Immobilization of hydrolases onto chitosan microparticles

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Chitosan microparticles were prepared by a simple coacervation method for immobilization of hydrolases: β-galactosidase from *Penicillium canescens*, alkaline phosphatase from *E. coli,* maltogenase from *Bacillus stearothermophilus* and pullulanase from *Bacillus acidopullulyticus*. A high yield of immobilization of β-galactosidase (98%) was achieved.

Key words: chitosan, glutaraldehyde, immobilization

INTRODUCTION

Enzymes have a wide variety of biotechnical, biomedical and pharmaceutical applications. An important application of enzymes is in industrial processes and products due to their high specificity, high rate of reactions, non-toxicity and water solubility, which are major advantages over inorganic catalysts. Enzyme immobilization is a method to keep enzyme molecules confined or localized in a certain defined region of space with retention of their catalytic activities. In comparison with their native form, immobilized enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of repeated usage, and continuous process technology. Different methods such as covalent binding, electrostatic binding, hydrophobic interactions, entrapment and encapsulation are often used for immobilization of enzymes. The most common method is the covalent binding onto an insoluble polymer such as cellulose and chitin, most abundant polysaccharides [1].

Chitosan is a linear polysaccharide obtained by the N-deacetylation of chitin in a strong alkali solution. Its structure is basically constituted of D-glucosamine units, with contents of N-acetyl-D-glucosamine in the range of 0–50%. Chitosan is known as an ideal support material for enzyme immobilization because of its many characteristics like hydrophilicity, biodegradability, biocompatibility and antibacterial properties [2]. It is non-toxic, available in different forms (powder, gel, fibers and membranes). It exhibits a high affinity toward proteins and has already been used for immobilization of lipase [3], alpha-amylase and invertase by entrapment [4]. Chitosan beads were prepared by ionic gelation using sodium tripolyphosphate as a cross-linking agent in the gelling solution. The entrapment efficiency of lipase was 43–50% for different chitosan levels. β-galactosidase from *B. singularis* was immobilized on tertiary amine groups containing chitosan by simple adsorption [5] and used for production of galacto-oligosaccharides. β-Glucosidase was adsorbed on chitosan and crosslinked with glutaraldehyde [6]. The enzyme exhibited a considerable affinity to chitosan, giving good immobilization yields (55–85%) while main-

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taining an optimum level of activity (550–850 U/g). Immobilization of enzymes by covalent binding to chitosan using glutaraldehyde as a cross-linking and activating reagent was used for immobilization of α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* [7], acid phosphatase and β-glucosidase [8]. The yields of immobilization were not high, but a good stability of immobilized enzymes was achieved.

The purpose of this study was to immobilize four hydrolases by covalent binding onto chitosan microparticles cross-linked and activated with glutaraldehyde; to study the effect of the chitosan molecular weight and the degree of deacetylation on enzyme loading, leaching and activity, and to characterize the microparticles.

EXPERIMENTAL

The fungus β-galactosidase from *Penicillium canescens* strain F-178 was a kind gift of Institute of Biotechnology (Moscow, Russia). Specific activity was 30–40 U/mg of protein. Maltogenase (4000L) from *Bacillus stearothermophilus* (activity 200 U/ml) and pullulanase from *Bacillus acidopullulyticus* (Promozyme 400L) (25000 U/ml) were obtained from Novo Nordisk (Denmark). Alkaline phosphatase from *E. coli* strain C4, specific activity 75 U/mg was a kind gift of Fermentas AB (Vilnius, Lithuania). 25% glutaraldehyde solution was obtained from Reanal (Budapest, Hungary).

Preparation of chitosan microparticles for enzyme immobilization

Chitosan of various molecular weights (M_w) and degrees of deacethylation (DD) was obtained from Fluka (M_w $7 \cdot 10^4$, DD 77%; M_w $7.5 \cdot 10^5$, DD 71%; M_w $2 \cdot 10^6$, DD 68%). The degree of deacetylation was calculated from the overall quantity of nitrogen (estimated by the Kyeldal method) and the quantity of nitrogen in primary amino groups (estimated by the direct titration method with HCl or reversible titration with HBr [9].

Microparticles of chitosan were prepared according to Budriene et al. [10]. Powdered chitosan was dissolved in 2% acetic acid. Into 20 ml of a 1% chitosan solution at 50 °C under stirring gradually 13.2 ml of 0.5 N KOH was added. 2.65 weight parts (w.p). of 25% glutaraldehyde (GA) were added after 10 min (the quantity of chitosan in the reaction mixture was 1 w.p.). Stirring was continued for additional 30 min.

Immobilization. Chitosan microparticles were washed with 0.1 M citrate-phosphate buffer (pH 6.5) or in the case of AP immobilization with 10 mM Tris-HCl buffer (pH 8.0). A solution of native enzyme was added, the mixture was stirred at 37 °C (or 40 °C in the case of maltogenase and pullulanase) for 30 min and then left at 4 °C overnight. Next day the immobilized enzyme was thoroughly washed with buffer until no enzyme activity was detected in the washes.

Enzyme assays. Activity of native β-galactosidase was assayed by incubation of 1 ml of enzyme solution with 2 ml of 7.5% lactose as a substrate in 0.1 M citrate-phosphate buffer (pH 4.5) at 37 °C for 30 min. The glucose produced in this reaction was determined by the glucoseoxidase method [11].

Activity of maltogenase and pullulanase was assayed by incubation of 1 ml of enzyme solution and with 10 ml of 5% starch as a substrate in 0.1 M citrate buffer (pH 5.0) at 40 °C for 10 min. The reduced sugars produced in this reaction were estimated by the Samogyi–Nelson method [12].

Activity of native alkaline phosphatase was determined by measuring spectrophotometrically at 410 nm the rate of p-nitrophenol formation from p-nitrophenolphosphate. 0.1 ml of enzyme solution was added to 0.9 ml of 1 mM p-nitrophenolphosphate solution in 0.5 M Tris-HCl buffer (pH 8.0) containing 1 mM of $MgCl_2$. The mixture was incubated at 37 °C for 10 min and then 2 ml of 0.5 M NaOH solution was added.

Activity of all four enzymes was expressed in activity units (U). One U is defined as the amount of enzyme that hydrolyses 1 μmol of substrate per minute at the optimal pH and temperature.

Activity of the immobilized enzymes was determined in the same way exept that the immobilized enzyme was added by weight (100–300 mg) to the substrate solution and incubation was carried out under intensive stirring.

Morphological characterization

The surface morphology of chitosan microparticles was observed by scanning electron microscopy (SEM). The microparticles were freeze-dried, coated with gold and observed microscopically (JEOL 50A, Japan).

RESULTS AND DISCUSSION

We used chitosan of various molecular weights for production of microparticles and investigated the possibility to use chitosan microparticles for immobilization of various hydrolases by covalent binding using GA as a cross-linking and activating agent. GA has been extensively used as an enzyme immobilizing agent for many years. Altough there are many discussions on the nature of the solution species and structures responsible for its properties, it is

usually accepted that reactions between the carbonyl group of GA and the amino groups of enzymes take place yielding Schiff bases (Scheme 1) [13]. Monsan et al. [14] have discussed the possibility of GA reaction also via the α, β-unsaturated double bonds originating from its polymerization (aldolic condensation), which is favored by an increase in pH so that as pH varies, the reactivity of GA changes. Richards et al. [15] demonstrated the existence of α, β-unsaturated aldehyde in aqueous GA solution by nuclear magnetic resonance spectrum. With amines, such materials give Michael-type adducts, which are stable even to acid hydrolysis. Oyrton et al. [16] have concluded that the cross-linking formed englobes 20 moles of GA for each monomeric unit of chitosan, and the process is due to glutaraldehyde polymerization.

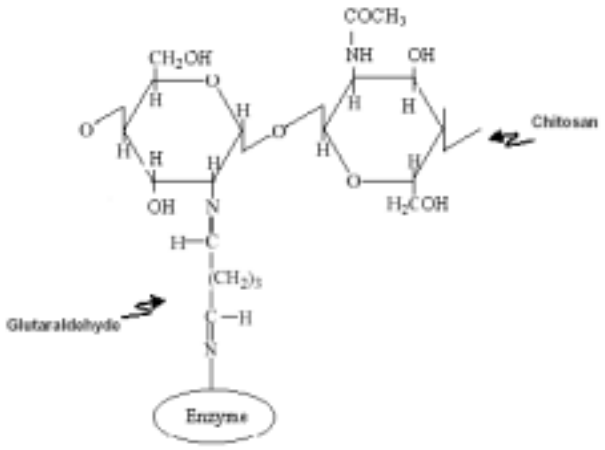
Table 1. **Immobilization of hydrolases**

No.	M_w of chitosan	Enzyme	Activity of immobilized enzyme, U/g wet carrier	Yield of immob.,%*
1.	70000	β-galactosidase	9.1	89.9
3.	750000	β-galactosidase	8.9	83.0
4.	2000000	β-galactosidase	11.1	79.2
5.	70000	alkaline phosphatase	20.6	38.0
7.	750000	alkaline phosphatase	29.7	49.5
8.	2000000	alkaline phosphatase	19.7	39.4
9.	70000	pullulanase	16.1	49.0
11.	750000	pullulanase	17.8	79.1
12.	2000000	pullulanase	25.5	42.0
13.	70000	maltogenase	82.4	54.4
15.	750000	maltogenase	104.3	61.6
16.	2000000	maltogenase	93.0	33.2

*Activity of immobilized enzyme in % from the activity of native enzyme used for immobilization. The given values of the yield are means of 2–4 immobilization experiments.

We have estimated that in alkaline medium the quantity of aldehyde groups of 25% GA solution became twice as low after 25 min showing GA polymerization. So, a variety of products may be produced by reacting GA with chitosan microparticles in alkaline solution.

The results of enzymes immobilization on chitosan microparticles are presented in Table 1. The yield of immobilization of β-galactosidase was higher in the case of chitosan of M_w $7 \cdot 10^4$ (Table 1, No. 1). For alkaline phosphatase, pullulanase and maltogenase the optimal M_w was $7.5 \cdot 10^5$ (Table 1, No. 7, 11, 15). Lower yields were obtained when using chitosan of M_w $2 \cdot 10^6$.



Scheme 1. Schematic illustration of enzyme immobilization by glutaraldehyde

The yield of β-galactosidase immobilization on chitosan depended also on the quantity of GA used for the preparation of microparticles (Fig. 1). The activity of enzyme increased with increasing GA concentrations up to 4.4 w.p. This was the optimal quantity of GA (yield of immobilization was 98% in that case). Nevertheless, activity of immobilized β-galactosidase gradually decreased at above 4.4 w.p. of GA. It is obvious that excess of GA exerts a negative effect on the enzyme. Results of immobilization of β-galactosidase listed in Table 1 were obtained by using 2.65 w.p. of GA for microparticle preparation. Therefore optimal yields of immobilization of β-galactosidase on chitosan are somewhat higher than those presented in Table 1. The optimal quantity of GA for pullulanase immobilization was 2.65 w.p. Alkaline phosphatase and maltogenase were immobilized on chitosan microparticles produced by using 2.65 w.p. of GA (1 w.p. of chitosan).

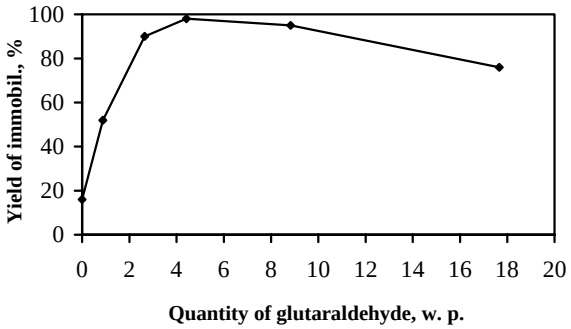


Fig. 1. Yield of β-galactosidase immobilization on chitosan (M_w = $7 \cdot 10^4$) as a function of the quantity of glutaraldehyde

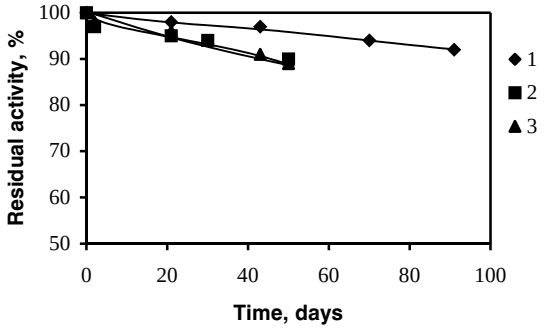


Fig. 2. Stability of β-galactosidase immobilized on chitosan during storage in 0.1 M citrate-phosphate buffer, pH 4.5, at 4 °C. Molecular weight of chitosan: 1 – $7 \cdot 10^4$, 2 – $7.5 \cdot 10^5$, 3 – $2 \cdot 10^6$

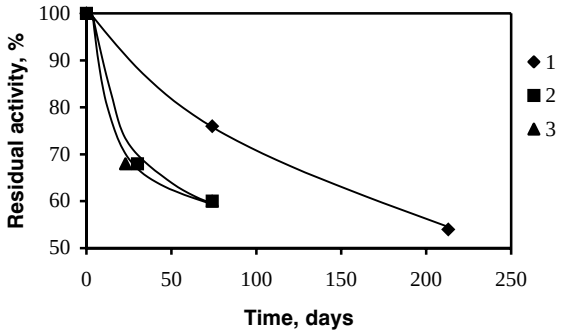


Fig. 3. Stability of maltogenase immobilized on chitosan during storage in 0.1 M citrate buffer, pH 6.5, at 4 °C. Molecular weight of chitosan: 1 – $7 \cdot 10^4$, 2 – $7.5 \cdot 10^5$, 3 – $2 \cdot 10^6$

The stability of β-galactosidase (Fig. 2) immobilized on chitosan during storage in buffer solution at 4 °C was sufficiently high. The stability of β-galactosidase was higher in the case of chitosan of M_w $7 \cdot 10^4$, β-galactosidase showed a 90% residual activity after 240 days of moist storage. The stability of alkaline phosphatase immobilized on chitosan of M_w $7.5 \cdot 10^5$ showed a 95% residual activity after 70 days of moist storage. The stability of immobilized maltogenase (Fig. 3) and pullulanase on chitosan microparticles was lower.

All freeze-dried chitosan microparticles cross-linked and activated with GA were not completely spherical in shape and had a rough, spongy surface (Fig. 4). The shape of microparticles from chitosan with M_w $2 \cdot 10^6$ were not spherical at all. The chitosan M_w affects the morphology of microparticle surface. Microparticles from chitosan with low M_w (DD 77%) and high M_w (DD 68%) had a smoother surface than those prepared with medium M_w (DD 71%). So, the yields of immobilization of hydrolases on chitosan with M_w $7.5 \cdot 10^5$ were highest in most cases (Table 1, No. 7, 11, 15). The size of microparticles ranged mainly between 200 μm and 800 μm. The porosity of microparticles was apparent, small pores were predominant on chitosan of M_w $7 \cdot 10^4$ and $2 \cdot 10^6$ (Fig. 4, a, c).

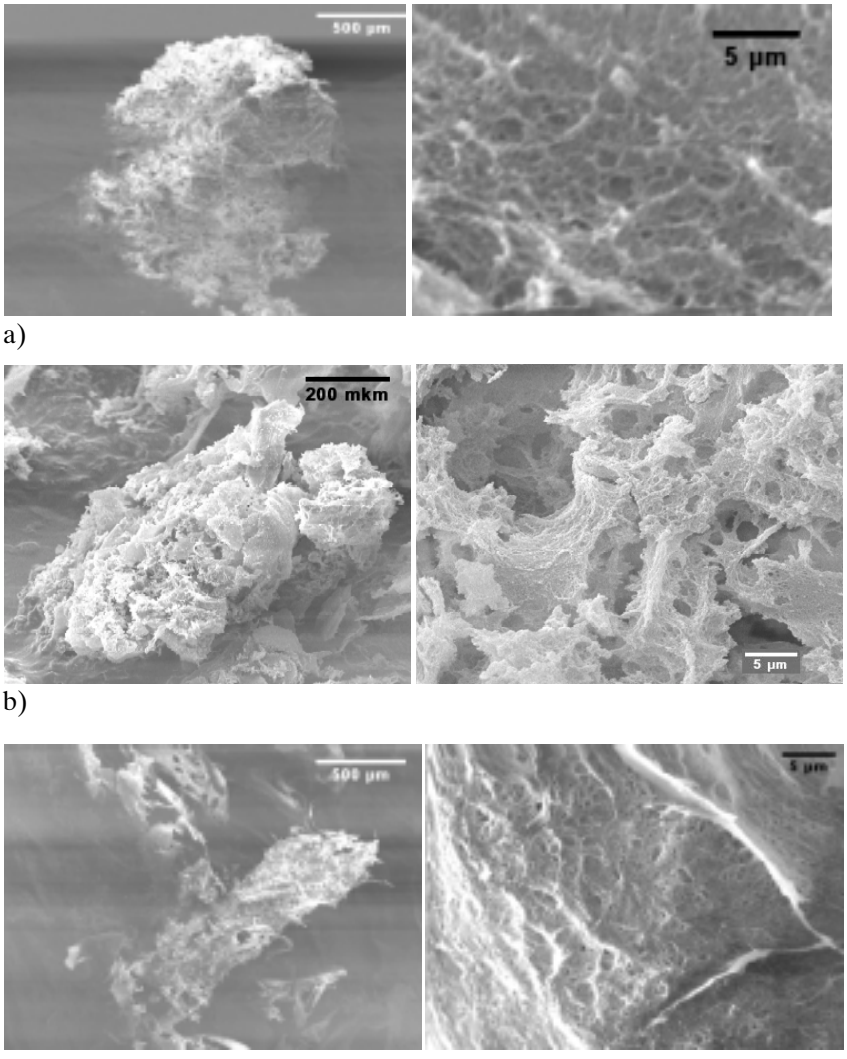


Fig. 4. SEM pictures of chitosan microparticles cross-linked and activated with GA. a) morphology of the chitosan microparticles prepared with chitosan M_w $7 \cdot 10^4$, b) chitosan M_w $7.5 \cdot 10^5$, c) chitosan M_w $2 \cdot 10^6$

CONCLUSIONS

Chitosan microparticles cross-linked and activated with glutaraldehyde were used for immobilization of β-galactosidase, alkaline phosphatase, pullulanase and maltogenase. The highest yield of β-galactosidase immobilization (98%) was obtained on chitosan with molecular weight 70000. Immobilized enzyme retained 90% of its original activity after 240 days at 4 °C. The highest yields of immobilization of alkaline phosphatase, maltogenase and pullulanase were obtained on chitosan with the molecular weight 750000 and exceed 50% in most cases. Chitosan microparticles were porous and had a rough surface.

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HIDROLAZIŲ IMOBILIZAVIMAS ANT CHITOZANO MIKRODALELIŲ

S a n t r a u k a

Paprastos koacervacijos būdu buvo gautos chitozano mikrodalelės, susiūtos ir aktyvuotos glutaro aldehidu. Ant šių mikrodalelių imobilizuotos 4 hidrolazės: β-galaktozidazė, šarminė fosfatazė, pululanazė ir maltogenazė. Didžiausia β-galaktozidazės imobilizavimo išeiga (98%) gauta ant chitozano, kurio molekulinė masė $7 \cdot 10^4$. Kitų hidrolazių imobilizavimo išeigos buvo didžiausios ant chitozano mikrodalelių, kurioms gauti naudotas vidutinės molekulinės masės chitozanas (M_w $7{,}5 \cdot 10^5$). SEM tyrimai parodė, jog chitozano (M_w $7{,}5 \cdot 10^5$) mikrodalelių poros yra didžiausios.