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Magnetically responsive enzyme powders

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ABSTRACT

Powdered enzymes were transformed into their insoluble magnetic derivatives retaining their catalytic activity. Enzyme powders (e.g., trypsin and lipase) were suspended in various liquid media not allowing their solubilization (e.g., saturated ammonium sulfate and highly concentrated polyethylene glycol solutions, ethanol, methanol, 2-propanol) and subsequently cross-linked with glutaraldehyde. Magnetic modification was successfully performed at low temperature in a freezer (−20 °C) using magnetic iron oxides nano- and microparticles prepared by microwave-assisted synthesis from ferrous sulfate. Magnetized cross-linked enzyme powders were stable at least for two months in water suspension without leakage of fixed magnetic particles. Operational stability of magnetically responsive enzymes during eight repeated reaction cycles was generally without loss of enzyme activity. Separation of magnetically modified cross-linked powdered enzymes from reaction mixtures was significantly simplified due to their magnetic properties.

1. Introduction

Enzymatic catalysis has gained considerable attention in recent years as an efficient tool in the preparation of fine chemicals, pharmaceuticals, food ingredients, natural products derivatives etc. The enzymatic reactions exhibit high selectivity and proceed under mild reaction conditions. In addition to "standard" reactions performed in water systems, alternative enzymatic reactions can be performed in appropriate organic solvents. For both types of enzymatic reactions immobilized (insolubilized) enzymes have been often used. Solid enzymes prepared by crystallization, lyophilization, or precipitation with acetone from aqueous solutions, and crude commercial powdered enzymes have been used as catalysts in organic solvents in suspension form. The way in which the enzyme powder is prepared can have dramatic effects on the exhibited catalytic activity. Enzymes lyophilization in the presence of salts or suitable imprinting agents (e.g., enzyme inhibitors or substrate analogs), and rinsing with propanol can give enzyme preparations with high activity. Additional cross-linking with a suitable cross-linking agent can be used for the modification of powdered enzyme preparations applicable for reactions in water systems [1].

Suspending of enzyme powders in appropriate organic reaction media (or cross-linked enzyme powders in water systems) is the simplest method to perform enzymatic reactions using insolubilized (immobilized) enzymes. After reaction the enzymes can be removed by filtration or centrifugation and reused afterwards. However, in some cases the powdered enzymes form an emulsion, the extraction and reuse of the enzyme is difficult, and hence a lot of potential enzyme is wasted [2,3].

In order to simplify the separation of powdered enzymes from the reaction media, preparation of magnetically responsive enzyme powders has been developed and described in this paper. The magnetization procedure is performed at low temperatures and enables to obtain "magnetic enzyme powders" with similar activities as the native enzyme powders.

2. Experimental section

2.1. Materials

Crystalline trypsin (bovine pancreas) was from Lachema, Czechoslovakia. Lipase (Lipolase 30 T) was from Novo Nordisk A/S, Denmark. 4-nitrophenyl butyrate, N_{α}-benzoyl-DL-arginine 4-nitroanilide hydrochloride, glutaraldehyde and 2-propanol were obtained from Sigma, USA. Ferrous sulfate heptahydrate, potassium hydroxide, ammonium sulfate, ethanol, methanol, dimethyl sulfoxide, trichloracetic acid and other common chemicals were from

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Penta, Czech Republic. Polyethylene glycol 20,000 was obtained from Serva, Germany. Different types of NdFeB permanent magnets (e.g., 15×15 mm^2, height 8 mm, nominal remanence 1.2 T) were used for magnetic separation. Azocasein was prepared in the laboratory according to the described procedure [4].

2.2. Cross-linking of enzyme powders

Enzyme powder (50 mg; in the case of Lipolase the enzyme granules were finely and gently ground in a mortar with a pestle into a powder) was suspended in various media (5 mL; 4.1 M ammonium sulfate and 25% (w/v) polyethylene glycol solution in 50 mM potassium phosphate buffer pH 7.5, or precooled (−18 °C) organic solvent, e.g., ethanol, methanol, 2-propanol). Then, this suspension of undissolved enzyme was cross-linked by dropwise addition of glutaraldehyde to final concentration 1.5% (v/v) in the suspension under gentle stirring on vortex (in case of polyethylene glycol solution, firstly glutaraldehyde was added and secondly the enzyme powder was thoroughly suspended in a few smaller doses). These samples were shaken on automatic rotator for 3 h at 4 °C (20 rpm). Then, cross-linked powdered enzymes were washed with water/appropriate buffer by centrifugation and pellet resuspension and then magnetized.

2.3. Magnetic modification of cross-linked powdered enzymes

Magnetic modification was performed using suspension of magnetic iron oxides particles prepared by microwave-assisted synthesis, as described previously [5,6]. Shortly, 1 g of ferrous sulfate heptahydrate was dissolved in 100 mL of distilled water in 800 mL-glass beaker. Then, pH was gradually increased up to value 11–12 by the dropwise addition of 1 M potassium hydroxide solution under stirring. This suspension with formed precipitate of iron hydroxides was diluted up to 200 mL with distilled water and treated in microwave oven (700 W, 2450 MHz) at maximum power for 10 min. The formed magnetic iron oxides particles were repeatedly washed with water using a permanent magnet.

Cross-linked enzyme powders were magnetically modified by iron oxides particles prepared by the above described microwave assisted synthesis. Cross-linked powdered enzyme was properly mixed with the suspension of magnetic particles (1 part of completely sedimented particles and 2 parts of 50 mM sodium acetate buffer, pH 4.0 with 20 mM $CaCl_2$ for trypsin/50 mM potassium phosphate buffer, pH 7.5 for lipase) in volume ratio 1 + 1 (v/v) in a test tube using a spatula. Then, excess of liquid was removed after centrifugation by pipette and mixed again. Finally, samples were put into the freezer (−20 °C) for at least 7 days to fix the particles on the surface or within the structure of cross-linked powdered enzymes. After this procedure, samples were completely dried up in a desiccator under reduced pressure. Magnetically modified cross-linked powdered enzymes were stored in a fridge in dry form.

2.4. Determination of enzymes activity

Activity of trypsin was determined by cleaving low-molecular weight substrate N_α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (1.25 mM; stock solution in dimethyl sulfoxide) in 50 mM Tris–HCl buffer, pH 8.5 with 20 mM $CaCl_2$ at 25 °C; the increase in concentration of yellow-colored reaction product 4-nitroaniline was measured spectrophotometrically at 405 nm. Proteolytic activity of trypsin was tested by digesting high-molecular weight substrate azocasein (0.7% (w/v); stock solution in 50 mM Tris–HCl buffer, pH 8.5 with 20 mM $CaCl_2$) at 37 °C; then the reaction was stopped by magnetic separation of enzyme and subsequent addition of 5% (w/v) trichloracetic acid into the supernatant (in the same volume ratio); after the centrifugation, the colored reaction product was measured in the supernatant spectrophotometrically at 366 nm.

Activity of lipase was determined by the hydrolysis of 4-nitrophenyl butyrate (0.5 mM; stock solution in ethanol) in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C; the increase in concentration of yellow-colored reaction product 4-nitrophenol was measured spectrophotometrically at 405 nm.

2.5. Operational stability of magnetic cross-linked powdered enzymes

Reusability of magnetically modified cross-linked powdered enzymes was tested as the operational stability during 8 repeated reaction cycles. Residual activity was compared with the initial activity in the first reaction cycle (taken as 100%).

2.6. Optical microscopy of magnetic cross-linked powdered enzymes

Structures of cross-linked powdered enzymes and fixed aggregates of magnetic particles on the surface of magnetically modified samples were studied using an optical microscope. The presence of Fe(III) ions (in iron oxides particles) was detected by Perls staining procedure [7].

3. Results and discussion

Two types of industrially important hydrolytic enzymes, namely protease (crystalline trypsin) and lipase (Lipolase) have been selected as model enzymes for the preparation of magnetically modified powdered enzymes. This is an alternative procedure in comparison to a classic process of immobilization of enzymes onto a magnetic solid carrier. During common immobilization procedures, enzyme is solubilized and then it is bound to the solid activated support. In our experiments, enzyme powders were firstly suspended in various environments not allowing their solubilization and then cross-linked. This insoluble cross-linked form of enzyme powders can be magnetically modified. Magnetization procedure was performed in a simple and gentle way with the respect to sensitive biomaterial, in this case enzyme.

In the first step, protease trypsin was suspended in various types of media. Saturated buffered aqueous solution of ammonium sulfate or highly concentrated buffered solution of water-soluble polymer polyethylene glycol, commonly used in biochemistry for precipitation of proteins, have been chosen for suspending of enzyme powder. In addition, selected organic solvents, e.g., ethanol, methanol and 2-propanol, were used in pre-cooled form. In the second step, suspended enzyme powder has to be transformed into the insoluble form, so cross-linking using glutaraldehyde has been performed. After this procedure, cross-linked powdered enzymes can be washed with water solutions (buffers) and further magnetically modified.

As mentioned above, it was necessary to choose an appropriate magnetization method when working with enzymes. Many previously described procedures are not suitable for modification of sensitive biomaterials because of extreme values of pH or high temperatures used during the magnetization process, for example treatment of non-magnetic materials by perchloric acid stabilized magnetic fluid [8], microwave irradiation of the magnetized materials in the presence of ferrous sulfate at high pH [9], mechanochemical synthesis of magnetic composites [10] or the alkaline precipitation of ferrous and ferric salts in the presence of the treated material followed by heating [11]. The procedure based on the direct treatment of non-magnetic materials by microwave-synthesized magnetic iron oxides nano- and microparticles uses