

Isolation of a Penicillin Acylase Producing *E.coli* and Kinetic Characterization of the Whole Cell Enzyme Activity

Sedigheh Javadpour¹, Dariush Norouzian^{*2}, Azim Akbarzadeh², Saeed Mirdamadi³ and Behrokh farahmand²

¹Dept. of Microbiology, Medical Sciences, Hormozgan University of Medical Science, Bandar Abbas; ²Dept. of Pilot Biotechnology, Pasteur Institute of Iran, Tehran; ³Biotechnology Research Center, IROST, Tehran, Iran

ABSTRACT

Penicillin acylase (EC 3.5.1.11) has been a target of study for a long time because of its pivotal role in the deacylation of the penicillin into the 6-aminopenicillanic acid (6-APA) and the side-chain organic acids. This property of penicillin acylase has been exploited commercially for large scale production of 6-APA, which is the key intermediate in the manufacture of semi-synthetic penicillins. Due to the worldwide demand for semi-synthetic penicillins, production of 6-APA has been increased up to 7000 tons in recent years. In this study, Sixty-five strains of *E. coli* were investigated for penicillin acylase activity using fluorescamine method. The 6-aminopenicillanic acid formed in the reaction mixture was developed on thin layer chromatography. One-minute beta-lactamase test was carried out to follow any trace of penicillinase activity. Only one sample designated as *E.coli* PPA78 was found to be penicillin acylase producer. The optimal pH and temperature of penicillin acylase activity of the whole cells were determined to be 8.0 and 57°C, respectively. Km value and activation energy of the enzymatic hydrolysis reaction of penicillin G by intracellular enzyme were estimated as 0.004 mmol and 6.2 Kcal/mol, respectively. *Iran. Biomed. J.* 6 (2 & 3): 93-96, 2002

Keywords: penicillin acylase, *E. coli*, Fluorescamine,

INTRODUCTION

Penicillin acylase (PA) or penicillin amidohydrolase (EC 3.5.1.11) is one of the most important enzyme applied in the pharmaceutical industry for large scale production of 6-aminopenicillanic acid (6-APA). This Enzyme is the starting material for the manufacture of penicillin derivatives, which are the most widely used beta-lactam antibiotics, with a share of about 19% of the estimated worldwide antibiotic market [1, 2]. Both natural and semi-synthetic penicillins contain an identical core ring structure termed 6-aminopenicillanic acid. Different penicillin types differ in their attached side chains. Semi-synthetic penicillin may be produced by enzymatic removal at the side chain of native penicillins with subsequent attachment of a novel side chain to the resultant 6-aminopenicillanic acid core [3]. According to the substrate specificity, the acylases are active against penicillin and are grouped into: ampicillin acylase, benzylpenicillin or penicillin G acylase (PGA), and phenoxyethyl penicillin or penicillin V acylase (PVA). Many genera of molds,

yeast and bacteria produce penicillin acylases. Among them, enzyme produced by *E. coli* is the most well-characterized and common one for industrial application. *E. coli* is known to produce an intracellular PGA that can be induced by phenylacetic acid (PAA) [4, 5]. Since the penicillin molecule is also susceptible to hydrolytic cleavage at the β -lactame ring, the process was carried out by the enzyme penicillinase rendering the antibiotic inactive. Hence, there is an unabated effort for over producing PA strains lacking penicillinase activity [6]. In this paper, the reaction of 6-APA with fluorescamine has been used for screening of penicillin acylase activity and one-minute beta-lactamase method has been carried out to detect penicillinase activity in 65 strains of *E. coli*.

MATERIALS AND METHODS

Benzylpenicillin was obtained from Jaber Ebne Hayyan Drug Manufacturing Co. (Tehran, Iran). Bacteriological peptone and yeast extract were supplied by Oxoid. Penicillinase, phenylacetic acid,

*Corresponding Author. E-mail: dnsa@institute.pasteur.ac.ir

ninhydrin, p-dimethyl amino benzaldehyde (PDAB) and fluorescamine were purchased from Sigma. All other reagents and chemicals were obtained from Merck.

Enzyme production. Sixty-five strains of *E. coli* obtained from a clinical laboratory were investigated for penicillin acylase activity by fluorescamine method [6]. The identification of all strains were confirmed by routine microbiological methods and biochemical tests. For Enzyme production, each strain was inoculated in 100-ml Erlenmeyer flasks containing 20 ml of fermentation medium described by Sato *et al.* [7], composed of 2.0% bacteriological peptone, 0.5% yeast extract, 0.02% MgSO₄, 0.3% KH₂PO₄, 0.7% K₂HPO₄, and 0.1% phenylacetic acid as the inducer agent. The flasks were then shaken (100 rpm) at 27°C. After 24 h, the cells were collected by centrifugation (12000 ×g, 10 min, 4°C) and the cells were assayed for enzyme activity.

Assay of penicillin acylase activity. In the screening stage, penicillin acylase activity was estimated essentially as described by Baker [8]. One millilitre of each cell suspension was mixed with 1 ml benzylpenicillin (10 mg/ml) prepared in phosphate buffer 0.1 M, pH 7.5 and incubated at 37°C for 60 min. The samples were diluted (1:10) by 0.5 M acetate buffer pH 4. Thereafter, 0.5 ml of fluorescamine reagent (15 mg/100 ml of acetone) was added to 1ml of each diluted sample. After one hour, the fluorescence was measured under UV light (366 nm). For kinetic investigations, the PDAB method was used to measure PA activity [9]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μM per min of 6-APA from Pen G at 57°C, pH 8.0 in 50 mM phosphate buffer.

Thin layer chromatography. In order to confirm the presence of 6-APA, a small amount of the cell free supernatant of reaction mixture was examined by silica gel thin-layer chromatography with butanol/acetic acid/water (4:1:1 v/v) as the running solvent. After 2 h, the chromatograms were air dried and benzylpenicillin and 6-APA were detected by reaction with ninhydrin [10].

Determination of penicillinase activity. The test reagent was constructed by dissolving 600 mg of penicillin G in 4.5 ml sterile distilled water plus 0.5 ml of 0.5% aqueous solution of phenol red. The test reagent was adjusted to pH 8.5 with 1 M NaOH

giving violet color to the solution. A loopful of the organism of choice grown on nutrient agar was emulsified in a test tube containing 0.5 ml sterile normal saline. Three drops of freshly made test reagent was added to the cell suspension. The tubes were mixed immediately. A tube containing 0.5 ml of sterile saline and another containing penicillinase, plus 3 drops of the test reagent served as a negative and positive control, respectively [11].

RESULTS AND DISCUSSION

Fluorescamine method has been considered as a specific and reliable procedure for screening penicillin acylase producing microorganisms [12,13]. Contrary to amino acids and polypeptides, 6-APA at pH 4, develops a deep fluorescence intensity with fluorescamine. In this study, among 65 strains of *E. coli*, isolated from urinary tract infection, only one sample showed penicillin acylase activity by fluorescamine method. However, Meevootisom *et al.* [10] have reported 20 positive PA strains among 411 isolates of *E. coli*, from fecal samples, using microbiological screening method with *Serratia marcescens* ATCC 27117. Thin layer chromatography of the reaction mixture showed the presence of benzyl penicillin and 6-APA (Rf 0.8 and 0.2, respectively). Most substrate of penicillins have higher Rf values than 6-APA [14]. Since beta-lactamase hydrolyzes amide bond of the beta-lactame ring of 6-APA and its derivatives transforming them into the penicilloic acid without any anti-bacterial activity, beta-lactamase (acidometric) assay was carried out to rule out the absence of this enzyme. Observation of a deep red color by penicillin-phenol red reagent, persisted for more than 48 h, was a convenient evidence for absence of β-lactamase in our isolate (coded *E. coli* PPA 78).

In order to determine the optimum temperature value, the hydrolysis of pen G by whole cells was carried out at different temperatures, ranged from 10 to 80°C. The highest activity was observed at 57°C. The optimum pH for pen G hydrolysis was found to be 8.0 at 57°C.

Since it is well-established that penicillin acylase is an aspecific enzyme, which can hydrolyse different substrates containing acyl groups [15], penicillin acylase activity of *E. coli* PPA78 cells was measured in the presence of penicillin G, penicillin V, and ampicillin, by PDAB method. Considering hydrolysis rate of penicillin G as,

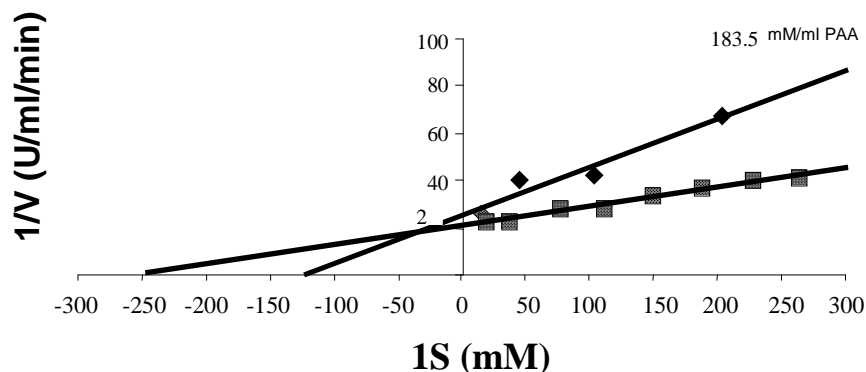


Fig. 1. Dixon plot to determine K_i value for phenyl acetic acid. The reaction was carried out in the presence of inhibitor with varying concentration of substrate and the end product was measured by PDAB method of Balasingham. \blacksquare —, K_m for determination of K_m ; \blacklozenge —, K_m for determination of K_i .

100%. The relative rates of deacylation of penicillin V and ampicillin were 54% and 23%, respectively.

K_m , the measure of enzyme affinity towards substrate, is one of the important characteristics of enzymatic reaction. The larger the value of K_m , the less affinity of enzyme-substrate binding. The K_m value of intracellular penicillin acylase was found to be 0.004 mM by Lineweaver Burk plot (Fig. 1). This K_m value is much lower than those reported by other investigators for cell bound penicillin acylase activity of different origins (Table 1). The maximum velocity for production of 6-aminopenicillanic acid was determined to be 0.048 U/ml/min. The competitive inhibition constant of phenylacetic acid was estimated as 6.31 mM (Fig. 1). The larger the value of K_i the less the capability of enzyme molecule to bind substrate in the presence of inhibitor. As it is illustrated in Figure 2, activation energy of the whole cells enzymatic activity, calculated from the slope of Arrhenius plot is 6.2 Kcal/mol.

Concerning kinetic properties of *E. coli* PPA78, it can be considered as an efficient penicillin acylase producing organism for further studies.

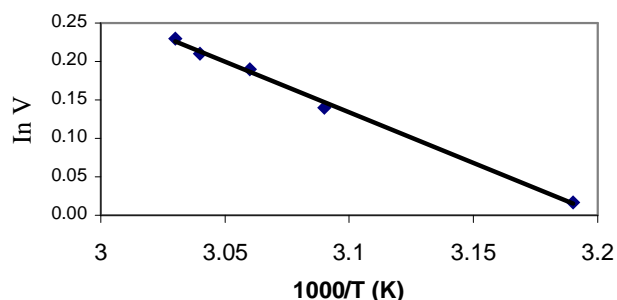


Fig. 2. Effect of temperature on initial reaction rate of *E. coli* PPA78 penicillin acylase activity. Enzyme activity was estimated at different temperature values, and the activation energy was calculated from the slope of Arrhenius plot.

Table 1: Comparison of optimal conditions and k_m values of different whole cells Penicillin acylase activity.

Microorganism	Optimum temp. (°C)	Optimum pH	K_m (mM)	Ref.
<i>E. coli</i> NCIB 9465	ND	5.5	4.000	15
<i>E. coli</i> spp	30-35	7.0	1.35-1.59	16
<i>E. coli</i> cell NCIB 87431	50	8.2	30.000	15
<i>E. coli</i> spp	ND	7.5	17.500	15
<i>E. coli</i> 5K (PHM12)	45	7.8	9-11	17
<i>E. coli</i> PPA78	57	8.0	0.004	*
<i>E. coli</i> ATCC 11105	45	8.0	4.050	18
<i>B. megaterium</i> ATCC 14945	ND	8.5	4.500	15

ND = Not determined, *Our isolate

REFERENCES

1. Parmar, A., Kumar, S., Marhava, J. and Kenedy, F. (2000) Advances in enzymatic transformation of penicillins to 6-aminopenicillanic acid. *Biotech. Adv.* 18: 289-301.
2. Chou, C.P., Lin, U.J., Kuo, B.Y. and Yu, C.C. (2000) Genetic strategies to enhance penicillin acylase production in *Escherichia coli*. *Enzyme. Microb. Technol.* 27: 266-273.
3. Walsh, G. and Headon, D. (1994) Protein biotechnology. John Wiley & Sons, England, pp. 342-345.
4. Shewale, J.G. and Sivaraman, H. (1986) Penicillin acylase: Enzyme production and its application in the manufacture of 6-APA. *Process Biochem.* 24 (4): 146-154
5. Chou, C.P., Tseng, J.H., Lin, M.I., Lin, H.K. and Yu, C.C. (1999) Manipulation of carbon assimilation with respect to expression of the pac gene for improving the production of penicillin acylase in *Escherichia coli*. *J. Biotechnol.* 69: 27-38.
6. Hamilton-Miller, J.M.T. (1966) Penicillin acylase. *Bacteriol. Rev.* 30 (4): 761-771.
7. Sato, T., Tosa, T. and Chibata, I. (1976) Continuous production of 6-amino penicillanic acid from penicillin by immobilized microbial cells. *Eur. J. Appl. Microbiol.* 2: 153-160.
8. Baker, W.L. (1983) Application of the fluorescamine reaction with 6-amino penicillanic acid to estimation and detection of penicillin acylase activity. *Antimicrob. Agents Chemother.* 23 (1): 26-30.
9. Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M.D. (1972) The isolation and kinetic properties of penicillin amidase from *Escherichia coli*. *Biochim. Biophys. Acta* 276: 250-256.
10. Meevootisom, V., Somsuk, P., Prachaktam, R. and Flegel, T.W. (1983) Simple screening method for isolation of penicillin acylase producing bacteria. *Appl. Environ. Microb.* 46: 1227-1229.
11. Escamilla, J. (1976) Susceptibility of *Haemophilus influenzae* to ampicillin as determined by use of a modified, one-minute beta-lactamase test. *Antimicrob. Agents Chemother.* 9 (1): 169-198.
12. Veronese, F.M., Franchi, D., Boccu, E., Gueroto, A. and Orsolini, P. (1981) *E. coli* penicillin acylase: extraction, purification and kinetic characterization by improved methods. *Farma. Ediz. Scien.* 36: 663-670.
13. Baker, W.L. (1984) A sensitive procedure for screening microorganisms for the presence of penicillin amidase. *Aus. J. Biol. Sci.* 37: 257-265.
14. Cole, M., Savidge, T. and Vanderhaeghe, H. (1975) Penicillin acylase *Methods Enzymol.* 43: 698-720.
15. Vandamme, E.J. and Voets, J.P. (1974) Microbial penicillin acylase. *Adv. Appl. Microbiol.* 17: 311-369.
16. Brandle, E. (1965) Kinetics of penicillin amidase. *Z. physiol. Chem.* 342: 86-92.
17. Savidge, T.A. and Cole, M. (1975) Penicillin acylase (bacterial). *Methods Enzymol.* 43: 705-721.
18. Hsiau, L.T., Lee, W.C. and Wang, F.S. (1997) Immobilization of whole cell penicillin acylase by entrapping within polymethyl acrylamide beads. *Appl. Biochem. Biotechnol.* 62: 303-315.