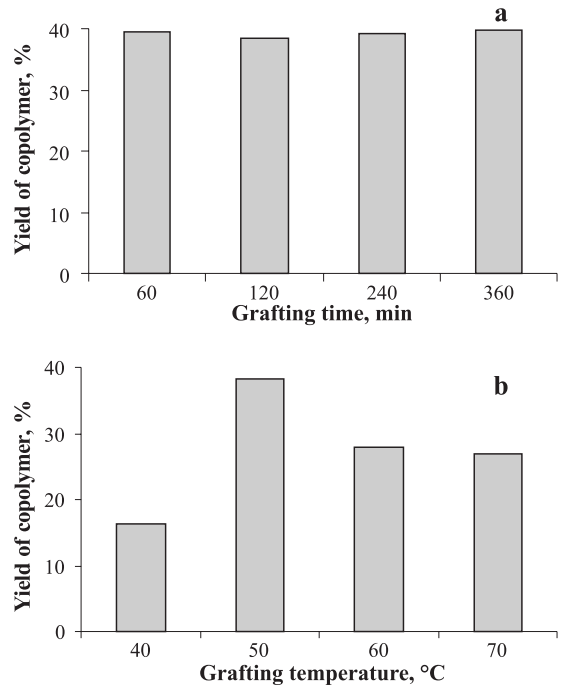


Fig. 3. Yield of copolymers as a function of grafting time (a, [Chs]:[PEGMEMA] = 1:3, 50 °C) and grafting temperature (b) ([Chs]:[PEGMEMA] = 1:3, 120 min)

Strong influence of CAN concentration on molecular weight and viscosity of the solutions of copolymers was noticed. Intrinsic viscosity of Chs-*g*-PEGMEMA copolymers is presented in Fig. 4. As it is known, grafting initiation with CAN causes the degradation of the polysaccharide backbone, thus giving rise to the grafted products with complicated and uncertain structures [18]. An increase in CAN concentration in the initial graft reaction mixture resulted in the reduction of the intrinsic viscosity and molecular weight of Chs-*g*-PEGMEMA copolymers (Fig. 4).

The molar composition of copolymers was estimated by determining the quantities of nitrogen, amino- or imino- and hydroxyl groups. The initial molar ratio of Chs and PEGMEMA in the reaction mixture had an influence on molar composition of graft copolymers. The quantities of the functional groups of Chs in graft copolymers were decreased with an increasing amount

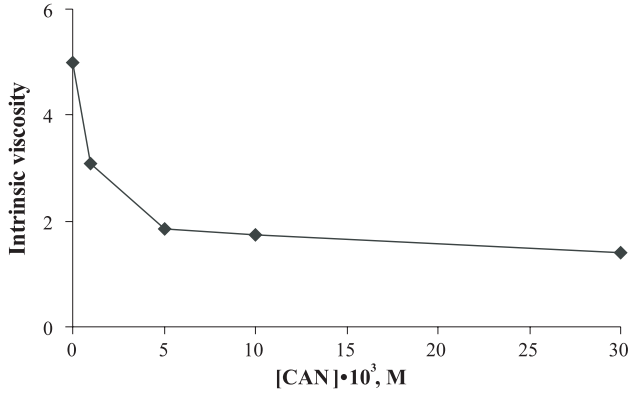


Fig. 4. Intrinsic viscosity as a function of CAN concentration used for synthesis of graft copolymers ([Chs]:[PEGMEMA] = 1:1, 50 °C, 120 min)

of PEGMEMA in the grafting reaction mixture. The quantity of PEGMEMA monomer units in the grafted copolymers was increased at the same time (Fig. 5a). However, the highest influence on molar composition of those copolymers was that of the concentration of the initiator (Fig. 5b). The increasing concentration of CAN in the reaction mixture caused the increased growth of the centres over which new monomer molecules could attach.

The increase in the reaction temperature and reaction time resulted in an increase in the quantity of monomer units in copolymers (Fig. 6a, b). The increase in temperature favoured the activation of backbone, leading to an increase of PEGMEMA monomeric units in copolymers.

There are several propositions of the grafting reaction mechanism onto Chs backbone with CAN initiator. According to the first mechanism [5], monomers (4-vinylpyridine [14], acrylamide [12], acrylic acid, methacrylic acid [13]) and *N*-isopropylacrylamide [9] were grafted onto Chs. The mechanism of the initiation of Chs is followed by a complex formation of Ce^{4+} with a primary amine and the hydroxylgroups at the C-2 and C-3 positions, respectively [6]. The monomers were grafted onto Chs via oxidation with ceric ion, which proceeded via a single electron transfer and pyranose ring decomposition with the formation of radicals on the Chs backbone for initiating graft polymerization of the monomers.

Second mechanism for the synthesis of Chs-*g*-*N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium copolymer was proposed. During a stepped oxidation of Chs, a pyranose ring decomposition with carbonyl group formation took place, and then the monomer was added over C-3 atom [8].

The third proposed mechanism for grafting onto Chs was presented in [13, 15]. The grafting of poly(ethylene glycol) diacrylate onto Chs was proceeded during the initiation with CAN, and monomer molecule was attached to Chs backbone via hydroxyl groups by C-6 atom.

In our case, the mechanism of PEGMEMA grafting onto Chs with CAN was followed in accordance with the first of the proposed mechanisms [9] because the quantities of the functional groups of Chs were proportionally decreased with an increasing quantity of monomeric units in the graft copolymers. It was also proved by FT-IR spectra methods. After the dissociation of Chs–Ce (IV) complex, Ce (IV) ion reduced to Ce (III), followed by the release of Ce (III) ion and pyranose ring cleavage at C2–C3 bond with the formation of a free radical on the polysaccharide backbone, which is capable of initiating graft copolymerization [19]. Free radical sites of PEGMEMA were produced on the Chs backbone (Scheme 1).

Immobilization of maltogenase onto Chs-g-PEGMEMA

The obtained copolymers were used for the immobilization of enzyme. Enzyme immobilization is the method to keep enzyme molecules confined or localized in a certain defined region of space with retention of their catalytic activity [20]. The microspheres from Chs grafted copolymers were activated and crosslinked with GA before the immobilization. The enzyme was immobilized onto the grafted Chs microspheres by covalent binding (Scheme 2). It is usually accepted, that reactions between the carbonyl group of GA and the amino groups of enzymes take place yielding Schiff bases [21].

In our case, enzyme maltogenase was used. Maltogenase from *Bacillus Stearothermophilus* is a maltogenic α-amylase, which catalyzes exohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides, such as amylase and amylopectin, which are constituents of starch. This enzyme is industrially used in the production of high-maltose syrups via saccharification of starch. A partially hydrolyzed starch solution was used as a substrate for the determination of the activity of the immobilized and native enzyme.

The immobilization results are presented in Fig. 7. The efficiency of maltogenase immobilization was estimated as a per-

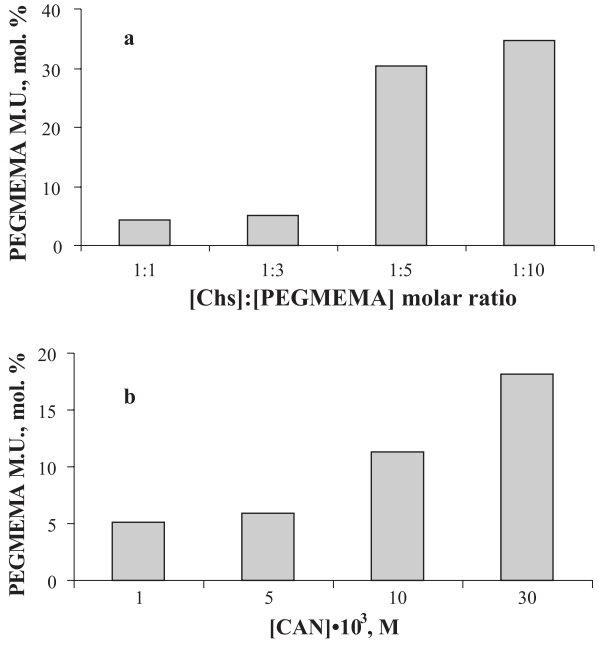


Fig. 5. PEGMEMA monomer units (M. U.) in copolymer as a function of Chs and PEGMEMA initial molar ratio (a, 50 °C, 120 min) and CAN concentration used for grafting (b), [Chs]:[PEGMEMA] = 1:1, 50 °C, 120 min))

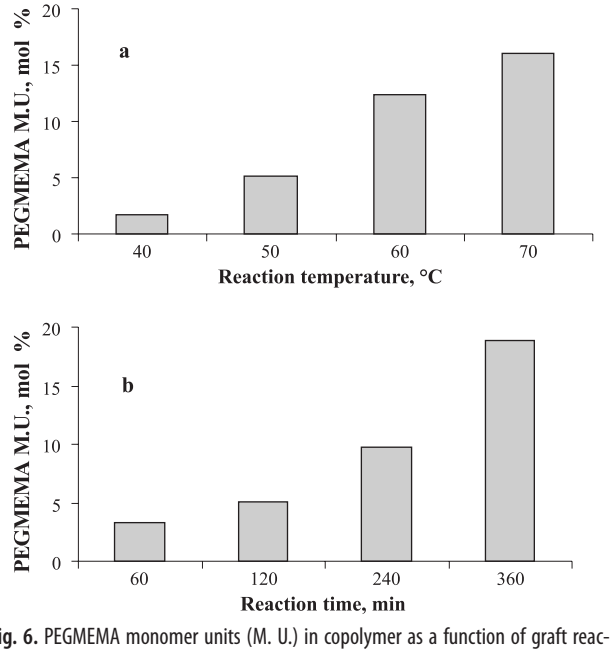
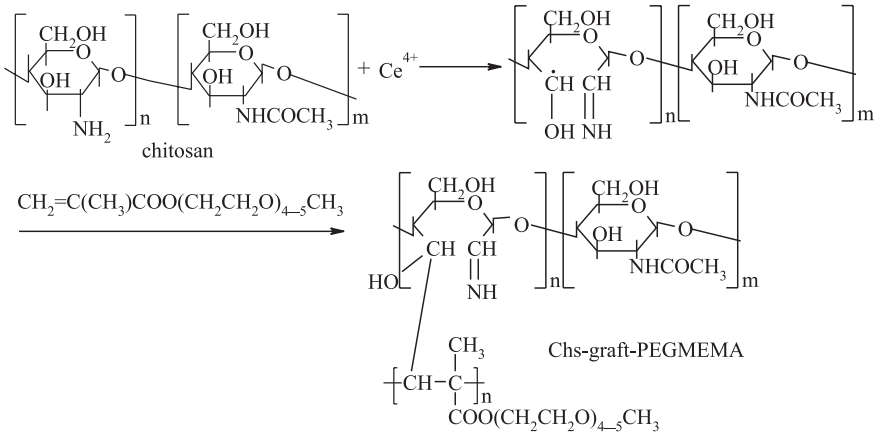
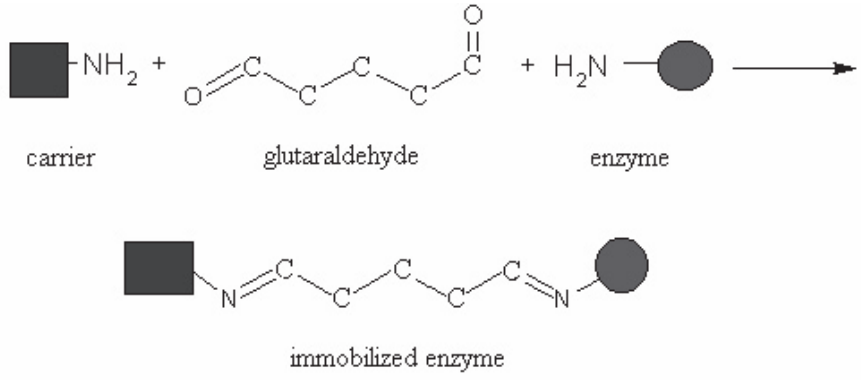


Fig. 6. PEGMEMA monomer units (M. U.) in copolymer as a function of graft reaction temperature (a, [Chs]:[PEGMEMA] = 1:3, 120 min) and graft reaction time (b, [Chs]:[PEGMEMA] = 1:3, 50 °C)



Scheme 1. Grafting reaction onto chitosan backbone



Scheme 2. Immobilization of enzyme via covalent binding onto Chs graft copolymers

centage ratio of the activity of enzyme after and before immobilization. Chs-*g*-PEGMEMA microspheres, using various initial molar ratios of Chs and PEGMEMA (reaction for 120 minutes at 50 °C), were used for the immobilization of maltogenase. In the cases when the initial molar ratio of Chs and PEGMEMA was 1:5 and 1:10, the efficiencies of the immobilization of enzyme onto those microspheres were higher than in the case when non-grafted Chs was used as a carrier. In the cases when Chs and PEGMEMA were 1:1 and 1:3, the efficiencies were lower. The reason of the decrease could be inappropriate size of pores of the microspheres.

The influence of the reaction time of carrier synthesis ([Chs]:[PEGMEMA] = 1:3, [CAN] = 0.03 M, 50 °C) on the immobilization results was studied. As it seems, an extended reaction time resulted in an increase in the efficiency of immobilization (Fig. 8). We suppose that it is concerned with the structure and porosity of the obtained microspheres.

The microspheres from Chs and PEGMEMA with the initial molar ratio 1:1 at 50 °C for 120 minutes were sinthesized in order to investigate the influence of CAN concentration on the efficiency of the immobilization. The results are presented in Fig. 9. The efficiency at first increased with increasing concentration of the initiator and after that gradually decreased. It is supposed that more PEGMEMA grafts in copolymers could form by further increasing the concentration of CAN, and as a result, too small pores were obtained in the prepared microspheres.

It was estimated that the best results of the immobilization of enzymes were achieved when the molar ratio of Chs and

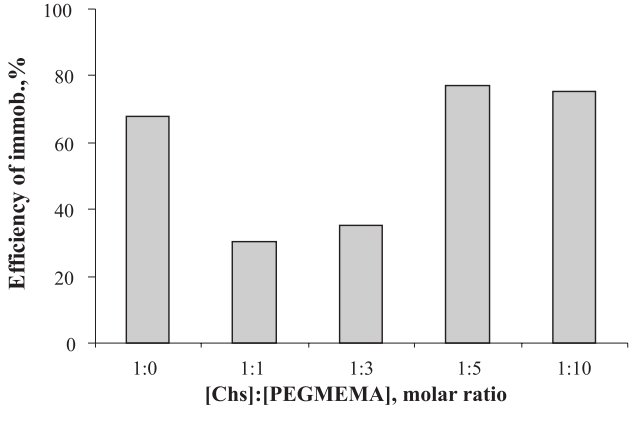


Fig. 7. Efficiency of immobilized maltogenase as a function of initial molar ratio of Chs and PEGMEMA used for synthesis of carrier

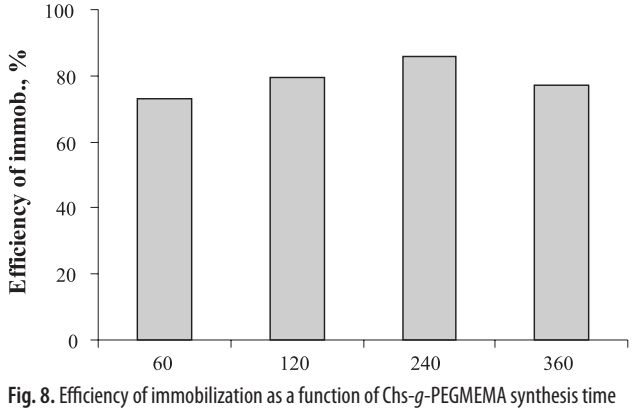


Fig. 8. Efficiency of immobilization as a function of Chs-*g*-PEGMEMA synthesis time

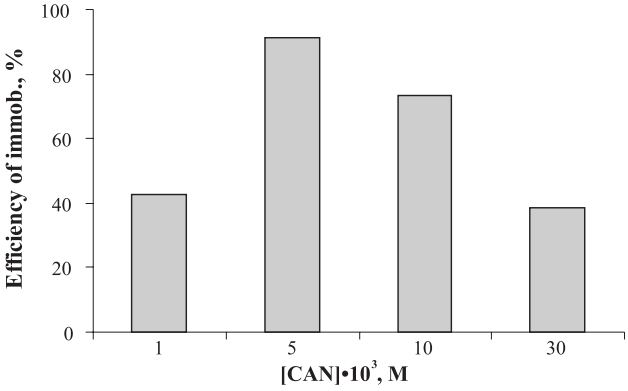


Fig. 9. Efficiency of immobilization as a function of CAN concentration used for synthesis of carrier

PEGMEMA was 1:5. In this case, the quantity of PEGMEMA monomer units in copolymer was 30.5 mol %. It was noticed that this grafted Chs was a better fit for immobilization (yield of immobilization was 77 %) in comparison with non-grafted Chs (67%). Furthermore, it was observed that because of special properties of graft Chs, the immobilized maltogenase had particularly good stability in time.

CONCLUSIONS

Chitosan-*graft*-poly(ethylene glycol) methyl ether methacrylate copolymers were obtained using cerium ammonium nitrate as an initiator under homogeneous conditions in acetic acid solution. The grafting reaction followed by pyranose ring decomposition and addition of monomer molecule over the third carbon atom. The structure and composition of copolymers were determined by FT-IR spectra and chemical analytical methods. The molar composition of copolymers under various reaction conditions were determined. The synthesized grafted copolymers were used as carriers for the immobilization of maltogenase. High efficiency of immobilization (77%) was achieved. A good stability of the enzyme immobilized onto the grafted chitosan was observed.

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CHITOZAN-*SKIEP*-POLI(ETILENGLIKOL)METILETER-METAKRILATO KOPOLIMERŲ SINTEZĖ IR JŲ TAIKYMAS MALTOGENAZĖS IMOBILIZAVIMUI

S a n t r a u k a

Susintetinti ir ištirti chitozano (Chz) ir poli(etilenglikol)metiletermetakrilato (PEGMEMA) skiepytieji kopolimerai, naudojant įvairius Chz ir PEGMEMA pradinius molinius santykius, sintezės trukmę, temperatūrą bei iniciatoriaus koncentraciją. Gautų kopolimerų sudėtis apskaičiuota iš nustatytų funkcinių grupių. Struktūra įrodyta FT-IR spektrais. PEGMEMA skiepijamas prie Chz grandinės oksidavimo metu skylant chitozano piranoziniam ciklui tarp 2 ir 3 anglies atomų ir prisijungiant monomerui prie 3 anglies atomo. Suformuotos skiepytųjų kopolimerų mikrosferos buvo sėkmingai panaudotos maltogenazės imobilizavimui. Imobilizavimo efektyvumas siekė 77%.