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Process Biochemistry 41 (2006) 1924–1931

Process Biochemistry

www.elsevier.com/locate/procbio

Low temperature effect on production of ampicillin and cephalexin in ethylene glycol medium with immobilized penicillin acylase

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Received 2 November 2005; received in revised form 28 March 2006; accepted 31 March 2006

Abstract

The effect of temperature was studied for the kinetically controlled synthesis of cephalexin and ampicillin with penicillin acylase immobilised in glyoxyl agarose. Yield increased at low temperatures in the absence and presence of ethylene glycol, while the initial ratio of synthesis to hydrolysis decreased. Arrhenius equations were used to describe the temperature dependency of the hydrolysis and synthesis rates. The effect of ethylene glycol was stronger over the yield of synthesis of cephalexin than ampicillin. In the case of cephalexin, yield increased from 82.8% in aqueous buffer to 97.6% in 50% (v/v) ethylene glycol medium at 0 °C, while at 20 °C an increase from 68.8% to 78.7% was obtained. The presence of ethylene glycol produced a greater increase in the energies of activation of the hydrolysis reactions than of the synthesis reactions, which explains the higher conversion yields obtained in the presence of the cosolvent, both for cephalexin and ampicillin. Cephalexin synthesis was optimized using an experimental design based on surface of response methodology.

Keywords: Penicillin acylase; Cephalexin; β-Lactam antibiotics; Enzymatic synthesis; Organic cosolvents; Energy of activation

1. Introduction

Biocatalysis is a valuable alternative to conventional, but cumbersome, chemical processes, not only for the well-established production of β-lactam nuclei from first-generation β-lactam antibiotics [1,2], but also for the production of semi-synthetic antibiotics derived from those leader molecules [3]. From the latter aspect, better enzymes and careful control of process conditions are required to compete with chemical synthesis in terms of product yield [4]. Penicillin acylase (penicillin amidohydrolase; E.C. 3.5.1.11) is a remarkably versatile enzyme which can conduct both hydrolytic and synthetic reactions over a wide range of compounds [5–7], playing a key role in the synthesis of β-lactam antibiotics [4]. Advances in the field of enzyme immobilization have been determinant for the industrial success in the production of β-lactam nuclei and, very recently, for the synthesis of β-lactam antibiotics as well [8–10].

New antibiotics have to be continuously developed to overcome the constant development of microbial resistance. Semi-synthetic cephalosporins (SSC) and penicillins (SSP) are important families of antibiotics, usually produced by chemical methods from precursor molecules, such as penicillin G (PG) and cephalosporin G [3,11]. Pharmaceutically relevant SSC can arise from 7-aminodesacetoxycephalosporanic acid (7-ADCA), such as cephalexin [12], cefadroxil [4] and cefachlor [13], or from 7-aminocephalosporanic acid (7-ACA), such as cephalotin [14] and cefamandole [15]. SSP, such as ampicillin [16] and amoxicillin [17], arise from 6-aminopenicillanic acid (6-APA). The enzymatic synthesis of SSC and SSP can be conducted either under thermodynamic or kinetic control. The latter, although requiring activated acyl donors, such as amides or esters, is usually a better strategy when product yield is the main issue, since it is not limited by the equilibrium of the reaction [18]. Kinetically controlled synthesis of β-lactam antibiotics has, however, some drawbacks since the synthesis reaction will occur simultaneously with the hydrolysis of both the activated acyl donor and the antibiotic product [19]. This can produce a rather sharp peak of antibiotic production [20]

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doi:10.1016/j.procbio.2006.03.045

and require an excess of activated acyl donor that can increase costs and hamper product recovery [4]. Conditions that depress hydrolytic reactions can increase product yield and facilitate product recovery at maximum yield.

Organic cosolvents are suitable media for performing the synthesis of β-lactam antibiotics, because they can favor synthesis by reducing water activity and increasing the proportion of reactive non-ionized species [21]. Polyols are especially suitable, since they strongly depress water activity and increase enzyme stability, those effects being correlated with the number of hydroxyl groups in the polyol moiety [16,22,23]. In the enzymatic synthesis of ampicillin and cephalexin with immobilized penicillin acylase, ethylene glycol (EG) and 1,2-propanediol (PD) were selected as the best among several poloyols tested [20,24]. Temperature was reported also to be a key variable, with a remarkable increase in yield at low temperatures [25,26] even though no explanation was given for this phenomenon.

A study of the temperature effect on the conversion yield in the synthesis of cephalexin and ampicillin with immobilized penicillin acylase in glyoxyl agarose was conducted, using 7-ADCA and 6-APA as nucleophiles and (*R*)-(−)-2-phenylglycine methyl ester (PGME) as acyl donor in 50% (v/v) EG medium and in aqueous buffer. The objective was to explain the higher conversion yields obtained at low temperatures in terms of the activation energies of the individual reactions that take place in the kinetically controlled mechanism of synthesis.

The kinetically controlled synthesis of cephalexin was optimized in terms of molar product yield using quadratic response surface methodology (RSM), considering pH, temperature and cosolvent concentration as the most relevant variables. Using this methodology, valuable information was obtained on the relevance of each variable and the interactions among them employing a limited number of experiments [27–29].

2. Materials and methods

2.1. Materials

Free penicillin G acylase (PA) from recombinant *Escherichia coli* was a product from Antibióticos SA (Spain), 7-aminodesacetoxycephalosporanic acid, (*R*)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure) (PGME), cephalexin (Cep) hydrate, ampicillin (Amp), penicillin G potassium salt (Pen G) and phenylglycine (PG) were from Sigma Chemical Company Inc. (St. Louis, MO, USA). Ethylene glycol (EG), and all other reagents were analytical grade either from Sigma–Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Immobilization of penicillin G acylase

IPA was obtained by immobilization of recombinant *E. coli* penicillin acylase (PA) through covalent multipoint attachment to activated agarose [30] with a specific activity of 256 IU_H/g.

2.3. Analysis

7-ADCA, Cep and PGME were identified and analyzed by HPLC using a system with a Lichrospher 100 RP-18 125 mm × 4 mm 5-μm column, a Bruker UV-detector at 220 nm, a Rheodyne 7125i 20-μL injector, and a Knauer 64 pump with a flow-rate of 1 mL/min. The eluant was composed of 30% (v/v) methanol in 20 mM phosphate buffer pH 6.0. Product concentrations were calculated from calibration curves using stock solutions. In the case of Pen G, methanol at 40% (v/v) in phosphate buffer was used.

One international unit of activity of hydrolysis (IU_H) was defined as the amount of IPA that hydrolyzes 1 μmol of Pen G per minute at 30 °C and pH 7.8 from 134 mM Pen G in 0.1 M phosphate buffer.

2.4. Hydrolysis of β-lactam antibiotics with IPA

Batch-wise hydrolysis reactions were performed at 30 mM Cep (or Amp), 125 IU_H/mmol 7-ADCA (or 6-APA) at pH 7.0 in a temperature range from 0 to 20 °C in 50% (v/v) ethylene glycol and aqueous buffer. In all cases initial rates of antibiotic hydrolysis (v_h) were measured.

2.5. Synthesis of cephalexin with IPA

Batch-wise synthesis reactions were performed at 30 mM 7-ADCA (6-APA), 90 mM PGME and 125 IU_H/mmol 7-ADCA (6-APA). The temperature effect on conversion yield (Y) and initial rate of antibiotics synthesis (v_s) were studied in the range from 0 to 20 °C, both in the presence (50%, v/v) and the absence of EG at pH 7.0. Yield was defined as the maximum molar conversion of 7-ADCA into Cep (%) and productivity (P) as the amount of antibiotic produced per unit time and unit reaction volume at maximum yield (mM/h).

For Cep optimization, temperature (T), pH and cosolvent concentration (Cs) varied according to the experimental design below. During synthesis, the pH and temperature were monitored and samples were taken to analyze product and substrates to determine yield and productivity. The amount of enzyme added at each temperature tested was corrected by a factor equivalent to the variation in initial reaction rate of penicillin G hydrolysis, using 30 °C and pH 7.8 as control. This is why the reaction of synthesis was optimized in terms of conversion yield, since the productivity will be affected by the introduction of the enzyme load as a new variable.

2.6. Energy of activation

Considering that the effect of the kinetic constants of hydrolysis k'_H and synthesis k'_S are proportional to the initial reaction rates (expressed by unit mass of IPA), when Arrhenius equations (Eqs. (1) and (2)) are applied, the apparent energies of activation of hydrolysis and synthesis (Ea'_H; Ea'_S) are obtained:

$$k'_H = A_H e^{-Ea'_H/RT} \quad (1)$$

$$k'_S = A_S e^{-Ea'_S/RT} \quad (2)$$

where A is the preexponential term, R the universal gas constant and T is the absolute temperature.

2.7. Experimental design

The effects of pH, T and ethylene glycol concentration (Cs) on the synthesis of Cep under the conditions defined above were determined by modulating the variables according to a full 2^k factorial design (three factors at two levels) considering also three central points to evaluate the experimental error. The design was further expanded to a circumscribed central composite design, introducing $2k$ additional runs, requiring a total of 17 experiments for this experimental design. A software package (Modde 4.0 for Windows, Umetri, Umeå, Sweden) was employed to fit the second order models using multiple linear regression, for the response (Y) with respect to T, pH and Cs, according to Eq. (3):

$$Y = \beta_o + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma\Sigma \beta_{ij} X_i X_j \quad (3)$$

where Y is the molar conversion yield, β_o the offset term, β_i the linear effect, β_{ii} the squared effect, β_{ij} the interaction effect and X_i and X_j are the independent variables or experimental factors (T, pH and Cs).

The determination coefficient (R^2) is the fraction of variation of the response explained by the model. The prediction coefficient (Q^2) is the fraction of