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Evaluation of kinetic parameters of immobilized penicillin G acylase subject to an inactivation and reactivation process

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ABSTRACT

Biocatalysts kinetic parameters are usually considered constant during reactor operation, which may not be the case if significant enzyme inactivation occurs and reactivation strategies are used to prolong biocatalyst lifespan. The effect of the partial inactivation and reactivation of penicillin G acylase from *Escherichia coli* immobilized in glyoxyl–agarose on the biocatalyst kinetic parameters was investigated. Apparent and intrinsic kinetic parameters were calculated in three biocatalyst states: native, partially inactivated and reactivated. Kinetic parameters were assessed from the initial rate of penicillin G hydrolysis at different penicillin G and phenylacetic acid (PAA) concentrations, the latter being a competitive inhibitor of the enzyme. The biocatalyst was inactivated down to $25 \pm 2\%$ of residual activity using 70% (v/v) of dioxane and afterwards it was reactivated. Biocatalyst kinetic behavior, reflected in the Michaelis constant (K_M) and inhibition constant PAA (K_I), could not be restored by reactivation and conformational changes induced by the organic solvent were reflected in different intrinsic kinetic parameters of the native and reactivated biocatalyst. The significant variation of biocatalyst kinetic parameters due to its inactivation and reactivation should be considered when the enzyme reactor performance is modeled, aspect which has not been given sufficient attention up to now.

1. Introduction

Enzyme inactivation during catalysis is one of the bottlenecks that avoid a more extended use of enzymes as process catalysts. Many efforts have been devoted to increase enzyme stability [1–4], among which enzyme immobilization outstands because not only contribute to stabilize the enzyme structure, but also allows its recovery after use and eventually its reactivation [5,6].

Information about the effect of inactivation and reactivation on enzyme kinetic parameters is lacking. There are only a couple of works where differences have been reported in the Michaelis constant (K_M) of the native and partially inactivated enzyme [7,8] and most information refers to the variation of enzyme activity along the process of inactivation and quite few during reactivation.

The purpose of this work is the evaluation of the effect of enzyme inactivation and reactivation on the values of the kinetic parameters of immobilized penicillin acylase, a very important industrial enzyme that catalyzes both the hydrolysis and formation of β-lactam structures [4,9–13]. This information is a valuable tool to improve the reactor design, by considering how the kinetic parameters of the enzyme vary during reactor operation as a consequence of biocatalyst inactivation.

2. Materials and methods

2.1. Materials

Penicillin G acylase (PGA) from *Escherichia coli*, with 362 ± 20 U_H/mL and 35 ± 2 mg of protein/mL, was kindly provided by Antibióticos S.A. (León, Spain). The enzyme was centrifuged and dialyzed prior to use and remained fully active at 5 °C during the whole working period. Phenyl acetic acid (PAA) was from Sigma (St Louis, MO, USA) and dioxane was from Merck (Darmstadt, Germany). Penicillin G potassium salt (PenG) was donated by Natsus S.A. (Lima, Perú). Cross-linked 6% agarose spherical beads (Sepharose 6B-CL) was a product from GE Healthcare (Uppsala, Sweden). All other reagents were of analytical grade.

2.2. Preparation of biocatalyst

Glyoxyl agarose (GA) was prepared as previously reported [14]. Immobilization of PGA in GA by multipoint covalent attachment (GA–PGA) was carried out as previously described [15,16], contacting 1 g of GA to 10 mL of an enzymatic solution (3.5 $mg_{protein}$/mL

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and 51.2 IU/mL) prepared in a mixture of 100 mM PAA in 25% (v/v) of glycerol in 100 mM sodium bicarbonate buffer pH 10 and gently stirring the mixture for 3 h at 25 °C. To reduce the Schiff's base formed between the enzyme and the support to an amine bond, solid sodium borohydride was added to a final concentration of 1 mg/mL. After 30 min, the preparation was washed with an excess of distilled water. During immobilization, samples of supernatant, suspension and enzyme solution incubated in the presence of the inert support were taken at different times, and the activity and/or the protein concentration were assayed. Immobilization yield (IY) was defined as the ratio of contacted and immobilized enzyme activity.

2.3. Determination of initial reaction rates

Initial reaction rates of penicillin G hydrolysis were determined at different substrate concentrations in 100 mM sodium phosphate buffer pH 7.8, 30 °C, and 800 rpm, using a pH-stat (Mettler Toledo, DL50) to titrate the H^+ produced by the hydrolysis of penicillin G as it is converted into PAA. 50 mM NaOH was employed as titrant solution. Initial rate measurements were carried out in triplicate, with an error lower than 5% in all cases. One international unit of hydrolytic activity of penicillin G acylase (IU) was defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G per minute from 10 mM penicillin G solution under the above conditions.

2.4. Determination of kinetic parameters

Kinetic parameters were conventionally determined from initial rate data and non-linear regression to the corresponding rate equations. The non-linear regression was carried out using the Solver function of Microsoft Excel. Penicillin G hydrolysis by PGA is subjected to competitive inhibition by PAA and non-competitive inhibition by 6-aminopenicillanic acid (6-APA) [17]. However, the latter is much less significant than the former; therefore, has not been considered in this study. Apparent Michaelis constants (K_M^{APP}) of all PGA biocatalysts were determined from initial reaction rate data, varying Penicillin G concentration. Experiences were done at 30 °C in 100 mM sodium phosphate buffer pH 7.8. Apparent inhibition constants (K_I^{APP}) were determined from initial rate of Penicillin G hydrolysis, varying PAA concentration.

Since GA–PGA is subjected to internal diffusional restrictions (IDR) [18], it was necessary to determine its intrinsic kinetic parameters (K_M and K_I) to discriminate the effect of mass transfer limitations from the conformational changes caused by inactivation (and reactivation) which is the purpose of this study. To assess the intrinsic parameters of the immobilized enzyme, the biocatalyst particles were subjected to intense mechanical rupture at 5 °C in order to reduce particles size until IDR are likely to be irrelevant and the kinetic parameters of such biocatalyst may be considered as intrinsic. The low temperature of the experiments precluded from biocatalyst inactivation. Values of kinetic parameters at different degrees of rupture were determined by subjecting the biocatalyst to mechanical attrition by high speed stirring (>1000 rpm) with magnetic bar up to 40 h.

2.5. Inactivation of GA–PGA

GA–PGA was incubated at 5 °C in 70% (v/v) dioxane in 10 mM phosphate buffer pH 7.4. The evolution of the residual hydrolytic activity of the catalyst was determined by periodical sampling until the desired level of inactivation was reached. A rather strong reaction condition was chosen to make inactivation more dramatic, allowing a better appraisal of the changes produced during inactivation and the subsequent reactivation stage.

2.6. Reactivation of partially inactivated biocatalysts

Two strategies were applied for the reactivation of biocatalysts partially inactivated by organic solvent, namely, direct incubation in aqueous reactivation medium and unfolding–refolding, in which enzyme unfolding is promoted by a chaotropic agent prior to incubation in reactivation medium [19–21].

GA–PGA was inactivated down to approximately 25% residual activity and then the inactivation medium was removed and the biocatalyst was recovered by filtration in a Gooch crucible fritted disc (Pyrex). In the strategy of direct incubation in aqueous reactivation media, the recovered biocatalyst was re-incubated in 0.1 M sodium phosphate buffer pH 7.4 at 25 °C to promote reactivation. Volume of re-incubation was the same as the one in the inactivation stage. Periodically, the recovered hydrolytic activity was determined as described above. In the unfolding–refolding strategy, the biocatalyst was incubated in 8 M guanidine for 1 h at 25 °C, then the unfolding agent was removed by filtering it out and the biocatalyst was incubated in phosphate buffer as above. Periodically, its hydrolytic activity was determined as described before.

3. Results and discussion

Expressed activity of GA–PGA was 372 IU/$g_{biocatalyst}$ with an IY of 73%, in agreement with the rage of values reported by Valencia et al. [18]. The immobilized biocatalyst was subject to an inactivation and reactivation process, determining its apparent and intrinsic kinetic parameters.

3.1. Inactivation of GA–PGA in the presence of dioxane and reactivation of partially inactivated biocatalysts

Time course of enzyme inactivation in presence of a high concentration of dioxane was determined until reaching a stage where the enzyme has lost 75% of its initial activity, as seen in Fig. 1A. The partially inactivated biocatalyst was then subjected to reactivation by unfolding–refolding and compared with results previously reported on direct incubation in aqueous medium, as seen in Fig. 1B [6].

The catalyst activity achieved after the reactivation process was 73% and 71% of the activity before inactivation for the direct incubation and unfolding–refolding strategies, respectively. Even though the levels of recovered activity were similar, kinetic parameters of the recovered biocatalysts were determined in both cases to evaluate possible differences between them. In the unfolding–refolding strategies, reactivation rate was very high, reaching the final reactivated state in a few minutes.

3.2. Evaluation of the impact of internal diffusional restrictions on GA–PGA

The use of a highly loaded catalyst is desirable for its further industrial application; however, under these conditions mass transfer limitations are unavoidable and must be assessed.

Apparent K_M of an immobilized enzyme (K_M^{APP}) is the value of K_M determined under the influence of mass transfer limitations and should increase according to the magnitude of IDR. Values of K_M^{APP} were then evaluated on progressively ruptured biocatalyst particles to examine the different impacts of IDR for the reaction of hydrolysis of penicillin G. Fig. 2 is an optical microscope photograph of the biocatalyst before and after 40 h of rupture. As may be seen, particles have been significantly reduced in size and