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Preparation and characterization of magnetic levan particles as matrix for trypsin immobilization

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ABSTRACT

Magnetic levan was synthesized by co-precipitating D-fructofuranosyl homopolysaccharide with a solution containing Fe^{2+} and Fe^{3+} in alkaline conditions at 100 °C. The magnetic levan particles were characterized by scanning electron microscopy (SEM), magnetization measurements, X-ray diffractometry (XRD) and infrared spectroscopy (IR). Afterwards, magnetic levan particles were functionalized by $NaIO_4$ oxidation and used as matrices for trypsin covalent immobilization. Magnetite and magnetic levan particles were both heterogeneous in shape and levan–magnetite presented bigger sizes compared to magnetite according to SEM images. Magnetic levan particles exhibited a magnetization 10 times lower as compared to magnetite ones, probably, due to the coating layer. XRD diffractogram showed that magnetite is the dominant phase in the magnetic levan. Infrared spectroscopy showed characteristics absorption bands of levan and magnetite (O–H, C–O–C and Fe–O bonds). The immobilized trypsin derivative was reused 10 times and lost 16% of its initial specific activity only. Therefore, these magnetic levan particles can be proposed as an alternative matrices for enzyme immobilization.

1. Introduction

Magnetic carrier technology has been applied as bioaffinity adsorbents for selective recovery from liquors containing other suspended solids; wastewater treatment via electrostatic adsorption; protein (enzyme) immobilization and in the preparation of immunological assays [1]. The magnetic properties of these particles play an important role in the effectiveness of the application and affect the behavior of the particles and ferrofluids in applied fields [2].

In biomedicine, the polymer shell ensures stability of the magnetic particles in physiological media providing non-toxicity by avoiding leakage of iron and enabling chemical modification for attachment of biologically active compounds [3]. The shells are biocompatible such as dextran, xylan, chitosan, PEG, etc. and possesses active groups, which can be conjugated to biomolecules such as proteins [4]. Magnetic particles are an attractive and efficient support for bioconversions using immobilized enzymes due to the following advantages: simplicity of the matrix synthesis and immobilization protocol and easy removal from the reaction medium by applying a magnetic field [5,6].

Magnetic carriers can be manufactured using inorganic materials or polymers. However, those based on polymers offers a variety of surface functional groups than can be tailored to specific applications [7]. The utilization of polysaccharides presents advantages due to a large number of derivable groups, wide range of molecular weights, low toxicity, biodegradability and high stability [8]. Most magnetic materials such as maghemite and magnetite are employed as the core of the supports [9]. The magnetite particles (Fe_3O_4) are preferred because of their greater saturation magnetization and no toxicity [10].

The application for biomolecules immobilization mainly based on the solid-phase magnetic feature which is able to achieve a rapidly easy separation and recovery from the reaction medium in an external magnetic field [11]. There are basically two main ways to immobilize protein on supports: physical adsorption and covalent immobilization. Comparatively, covalent immobilization presents the benefits of eliminating or reducing protein leakage (a stronger linkage is formed) and usually increases protein tertiary structure stability [12–16]. Immobilization of biomolecules onto

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insoluble supports is an important tool for the fabrication of a diverse range of functional materials or devices [17]. It provides many distinct advantages including enhanced stability, easy separation from reaction mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth [18].

In our lab magnetic levan composite was previously used to purify using affinity binding lectins from *Canavalia ensiformis* (Con A) and *Cratylia mollis* seeds (Cramoll 1 and Cramoll 1, 4) [19]. Cramoll 1 was purified using this procedure in two steps instead of a preceding three-step protocol employing ammonium sulfate fractionation, affinity chromatography on Sephadex G-75, and ion exchange chromatography through a CM-cellulose column [19]. Here, these magnetic particles were characterized regarding structural, microstructural and magnetic properties and further used as matrix to immobilize trypsin.

2. Experimental

2.1. Materials

Levan from *Zymomonas mobilis* strain ZAG–12 (Molecular weight average equal to 300 kDa) was precipitated by addition of ethanol to 70% (v/v) at low temperature according to Calazans et al. [20]. Ferric chloride hexahydrate and ferrous chloride tetrahydrate were purchased from Merck (Germany) whereas ammonium hydroxide was from Vetec Chemical (Brazil). All other reagents were of analytical grade.

2.2. Preparation of magnetic particles of levan

An aqueous mixture with 5 mL of 1.1 M $FeCl_3.6\,H_2O$ and 5 mL of 0.6 M $FeCl_2.4\,H_2O$ were added to 50 mL of 2.0% w/v levan (prepared in distilled water). Ammonium hydroxide was then added to achieve a pH of 11. The mixture was then heated up to 85 ± 3 °C and kept for 30 min with vigorous stirring. The magnetic levan particles synthesized were thoroughly washed with distilled water to a neutral pH. The material was dried and kept at 25 °C. This procedure was according to Carneiro Leão et al. [21], except by incubation time (30 min), temperature (85 °C) and final pH of the mixture (11).

2.3. Matrix characterization

The particle size and morphology of the samples were established by scanning electronic microscopy (SEM), utilizing a JEOL Model JSN—5900 electron microscope. Magnetization measurements were obtained at 293 K and 313 K in magnetic fields from 0 to 5.0 T using a SQUID magnetometer (Quantum Design Model MPMS-5). The structural properties of the magnetic particles were characterized by X-ray powder diffraction, which was carried out in an X-ray diffractometer Siemens D5000. Representative powder samples were analyzed in the range $10^\circ < 2\theta < 90^\circ$ using CuKα radiation ($\lambda = 1.5406$ Å), in steps of 0.02°, and with a counting time of 1.0 s per step. Fourier transform infrared (FTIR) spectrum from the KBr pellet method in the range of 4000–400 cm^{-1} with particles-coated was recorded in a BRUKER instrument model IFS 66. Magnetite, magnetic levan and levan (2 mg each) were mixed with KBr (200 mg) and disks obtained under pressure at 490 atm. Scan (100 scans) was recorded with a 4 cm^{-1} resolution.

2.4. Magnetic levan particles Functionalization, trypsin immobilization and reuse of the enzymatic derivative

Magnetic levan particles (10 mg) and sodium metaperiodate (10 mg) were mixed in 3 ml distilled water [22]. This mixture was constantly stirred in the dark at 25° C for 7 h. The magnetic particles containing the partially oxidized levan were collected by a magnetic field of 0.6 T and washed with 0.1 M Tris-HCl buffer pH 8.0 (1.0 mL, 10 times). After this procedure, it was incubated with trypsin (0.2 mg/mL, 1.0 mL) for 16 h at 4 °C under mild stirring. The enzymatic derivative was collected by magnetic field of 0.6 T and supernatant and washings were collected for protein determination [23] using trypsin as standard. Sodium borohydride (0.03 M, 1 mL) was added to trypsin–magnetic levan particles and slightly mixed for 2 h at 4 °C. Then they were washed 10 times with the aforementioned buffer and kept in the buffer at 4 °C until use. The retained protein was estimated by the difference between the offered protein (200 μg) and that found in the supernatant and washings. The reuse was carried out by assaying of the same trypsin–magnetic levan particles preparation with BAPNA for 10 times intercalating each successive use by washing the immobilized enzymatic derivative 10 times with 0.1 M Tris-HCl buffer, pH 8. The activity of trypsin for the free and immobilized enzyme was measured as described by Amaral et al. [24]. The activity (unit) was defined as μmol BAPNA hydrolyzed during 1 min using an absorption coefficient of ε_{405} 9100 $M^{-1}\cdot cm^{-1}$. All of the experiments were carried out in duplicate.

3. Results and discussion

3.1. Preparation, size and morphology of the magnetic particles

The co-precipitation process to obtain magnetic levan was carried out in an alkaline aqueous medium and the final product obtained from this process yielded a dense, black and magnetic powder. These particles exhibited a magnetization in the presence of a magnetic field 0.6 T. SEM images shown in Fig. 1 reveal

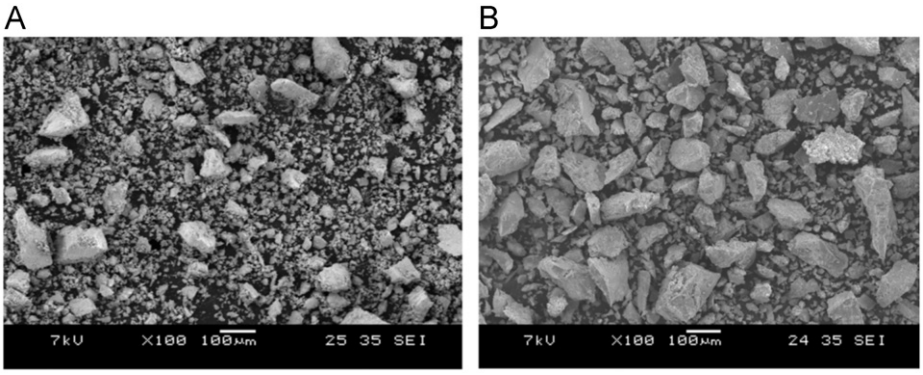

Fig. 1. Scanning electron microscopy images of magnetite (A) and magnetic levan (B) particles.