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Immobilization of trypsin on chitosan gels: Use of different activation protocols and comparison with other supports

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Abstract

Trypsin was immobilized on chitosan gels coagulated with 0.1 or 1 M NaOH and activated with glutaraldehyde or glycidol. The derivatives were characterized by their recovered activity, thermal (40, 55 and 70 °C) and alkaline (pH 11) stabilities, amount of enzyme immobilized on gels for several enzyme loads (8–14 $mg_{protein}/g_{Gel}$) and compared to agarose derivatives. Enzyme loads higher than 14 $mg_{protein}/g_{Gel}$ can be immobilized on glutaraldehyde derivatives, which showed 100% immobilization yield and, for loads up to 8 $mg_{protein}/g_{Gel}$, 100% recovered activity. Activation with glycidol led to lower immobilization yields than the ones obtained with glutaraldehyde, 61% for agarose-glyoxyl (AgGly) with low grade of activation and 16% for the chitosan-glyoxyl (ChGly), but allowed obtaining the most stable derivative (ChGly), that was 660-fold more stable than the soluble enzyme at 55 and 70 °C—approximately threefold more stable than AgGly. The ChGly derivative presented also the highest stability during incubation at pH 11. Analyses of lysine residue contents in soluble and immobilized trypsin indicated formation of multipoint bonds between enzyme and support, for glyoxyl derivatives.

Keywords: Trypsin; Chitosan; Enzyme immobilization; Multipoint attachment

1. Introduction

Although enzymes are potent and specific biocatalysts, they are soluble in aqueous medium and operationally fragile. Therefore, immobilization and stabilization of enzymes in industrial-scale applications are important, in order to reduce the biocatalyst cost. The covalent multipoint immobilization of the enzyme on insoluble supports, besides allowing an easy recovery and re-utilization of the enzyme, can also improve its stability. Although there are several methods to immobilize enzymes, their immobilization/stabilization by multipoint covalent attachment to pre-existing supports presents some practical advantages when compared with other immobilization methods: the immobilization process can be easily controlled and different supports can be tested without difficulty. Furthermore, the enzyme molecules become more rigid, and thus more resistant to conformational changes induced by heat and organic solvents than the corresponding unmodified ones [1]. A great increase in the stability of several enzymes after their multipoint immobilization on agarose activated with an epoxyde reactant (glycidol), followed by oxidation with sodium periodate generating glyoxyl groups in the support, has been reported for different enzymes. Penicillin G acylase became 10,000-fold more stable than soluble enzyme [1]; trypsin, 5000-fold, at 50 °C using agarose 6-BCL [2]; alcalase®, 500-fold [3] and carboxypeptidase A, 260-fold [4] to 1000-fold [5]. The stability factor for each enzyme depends on the enzyme structure, on the number of aldehyde groups generated in the support and on the immobilization conditions.

Trypsin is an important proteolytic enzyme, already used in large-scale processes by the detergent and dairy industries [6]. The industrial use of this enzyme in the immobilized form is a trade-off between the reduction of the enzyme cost (since the catalyst can be reutilized) and the immobilization cost. The latter one includes the cost of the support. Besides agarose, trypsin has been already immobilized on silica, porous glass and celite™

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[7–9], but there are few reports about its immobilization on chitosan in the literature [10,11].

Chitosan is a linear polysaccharide of high molecular mass composed of repeating units of β-(1-4)-2-amino-2-deoxy-D-glucopyranose (D-glucosamine). It is obtained via deacetylation of chitin (extracted from crab and prawn shells, for instance) using concentrated alkali. Thus, chitosan is usually referred as the deacetylated derivative of the natural polymer chitin. Chitosan is an important by-product of the fishing industry and presents advantages in relation to other materials, mainly due to its great versatility, relative low cost and broad availability [12]. Although the immobilization of trypsin on agarose-glyoxyl had led to very good stability factors, agarose-glyoxyl is an expensive support. The presence of reactive amine groups in chitosan may reduce the number of steps for support activation and this fact allied to the low cost of this matrix may decrease the overall trypsin immobilization costs. Therefore, if a high stability factor could be achieved through the immobilization of trypsin on chitosan, the trypsin–chitosan derivative could be a competitive choice for large-scale process.

In this work different trypsin–chitosan derivatives were produced, changing the conditions for coagulation and activation of the support and for enzyme immobilization, aiming at obtaining very active and stable chitosan derivatives. The trypsin–chitosan derivatives were characterized and compared with trypsin–agarose.

2. Materials and methods

2.1. Materials

Chitosan (powder) was purchased from Polymar Ind. Com. Exp. Ltda (Ceará, Brazil). Sepharose 6B-CL was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Trypsin (EC 3.4.21.4), from bovine pancreas, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark), with 0.2 mg protein/mg product and 1.51 UBA_pNA/mg of protein. Benzoyl-L-arginine-*p*-nitroanilide (BA_pNA) was purchased from Bachem AG (Budendorf, Switzerland). Glutaraldehyde and sodium borohydride were purchased from Quimibrás Indústrias Químicas S.A. (Brazil) and Nuclear (Brazil), respectively. All other reagents were of analytical grade from several trademarks.

2.2. Methods

2.2.1. Preparation of chitosan gel

A 2.5% (mass basis) solution of chitosan was prepared dissolving chitosan powder into acetic acid 5% (mass basis). This solution was filtered to remove impurities (small particles, residues of the chitin deacetylation process). After that, the filtrate was dispersed through a nozzle into a coagulation solution (0.1 or 1 M NaOH). The system was kept under stirring until complete coagulation of the particles. The resulting gel was washed with distilled water until pH 7 was reached and classified using stainless steel sieves. This methodology followed the protocol described by Arruda and Santana [13].

2.2.2. Activation of chitosan gel

(a) Chitosan gel coagulated with 1 M NaOH was activated under two different conditions: (i) glutaraldehyde 5% (mass basis) at 25 °C and pH 7.00 (50 mM sodium phosphate buffer) for 1 h and (ii) glutaraldehyde 5% (mass basis) at 25 °C and pH 10.00 (100 mM sodium bicarbonate buffer) for 1 h, both under mild stirring. Particles previously separated using stainless steel sieves with diameter in the range 177–300 μm were used.

(b) Chitosan gel coagulated with 0.1 M NaOH, with particle diameter in the range 60–177 μm, was also activated under two different conditions: (i) glutaraldehyde 5% (mass basis) at 25 °C and pH 7.00 (50 mM sodium phosphate buffer) for 1 h and (ii) glycidol (1 mL/g_{Gel}) was added dropwise, in an ice bath, to the suspension containing chitosan gel, water (286 $\mu L/g_{Gel}$), cold NaOH 1,7N solution (476 $\mu L/g_{Gel}$, previously prepared and containing sodium borohydride solution, 28.5 mg/mL). The suspension was kept under mild stirring, at room temperature, for 12–15 h. After the etherification step, the glyceril support was washed with distilled water, dried under vacuum and re-suspended in water, $V_{support}/V_{suspension} = 1/10$. For each mL of support, it was added to the suspension 214 μg of sodium periodate/μequiv. of aldehyde groups that were intended to be produced in the support (for agarose 6% BCL, the maximum amount is 70 $\mu mol/mL_{Gel}$). After 2 h, the glyoxyl support was washed with distilled water, dried under vacuum and stored at 4 °C, following Guisán [1].

2.2.3. Preparation of glyoxyl-agarose and agarose-amine-glutaraldehyde gels

Agarose was activated with glycidol following the protocol to activate chitosan, except for using 343 $\mu L/g_{Gel}$. Glyoxyl-agarose gel poorly activated was prepared by oxidating agarose with sodium periodate, without the previous step of etherification with glycidol, and offering the amount of sodium periodate needed to produce 18 μequiv. of aldehyde groups/mL of gel. Agarose-amine-glutaraldehyde gel was prepared according to Fernández-Lafuente et al. [14]. Agarose, activated with the maximum amount of aldehyde groups, was reacted with ethylenediamine and then activated with glutaraldehyde. For each g of gel, it was added 5.72 mL of ethylenediamine 2 M, pH 10 (cold, previously prepared). After mild agitation for 2 h, at room temperature, 57.14 mg of sodium borohydride were added and the suspension was kept for two more hours under stirring. The amine-support was washed with 100 M sodium acetate, pH 4 (30 mL/g of support), 0.1 M sodium borate, pH 9.00 (30 mL/g of support) and distilled water. For each g of the aminated support, 1.12 mL of 200 mM phosphate buffer, pH 7.0 and 1.68 mL of glutaraldehyde 25% were added. After adjusting the pH at 7.0, the suspension (with the flask protected with aluminum paper) was kept under mild stirring, for 12–14 h. The glutaraldehyde support was washed with distilled water and used for the enzyme immobilization.