PENICILLIN G ACYLASe FROM KLUYVERA CITROPHILA
NEW CHOICE AS INDUSTRIAL ENZYME.

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SUMMARY

We propose a new and integrated method for the evaluation of industrial enzymes. The application of this method to the enzyme penicillin G acylase from Kluyvera citrophila shows very interesting industrial prospects. This acylase presents a much better stability against heat, pH or organic cosolvents as compared with the more popular enzyme from Escherichia coli. In addition, this enzyme is very easy to immobilize through its amine groups and to stabilize through multipoint covalent attachment on activated pre-existing supports.

INTRODUCTION

The existence of different enzymes from different microbial sources which are able to catalyze similar industrial processes offers great biotechnological possibilities. In this way, nature allows us the possibility of selecting the most adequate industrial enzyme. An accurate selection of a given native enzyme may help us to overcome a number of obstacles which hinder a massive implantation of enzyme derivatives as industrial catalysts. However, comparative studies of similar industrial enzymes are not very common in scientific literature. Even when such studies are reported, they are mainly focussed to conventional kinetic studies (e.g. at low concentration of model substrates under standard conditions) which are clearly insufficient from an industrial point of view. In our opinion, the selection of industrial enzymes should be made as a "compromise solution" taking into account a number of industrial parameters: i.- Kinetic Studies under Industrial Conditions (e.g. very high conversions of very high substrate concentrations under experimental conditions of industrial interest), ii.- Stability against different agents (heat, organic solvents, antimicrobial agents..), iii.- Easiness for immobilization on different supports (e.g., fast processes yielding non-distorted immobilized enzyme molecules and very high loaded derivatives), iv.- Possibility to develop additional strategies for enzyme stabilization (e.g. immobilization through multipoint covalent attachment, chemical modifications..) and so on.

The enzymes penicillin G acylases (E.C. 3.5.1.11) have a very great biotechnological interest. In the field of the antibiotics industry they are currently used to hydrolyze the penicillin
G (production of the "key intermediate" 6APA) and they are also potentially useful as synthetic catalysts for the N acylation of antibiotic nuclei to yield the corresponding semisynthetic antibiotic. A number of penicillin G acylases from a wide variety of microorganisms have been described. It should be noted however, that comparative studies among these acylases are insignificant and the first and most popular one (the enzyme from E. coli) is still the most widely used both at industrial as well as academic level (Savidge, 1984). In this paper, we present a novel and integrated evaluation of the penicillin G acylase (PGA) from K. citrophila as a possible industrial alternative to the enzyme from E. coli.

MATERIALS AND METHODS

Materials. 10 % CL-Agarose gels were obtained from HISPANAGAR S.A. (Burgos, Spain). Extracts (containing 25 % of pure enzyme) of penicillin G acylase from K. citrophila and the enzyme inhibitor penicillin G sulfoxide were generously donated by Antibióticos S.A., León, Spain. Extracts of penicillin G acylase from E. coli with similar purity degree were a generous gift from Boehringer Mannheim GmbH (Penzberg, Germany). Penicillin G, phenylacetic acid, 6-amine penicillanic acid (6-APA) were purchased from Sigma Chem. Co., St Louis, Mo.

Preparation of penicillin G acylase-agarose derivatives. Glyoxyl-Sepharose CL gels were prepared by etherification of agarose gels with glyc oxidol (2,3-epoxypropanol) and further oxidation with periodate (Guisán, 1988). The PGA(amine)-agarose(alkyl) derivatives from K. citrophila were prepared as we have been previously reported for the preparation of PGA-derivatives from E. coli (Alvaro et al. 1990). The process of immobilization-stabilization was performed at pH 10.0 and the concentration of buffer was 50 mM, the rest of the conditions are given in table 1. The amount of enzyme offered to the activated support was ranged between 5 and 600 IU of acylase per mL of support and a maximum of 500 IU were immobilized.

Enzymatic assays

1. Hydrolytic assays. i.- Hydrolytic initial rates: Soluble and immobilized enzymes were assayed with penicillin G as substrate, titrating the released phenylacetic acid with 0.01 M NaOH, 0.1 M ClNa in a pH-stat (model TTT80 Radiometer, Denmark). Experiments were carried out at pH 8.0 at 37°C and using 20 mL of 30 mM of penicillin G in 0.1 M ClNa, 50 mM phosphate as assay mixture. 0.1-0.2 IU of enzyme were used in each experiment. An International Unit of PGA is defined as the amount of enzyme which hydrolyzed 1 μmole of penicillin G per minute under the conditions described above (standard assay). ii.- Complete courses of hydrolysis: Soluble PGA from K. citrophila and from E. coli was assayed with 140.5 mM penicillin G as substrate, titrating the released phenylacetic acid as it has been described above.

2. Synthetic assays. A jacketed column was used as a packed bed reactor. A small 3 mL bed of PGA-agarose derivative was used. The reaction solution was constituted by an equimolar mixture of phenylacetic acid and 6-APA (20 mM) dissolved in water-DMF (50:50) (v/v) mixtures containing 25 mM of acetate buffer. The final pH was adjusted after addition of DMF and it is given as pH_{app}. Substrates and product (penicillin G) were identified and analyzed by HPLC. Samples were eluted with 35% MeOH, 65% 0.067 M KH₂PO₄ (v/v) final pH_{app} 4.7.

Irreversible deactivations. Samples of derivatives or soluble PGA were incubated in 50 mM phosphate, pH 8.0 at 58°C. At different times aliquots were withdrawn and assayed at pH 8.0 and 37°C as described above. Stabilization is defined as the ratio between the half-life time of each derivative and that for soluble enzyme in irreversible thermal deactivations.

RESULTS AND DISCUSSION

HYDROLYSIS OF PENICILLIN G. We have compared the properties of soluble PGAs from K. citrophila and from E. coli as catalyst for the hydrolysis of penicillin G. The kinetic constants