Process Biochemistry 44 (2009) 322–326

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Effect of the degree of cross-linking on the properties of different CLEAs of penicillin acylase

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ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 1 October 2008
Accepted 12 November 2008

Keywords:
CLEAs
Enzyme stabilization
Penicillin acylase
Organic cosolvents
Cephalexin
Saline microenvironment

ABSTRACT

Cross-linked enzyme aggregates (CLEAs) are novel type biocatalysts well suited to catalyze reactions of organic synthesis. Penicillin acylase is a versatile enzyme that can both hydrolyze and synthesize β-lactam antibiotics. CLEAs and CLEAs covered with polyionic polymers (polyethyleneimine and dextran sulfate at two different enzyme to polymer ratios) were prepared at varying cross-linking agent to enzyme ratio: 0.15 and 0.25. Results are presented on the effect of such variables on immobilization yield, specific activity, stability and performance of penicillin acylase CLEAs in the kinetically controlled synthesis of cephalexin. The cross-linking agent to enzyme ratio had no significant effect on the specific activity of the CLEAs, but affected immobilization yield, stability in ethylene glycol medium and conversion yield and productivity in the synthesis of cephalexin, being always higher at the lower cross-linking agent to enzyme ratio. Best results were obtained with CLEAs at 0.15 glutaraldehyde to enzyme protein ratio: specific activity of hydrolysis and synthesis was 708 and 325 UI/g_{CLEA} respectively, conversion yield was 87%, specific productivity was 5.4 mmol cephalexin/(g_{CLEA}·h) and 90% of the enzyme remained active after 170 h at operating conditions.

1. Introduction

The large-scale production of 6-aminopenicillanic acid, a key intermediate for the synthesis of semi-synthetic penicillins, using immobilized penicillin acylase (penicillin amidohydrolase; E.C. 3.5.1.11) is already a mature technology [1–3], having displaced almost completely the former chemical process [4,5]. However, penicillin acylase is a remarkably versatile enzyme that can conduct both hydrolytic and synthetic reactions over a wide range of compounds [6–10], including the synthesis of derived penicillins and cephalosporins from the corresponding β-lactam nuclei and suitable acyl donors [11–16].

Synthesis of both derived penicillins and cephalosporins have been thoroughly studied with immobilized penicillin acylase and several strategies have been envisaged to increase conversion yield, namely the use of organic cosolvents [17,18], precipitation driven synthesis [19], biphasic systems [20,21] and very high substrates concentrations [22–25]. Organic cosolvents improve yield without affecting productivity and among them, polyols, and particularly ethylene glycol has been proven as very effective [26,15].

Advances in the field of enzyme immobilization have been determinant for industrial success in the production of β-lactam nuclei and, more recently, for the synthesis of β-lactam antibiotics from those nuclei as well [5,27,28]. Cross-linked enzyme aggregates (CLEAs) are new generation enzyme biocatalysts [29] particularly promising for performing in organic synthesis [30–32]. These non-supported recoverable biocatalysts compare quite favorably with carrier-bound enzymes in terms of kinetic properties and costs [33]. They share the good properties of cross-linked enzyme crystals [34,35], being simpler and cheaper to produce and not requiring the protein purity inherent to crystallization. CLEAs have been proposed as suitable forms of biocatalyst for penicillin acylase [36,37] and have been preliminarily evaluated in the synthesis of cephalexin in organic medium at high substrate concentrations [38]. Combination of CLEAs with polymeric co-aggregates [39] and encapsulated CLEAs into hydrophilic gels [40,41] have also been proposed as suitable forms of biocatalysts for performing organic synthesis with penicillin acylase.

One key variable in the production of CLEAs is the cross-linking ratio, defined as the mass ratio between cross-linking agent (glutaraldehyde, in this case) and enzyme protein. The amount of glutaraldehyde used in the preparation of CLEAs has shown to produce different effects on the properties of the biocatalyst obtained. The optimum degree of cross-linking will very much depend on the biocatalyst application so that no general rule can be

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doi:10.1016/j.procbio.2008.11.010

adopted for optimization [42], but certainly high concentrations of cross-linking agent may produce biocatalyst inactivation [43]. The effect of cross-linking agent concentration has been studied in different systems. In the case of lipases rather high degrees of cross-linking have produced hyperactivation [31] and CLEAs of the same lipase may exhibit quite different properties according to the level of cross-linking. In the case of CLEAs of *B. cepacia*, three different cross-linking agent concentrations were studied, being the one with the higher level of cross-linking the more stable in aqueous medium, while the less cross-linked was the best for enantioselective synthesis and the non-cross-linked derivative the one with the higher rate of transesterification [42]. An optimum concentration of cross-linking agent has been reported for CLEAs of *B. cepacia* lipase coprecipitated with bovine serum albumin in terms of transesterification activity [44]. The level of cross-linking has also been reported to affect biocatalyst morphology [42–45] as shown for CLEAs of *Candida rugosa* lipase where particle size increased with the level of cross-linking [45]. In the case of CLEAs with polymeric co-aggregates [39], the amino groups of the polymer (i.e. polyethyleneimine) may react with glutaraldehyde so that enzyme–enzyme, polymer–polymer and enzyme–polymer linkages may be established. In this case, the effect of cross-linking agent concentration may well affect biocatalyst stability and activity by promoting a high number of bonds, being also the polymer concentration a variable to consider. Recent studies have demonstrated that a correlation exists between morphology and catalytic properties of CLEAs and the time elapsed between precipitation and cross-linking. The suggestion was made that the size of the aggregate might regulate the extent of covalent modification of Penicillin acylase (PA), thereby influencing the catalytic properties of CLEA. [46]

CLEAs and CLEAs covered with polyionic polymers were prepared and results are presented on the effect of two cross-linking ratios on biocatalysts stability and performance. Results are compared for both types of CLEAs in the kinetically controlled synthesis of cephalexin. Two enzyme to polymers ratios were studied in the case of CLEAs covered with polyionic polymers.

2. Materials and methods

2.1. Materials

Penicillin acylase from *Escherichia coli*, with 220 ± 20 IU_H/mL and 21 ± 2 mg/mL protein, was a product from Antibióticos S.A. (León, Spain), kindly provided by Dr. José Manuel Guisán (Instituto de Catálisis, CSIC, Madrid, Spain). The enzyme was centrifuged and dialysed prior to use and remained fully active at 5 °C during the whole working period. Polyethyleneimine (25,000 Da) was purchased from Aldrich (Milwaukee, USA); dextran sulfate (10,000 Da) and sodium borohydride were from Sigma (St. Louis, MO, USA); *tert*-butyl alcohol was from Merck (Darmstadt, Germany); glutaraldehyde solution was from Fluka; (R)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure); ethylene glycol and cephalexin hydrate were from Sigma Chemical Company Inc. (St. Louis, MO, USA); 7-amino 3-desacetoxicephalosporanic acid (7ADCA) was kindly provided by Antibióticos S.A. (León, Spain) and penicillin G potassium salt (PenGK) was donated by Natsus S.A. (Lima, Perú). All other reagents were of analytical grade.

2.2. Determination of enzyme hydrolytic activity

Enzyme activity was determined using a pHstat (Mettler Toledo, DL50) to titrate the H^+ produced by the hydrolysis of 10-mM PenGK in sodium phosphate buffer 0.1 M, pH 7.8 and 30 °C. 50 mM NaOH was employed as titrant solution. One international unit of hydrolytic activity (IU_H) of PA was defined as the amount of enzyme that hydrolyzes one μmol of PenGK per minute under the above conditions. Specific activity of hydrolysis is defined as the IU_H per unit mass of biocatalyst.

2.3. Determination of enzyme synthetic activity

Synthetic activity of CLEAs of penicillin acylase was determined from the initial slope of the reaction of synthesis of cephalexin. One international unit of PA activity of synthesis (IU_S) was defined as the amount of enzyme that produces 1 μmol of cephalexin per minute under the conditions of synthesis. Specific activity of synthesis is defined as the IU_S per unit mass of biocatalyst. Substrates and products of synthesis were identified and analyzed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV UV–vis detector and a CBM-101 Shimadzu HPLC/PC integrator. The column used was a μ-Bondapack C_{18} (300 mm × 3.9 mm) from Waters (Milford, MA, USA). Samples were eluted isocratically with a sonicated mixture of 70% (v/v) 20 mM phosphate buffer pH 7.0 and 30% (v/v) methanol at a flow rate of 1 mL/min, and analyzed in the UV detector at 214 nm. Elution times were 2.8, 3.5, 6.3 and 12.7 min for 7ADCA, phenylglycine (PG), cephalexin and phenylglycine methyl ester (PGME) respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions of enzyme that produces one μmol of cephalexin per minute under the conditions of synthesis.

2.4. Preparation of glutaraldehyde cross-linked aggregates of PA

Glutaraldehyde cross-linked aggregates of PA (CLEA–G) were prepared by adding 6 mL of *tert*-butyl alcohol under agitation to 4 mL of PA solution (pH 7.0) to precipitate the enzyme. After 30 min, 0.6 or 1 mL of glutaraldehyde solution (25%, v/v), corresponding to glutaraldehyde to enzyme protein mass ratio (G/E) of 0.15 or 0.25 respectively, were added to cross-link the enzyme precipitate, and the mixture was kept under stirring for 1 h. Glutaraldehyde tends to polymerize at high pH, but this does not occur at pH 7 at which the biocatalyst is prepared. Next step after cross-linking is reduction; therefore, the volume was duplicated by adding 100 mM sodium bicarbonate buffer at pH 10, and 1 mg/mL of sodium borohydride was added. After 15 min, another 1 mg/mL of sodium borohydride was added and allowed to react for an additional 15 min. Finally, the resulted CLEA was centrifuged at 12,000 rpm for 15 min and washed with 100 mM sodium phosphate buffer at pH 7.0; this procedure was repeated five fold. All the steps were performed in an ice bath at approximately 2 °C. Immobilization yield in terms of protein (Y_P) was determined as the ratio of protein retained in the CLEA (measured as the difference between contacted protein and protein remaining in the supernatant) to the protein contacted; immobilization yield in terms of enzyme activity (Y_E) was determined as the ratio of the PA activity expressed in the biocatalyst to the activity contacted.

2.5. Preparation of glutaraldehyde cross-linked aggregates of PA CLEA with polyionic polymers

CLEAs with saline hydrophilic microenvironment produced by the addition of polyionic polymers (CLEA–GPD) were prepared according to the same procedure as above, but before adding the precipitant (*tert*-butyl alcohol) to the PA solution, 0.63 mL of dextran sulfate solution (100 mg/mL) was added under stirring for 15 min and then 0.63 mL of polyethyleneimine solution (100 mg/mL) was added under stirring for 10 min, to prepare a CLEAs with a 1:1:1 enzyme protein to polyethyleneimine to dextran sulfate mass ratio (E:P:D). In the case of a 1:2:1 (E:P:D) CLEA, 1.26 mL of polyethyleneimine solution (100 mg/mL) were used instead. Dextran sulfate and polyethyleneimine solutions were adjusted to pH 7.0 prior to use. All operations were carried out in an ice bath at approximately 2 °C.

2.6. Stability of CLEAs

The different CLEAs were incubated at 14 °C in a mixture composed by 40% (v/v) ethylene glycol and 60% (v/v) 5 mM sodium phosphate buffer at pH 7.0. This cosolvent concentration was selected for being the best in terms of conversion yield [15]. Periodically, the residual hydrolytic PA activity was determined as described in Section 2.2. The experiments were carried out in triplicate and error was always below 5%.

In the case of thermal inactivation, CLEAs were incubated at 50 °C in 100 mM sodium phosphate buffer at pH 7.0 and the residual hydrolytic PA activity of each sample was determined.

Inactivation by cosolvent and thermal inactivation were modelled based on the deactivation theory proposed by Henley and Sadana [47]. Inactivation parameters were determined from the best-fit model of the experimental data.

2.7. Synthesis of cephalexin

Syntheses were performed batch-wise with temperature and pH control in 50 mL Pyrex glass reactors with a working volume of 30 mL, equipped with a paddle impeller to keep biocatalyst particles in suspension. Samples were taken at intervals and were properly diluted prior to be assayed by HPLC as described in Section 2.3.

Syntheses of cephalexin with CLEAs were conducted under kinetic control at 40% (v/v) ethylene glycol medium using 600 mM PGME as acyl donor and 200 mM 7ADCA as the limiting substrate at pH 7.4, 14 °C and 62.5 IU_H/mmol 7ADCA. These conditions correspond to those previously determined as optimum for the same enzyme immobilized in glyoxyl agarose by multi-point covalent attachment [15]. Molar conversion yield (Y) was defined as the maximum molar conversion of 7ADCA into cephalexin (%). Specific productivity (q) (mmoles/h g_{CLEA}) was determined as the amounts of cephalexin produced per unit time and unit mass of biocatalyst at maximum yield. Experiments were done in duplicate and samples assayed in triplicate with variations below 5% among them.