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Improvement of thermal stability of β-galactosidase from *Bacillus circulans* by multipoint covalent immobilization in hierarchical macro-mesoporous silica

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ABSTRACT

β-Galactosidase from *Bacillus circulans* was immobilized on hierarchical macro-mesoporous silica by multipoint covalent attachment by formation of Schiff bases between enzyme and support. The enzyme was effectively immobilized with high yields (around 60–80%) and expressed activity (around 50–80%) depending on the concentration of aldehyde groups in the carrier. Immobilization of β-galactosidase in chemically modified silica conferred excellent thermal stability to the biocatalyst and enzyme leaching was completely avoided. The effect of the concentration of functional groups in the silica surface was studied on the activity and thermal stability of the biocatalyst. The best hybrid catalyst was 370-fold more stable than the soluble enzyme at pH 6 and 55 °C.

1. Introduction

β-Galactosidase (E.C. 3.2.1.23, β-gal), also called lactase, catalyzes the hydrolysis of the β-galactosidic linkages in lactose, producing glucose and galactose. The physicochemical properties and intrinsic pH and temperature stability of β-gal varies according to the producing microorganism. The β-gal from *Bacillus circulans* (*B. circulans*) is a mixture of three isoenzymes. Isoenzyme I, which predominates in the preparation used in this work, has a molecular weight of 212 kDa, optimum pH of 6.0 and optimum temperature of 45 °C [1].

β-Gal has been used for lactose hydrolysis in milk, producing dairies for people with lactose intolerance and also for lactose conversion in whey reclamation, so reducing the pollution caused by whey disposal into water courses. Nowadays, most studies with this enzyme are focused on its transglycosylation activity for galacto-oligosaccharides (GOS) production from lactose. GOS are an important class of food-grade oligosaccharides, which are considered prebiotics [2]. The enzyme catalyzed reaction of GOS synthesis from lactose is under kinetic control, therefore it can be modulated by changes in lactose concentration [3], pH [4] and temperature of the reaction medium [5].

Recent researches in GOS synthesis with β-gal have shown that temperature increase generates a significant increase in GOS conversion [5,6], thereby it is necessary to obtain new active heterogeneous biocatalysts exhibiting high thermal stability and able to be reused. These objectives can be achieved by enzyme immobilization on solid supports, which also allows the development of continuous and sequential batch processes [7]. β-gal from *B. circulans* has been immobilized by physical adsorption on Duolite ES-762 [8] and by covalent attachment to acrylic supports [9], polymeric membranes [10], Eupergit C [11,12] and agarose modified with cyanogen bromide groups [13]. The main disadvantage of these methodologies is related with the low mechanical resistance shown by the organic supports, especially under high pressure, at which they lose their structural characteristics affecting the enzyme catalytic efficiency. Another drawback is that bonding between enzyme and support is frequently done by one-point covalent attachment or by adsorption, which does not favor enzyme thermal stability.

The inorganic materials with controlled porous structures, such as porous silica, offer some special features for their use as enzyme supports due to their high surface area, thermal and mechanical stability, non toxicity and high resistance against microbial attack and organic solvents. Their pore size can be also adjusted for the immobilization of a variety of biomolecules, via hydrogen bonding and electrostatic interactions through their surface silanol groups, or covalent attachment when the silica surface is modified with functional groups. The last strategy is desirable because it improves the biocatalyst thermal stability and avoids enzyme leaching [14].

Supports activated with glyoxyl groups have demonstrated to be quite suitable for immobilizing and stabilizing proteins because they are highly reactive with non-ionized amino groups; they present very low steric hindrances in their reaction with the proteins and are quite stable even at alkaline pHs allowing strong enzyme–support interactions, which produce extra stiffening to the enzyme and, therefore, high thermal stabilization [15]. The organic grafting process for obtaining chemically modified silica is

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carried out by using alkoxysilane reactants ($(R'O)_3SiR$): the target organo-functional groups (R) is introduced to the silica surface via condensation of silanol groups (≡Si—OH) with the alkoxy groups (—OR') of the coupling agent. Although the grafting of epoxide groups onto the silica surface has been reported [16], their conversion to glyoxyl groups for enzyme immobilization has not been widely explored. This conversion is made through ring epoxide opening and diol oxidation [17].

Supports activated with glyoxyl groups have demonstrated to be very suitable to immobilize and stabilize proteins because they are highly reactive with non-ionized amine groups; they have very low steric hindrances in their reaction with the proteins and they are quite stable even at alkaline pHs allowing long enzyme–support interactions, which produce extra stiffening to the enzyme and therefore, high thermal stabilization [15]. The organic grafting process for obtaining chemically modified silica is carried out by using alkoxysilane reactants ($(R'O)_3SiR$): the target organo-functional groups (R) is introduced to the silica surface via condensation of silanol groups (≡Si—OH) with the alkoxy groups (—OR') of the coupling agent. Although the grafting of epoxide groups onto the silica surface has been reported [16], their conversion to glyoxyl groups for enzyme immobilization has not been widely explored. This conversion is made through ring epoxide opening and diol oxidation [17].

The objective of this study was to improve the thermal stability of the β-gal from *B. circulans*, by multipoint covalent immobilization in hierarchical macro-mesoporous silica modified with glyoxyl groups, which is a low cost support that exhibits high mechanical stability. The thermal stability of this immobilized β-gal was highly dependent on the concentration of the glyoxyl groups on the silica support. Temperature and pH for maximum activity were also evaluated and compared with the values obtained for the same β-gal immobilized in commercial agarose support, with grafted glyoxyl groups. The hybrid β-gal/silica biocatalysts prepared in this study are suitable for GOS synthesis, especially at high temperatures.

2. Experimental

2.1. Reactants

The following analytical grade reactants were used without further modification: sodium silicate (25–29% SiO_2 and 7.5–9.5% Na_2O), ethylacetate (EtAc), sulfuric acid (98%), all purchased from Merck. Cetyltrimethylammonium bromide (CTAB, Sigma), 3-glycidyloxypropyltrimethoxysilane (GPTMS 99.7%, Aldrich), sodium periodate (Fluka), lactose (Sigma), sodium borohydride (Sigma), glycerin (Sigma) all purchased from MO, USA. The β-galactosidase (β-gal) from *B. circulans* (BIOLACTASA NTL X2, 1250 IU/mL) is purchased to Biocom (Barcelona, Spain). Sepharose CL-6B bead (6% agarose, cross-linked with epichlorohydrin, GE Healthcare, Spain), Glycidol (Sigma, MO, USA). The glucose kit (from Byosystem, Spain) was used as color-developer reagent (glucose oxidase-peroxidase) for measuring the catalytic activity.

2.2. Synthesis, chemical modification and characterization of siliceous supports

Synthesis of silica was carried out from a reaction mixture with the following molar composition: SiO_2: 0.30 Na_2O: 0.24 CTAB: 7.2 EtAc: 193 H_2O. This mixture was heated at 80 °C by 48 h, under quiescent conditions. The solid obtained was calcined at 540 °C (heating rate: 1.5 °C min^{-1}) for 3 h. The details of synthesis were reported elsewhere [18]. This material was identified with the code "S".

For the modification with glyoxyl groups, 1.0 g silica (activated under vacuum at 200 °C) was contacted with 30 mL of 10% GPTMS aqueous solution, at pH 8.5 and 94 °C, during 6 h, under gentle stirring. The hydrolysis of epoxy groups was done with 0.1 M H_2SO_4 for 2 h at 85 °C according to Porsch [19]. After filtration, washing with water/acetone and drying, the oxidation proceeded by 2 h at room temperature, with 0.003–0.1 M $NaIO_4$ solution. These materials were identified with the code "S_x", where *x* indicates the resultant glyoxyl concentration (mmol) in the material.

The porous characteristics for calcined and chemically modified samples (specific surface area, mesoporous volume, diameter size) were calculated from the nitrogen adsorption/desorption isotherms measured in the ASAP2010 instrument (Micromeritics) by applying known models [20]. The measurements were carried out in duplicate and the standard deviation was calculated. The morphology of silica was determined by Scanning Electron Microscopy, SEM (JEOL JSM-6490), before and after to be submitted to orbital agitation in aqueous solution, at pH 6.0 and room temperature. The macroporosity was estimated from these SEM micrographs.

The quantification of glyoxyl groups on the chemically modified silica was carried out by backtitration with $NaHCO_3$/KI, measuring the absorbance difference at 405 nm of the supernatant before and after epoxy oxidation process [21] (Lambda35 UV/Vis spectrophotometer). The silica surface modification was confirmed qualitatively from ^{29}Si CP/MAS NMR spectra, recorded at 79.49 MHz, using a 4 mm zirconia rotor spinning at 7 kHz (sample size ~50 mg), 120 s of recycle delay, 5 ms of contact time and ~700 scans.

2.3. Enzyme assay

The specific activity of the soluble and immobilized β-gal was measured by using visible spectroscopy (Lambda35 UV/Vis spectrophotometer), under controlled temperature and stirring. The soluble or immobilized enzyme was suspended in a potassium phosphate buffer solution (100 mM, pH 7). This enzymatic solution (20 μL) was added to a cell containing lactose (1 mL, 80 mM) in potassium phosphate buffer at pH 7.0 and a commercial glucose kit (1 mL). The increase of the absorbance at 500 nm, corresponding to the release of quinoneimine, was recorded [22]. The light dispersion produced by the presence of silica particles was corrected by subtracting the radiation-intensity loss measured for a silica dispersion. The experiments were carried out in triplicate and the standard deviation was calculated. The specific activity is expressed in international units of activity (IU). One IU is defined as the amount of enzyme that hydrolyzes 1 μmol of lactose per minute under the conditions described above.

2.4. Immobilization process in agarose (A) or silica (S_x) supports

The Sepharose CL-6B was activated with glycidol, followed by oxidation with $NaIO_4$, as previously described by Guisán [21] and Mateo et al. [17], before being used as enzyme support.

The immobilization was carried out by suspending 1.0 g of support in 5 mL of 100 mM potassium bicarbonate solution at pH 10.05, which contained β-gal solution (less than 100 IU/mL of support in order to avoid the diffusion problems that could disturb the apparent enzymatic activity and stability). The suspension was incubated at 4 °C during several hours (1–24 h) with 40% glycerol, under mild stirring. Finally, the derivative was reduced for 30 min at the immobilization temperature with 4 mg of sodium borohydride as previously described in [17]. After this period of time, the hybrid catalyst was washed with water. In a parallel experiment, the soluble enzyme was left under the same conditions in order to check the influence of the support (enzyme blank). The