



Short communication

Low-cost, easy-to-prepare magnetic chitosan microparticles for enzymes immobilization

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ABSTRACT

Extremely simple procedures for the preparation of magnetic chitosan enzyme carriers have been developed and used for the immobilization of lipase and β -galactosidase as model enzymes. In the first case commercially available magnetite microparticles were entrapped in chitosan gel, while in the second case magnetic iron oxides microparticles were synthesized in chitosan matrix from ferrous sulfate using microwave irradiation. Immobilized enzymes showed long-term stability without leaching of enzyme from the support and enabled their repeated use without significant loss of activity.

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1. Introduction

Immobilized enzymes have many benefits over soluble enzymes in many laboratory-scale and biotechnology applications. The number of applications of immobilized enzymes is increasing steadily. Easy separation of immobilized enzymes from the product simplifies biocatalysts applications. In addition, reuse of immobilized enzymes provides cost advantages of enzyme-catalyzed processes.

Many different carriers have been used for enzymes immobilization (Xie et al., 2009). Among them, chitosan (a linear polysaccharide composed of randomly distributed β -(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine) has been often used (Krajewska, 2004). Chitosan exhibits many interesting properties, namely biocompatibility, availability of reactive functional groups for chemical modifications, hydrophilicity, mechanical stability, regenerability, and ease of preparation in different geometrical configurations suitable for a chosen biotransformation. In addition, chitosan is an inexpensive material enabling to prepare low-cost carriers for large scale applications. Enzyme immobilization can be performed in a simple way, after chitosan cross-linking and activation by glutaraldehyde.

Magnetic carriers enable simple magnetic separation of immobilized enzymes (Safarik & Safarikova, 2009). Magnetic chitosan derivatives have been already prepared and used for the immobilization of various enzymes (Ju et al., 2012; Peniche, Osorio, Acosta, de la Campa, & Peniche, 2005; Wu, Wang, Luo, & Dai, 2009; Yang, Xu, & Su, 2010). In many cases the processes for magnetic chitosan particles preparations are complicated, and not applicable for large-scale synthesis.

In this paper we describe two extremely simple procedures for the large-scale preparation of magnetic chitosan microparticles. Their applicability has been tested by immobilization of two enzymes, namely lipase and lactase. The results have shown that inexpensive carriers prepared by extremely simple procedures can be used for efficient enzyme immobilization.

2. Materials and methods

2.1. Materials

Chitosan (medium molecular weight, 75–85% deacetylated, molecular weight ca 400 000) was from Fluka, Switzerland while magnetite (iron(II,III) oxide, nominal particle size <5 μ m) was from Aldrich, USA. *Candida rugosa* lipase (EC 3.1.1.3; catalog no. L1754; 153 nkat mg^{−1}), *Kluyveromyces lactis* β -galactosidase (lactase; EC 3.2.1.23; catalog no. G3665; 2430 nkat mg^{−1}), 4-nitrophenyl butyrate, 2-nitrophenyl β -D-galactopyranoside and glutaraldehyde were obtained from Sigma. Iron(II) sulfate heptahydrate, sodium hydroxide and common chemicals were from

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Lach-Ner, Czech Republic. Regular kitchen microwave oven (700 W, 2450 MHz) was obtained locally.

2.2. Standard preparation of magnetic chitosan microparticles with entrapped magnetite (ENCH)

Four grams of chitosan were dissolved in 200 mL of 0.2 M acetic acid and then 8 g of commercial magnetite microparticles were added. After thorough mixing an excess of 1 M sodium hydroxide was added to convert solubilized chitosan into insoluble magnetic chitosan gel. The chitosan gel containing entrapped magnetite microparticles was cut into smaller pieces, washed with water several times and then thoroughly homogenized in a standard kitchen mixer. If necessary, the suspension was sieved through a 100 μm sieve (Safarik, Horska, Martinez, & Safarikova, 2010).

2.3. Microwave assisted preparation of magnetic chitosan microparticles (MWCH)

1 g of chitosan was dissolved in 250 mL of 5% (v/v) acetic acid solution under stirring with a mechanical overhead stirrer (300 rpm; RZR 2041, Heidolph). After dissolution 250 mL of water was added, followed by 100 mL of 3.6% (w/v) solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Then 10% (w/v) NaOH was added dropwise under intense stirring (1000 rpm) until the dark precipitate occurred and the pH of the suspension was at least 10. Then portions of the suspension (ca. 200–250 mL) were transferred into 800–1000 mL beakers and the suspension underwent a microwave treatment for 10 min at the maximum power (700 W). Magnetically responsive microparticles formed were repeatedly washed with water.

2.4. Enzymes immobilization

Lipase and β -galactosidase (lactase) were immobilized on both types of magnetic chitosan microparticles (ENCH and MWCH). 300 mg of each material (wet weight) was repeatedly washed with distilled water and modified by 4.5 mL of 5% (v/v) glutaraldehyde solution in a 5 mL-plastic micro test tube and shaken for 3 h at room temperature using an automatic rotator (20 rpm). Then these particles were magnetically separated, glutaraldehyde solution was washed out and particles were carefully washed with distilled water. Subsequently, 4.5 mL of the enzyme solution in buffer was added (lipase: 1 mg mL^{-1} , in 50 mM phosphate buffer pH 7.5; β -galactosidase: 450 μL in 4.05 mL of 100 mM phosphate buffer pH 7.0 containing 5 mM MgCl_2) and shaken for 20 h at 4 °C. Then the supernatant with unbound enzyme was removed and chitosan microparticles were washed with appropriate buffer until no enzyme activity in supernatant was detected.

2.5. Lipase and β -galactosidase assays

Activities of hydrolases immobilized on magnetic chitosan particles were determined spectrophotometrically using artificial substrates 4-nitrophenyl butyrate and 2-nitrophenyl β -D-galactopyranoside. Particles of magnetic chitosan with attached enzyme were stirred during the reaction in buffer containing the substrate, then magnetically separated to the bottom of the cuvette to stop the reaction and increasing amount of yellow-colored 4-nitrophenol or 2-nitrophenol was measured spectrophotometrically exactly as described recently (Safarik, Horska, Pospiskova, & Safarikova, 2012).

2.6. Operational and time stabilities of immobilized lipase and β -galactosidase

Operation stability of immobilized enzymes was tested during 8 cycles; particles with attached enzyme were washed with buffer between each cycle. Activities of hydrolases were measured and residual activities of enzymes after each cycle were determined and compared taking the initial activity in the first cycle as 100%. The time stability was tested after storage of immobilized enzymes in buffer at 4 °C for 28 days (Safarik et al., 2012).

3. Results and discussion

Chitosan, the principal derivative of chitin, is obtained by N-deacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetyl-D-glucosamine and D-glucosamine. Chitosan can be chemically considered as an analog of cellulose, in which the hydroxyl at carbon-2 has been replaced by amino groups. Chitosan is insoluble in water, but the presence of amino groups causes its solubility in acidic solutions below pH about 6.5. It is important to note that chitosan is not a single chemical entity, but vary in composition depending on the origin and manufacture process. Chitosan can be defined as chitin sufficiently deacetylated to form soluble amine salts, the degree of deacetylation necessary to obtain a soluble product being 80–85% or higher (Krajewska, 2004).

Chitosan, both in nonmagnetic and magnetic form, has been frequently used for immobilization of enzymes and other biologically active compounds. Magnetic chitosan derivatives enable simple separation of the immobilized compounds using simple magnetic separators. Recently several types of magnetic chitosan carriers have been synthesized and used for enzymes immobilization; however, in many cases rather complicated procedures for the magnetic chitosan carriers synthesis have been used. Biotechnology and other large-scale technologies require easy-to-prepare, low cost carriers which can be prepared in standard chemical labs.

Extremely simple procedures for the rapid, low-cost preparation of magnetic chitosan microparticles have been developed and described in this paper (see Section 2). In the first case commercially available magnetite microparticles have been used as a magnetic component of chitosan microparticles; this material was originally used for magnetic affinity separation of potato lectin; the particles diameters ranged between 20 and 100 μm (Safarik et al., 2010). In the second case microwave assisted synthesis has employed ferrous sulfate as magnetic iron oxides precursor (Zheng, Zhang, Xiao, Jin, & Choi, 2010). During the pH increase of the ferrous sulfate/chitosan solution under intensive stirring ferrous hydroxide/insoluble chitosan composite microparticles were formed. During the subsequent microwave treatment ferrous hydroxide was converted into magnetic iron oxides nano- and microparticles present within the chitosan matrix. The use of inexpensive ferrous sulfate as a precursor resulted in even cheaper production of magnetic chitosan microparticles; the diameters of majority of particles ranged between 10 and 200 μm (see Fig. 1). Both magnetic chitosan derivatives could be very easily and efficiently separated from the suspension using a NdFeB permanent magnet or any appropriate magnetic separator (Fig. 2).

Both magnetic chitosan derivatives were used as carriers for immobilization of two important enzymes, namely lipase and β -galactosidase (lactase). The studied enzymes were immobilized on glutaraldehyde crosslinked/activated chitosan microparticles. Table 1 shows the activities of immobilized enzymes on 1 mg of magnetic chitosan microparticles. During the study of operational stability, lipase and lactase immobilized on both types of chitosan magnetic microparticles retained practically 100% of initial activity

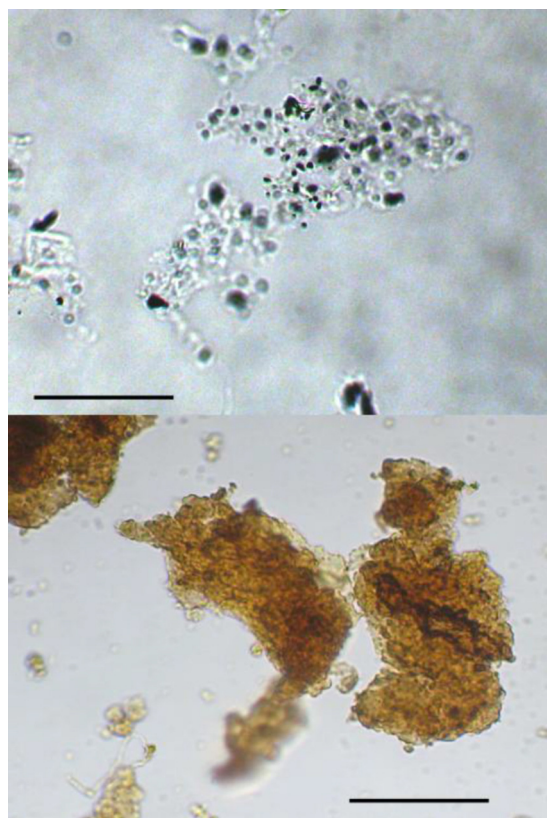


Fig. 1. Optical microscopy of magnetic chitosan microparticles with entrapped magnetite (ENCH, top) and of magnetic chitosan microparticles prepared by microwave assisted synthesis (MWCH, bottom). The bars correspond to 100 μm .

after 8 cycles. Lipase and lactase immobilized on magnetic chitosan microparticles prepared by microwave assisted synthesis were very stable for 28 days without loss of activity; on the other hand these enzymes immobilized on chitosan microparticles with entrapped magnetite retained more than 90% of activity during the same time period (Fig. 3). No significant leaching of enzymes from the supports was detected.



Fig. 2. Demonstration of magnetic separation of magnetic chitosan microparticles prepared by microwave assisted synthesis (MWCH).

Table 1

Activities of lipase and lactase immobilized on two types of magnetic chitosan microparticles using glutaraldehyde activation and retention of enzyme activity on chitosan microparticles.

Chitosan microparticles with immobilized enzyme	Activity of enzyme on a mass unit (wet weight) of chitosan microparticles (nkat mg^{-1})	Enzyme activity retention on chitosan microparticles (%)
MWCH-Lipase	0.45	86
ENCH-Lipase	0.50	94
MWCH-Lactase	4.40	80
ENCH-Lactase	4.75	67

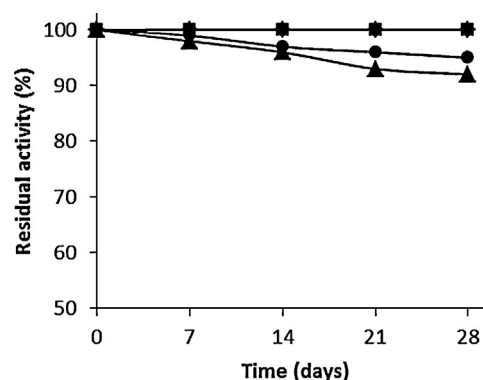


Fig. 3. Time stability of lipase and lactase immobilized on two types of magnetic chitosan microparticles using glutaraldehyde activation. Dependence of relative residual activity of immobilized enzyme (%) during the storage at 4 °C for 4 weeks. (■) MWCH-Lipase; (◆) MWCH-Lactase; (▲) ENCH-Lipase; (●) ENCH-Lactase.

4. Conclusions

As can be seen from the results, two extremely simple procedures for the preparation of magnetic chitosan microparticles, applicable as enzymes carriers, have been developed.

Two industrially important hydrolases (lipase and β -galactosidase) immobilized on the developed magnetic chitosan carriers showed long-term stability without leaching of enzyme from the support; the enzymes could be used repeatedly without significant loss of their activity.

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