ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 103–107. © Allerton Press, Inc., 2008.
Original Russian Text © A.S. Yasnaya, O.V. Yamskova, D.T. Guranda, T.A. Shcherbakova, V.I. Tishkov, V.K. Švedas, 2008, published in Vestnik Moskovskogo Universiteta. Khimiya, 2008, No. 2, pp. 127–133.

Cloning of Penicillin Acylase from *Escherichia coli*: Catalytic Properties of Recombinant Enzymes

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Received November 23, 2007

Abstract—The gene of penicillin acylase (PA) from *Escherichia coli* has been cloned from a PA producer strain that is an analogue of strain ATCC 11105. Optimization of the cultivation conditions made it possible to obtain up to 130 mg of active enzyme per liter of culture broth. A number of single, double, and triple mutants were obtained by the method of site-specific mutagenesis using PCR. As a result of isolation and purification procedures, homogeneous preparations of the wild-type enzyme and its mutants were obtained. Studies showed that (1) the obtained enzymes have the correctly folded structure; (2) complexing agents and metal cations do not inhibit their catalytic activity; (3) mutant PAs, like the wild type, are efficiently inactivated by phenylmethylsulfonyl fluoride (PMSF), which makes it possible to titrate their active sites; and (4) the obtained mutants are characterized by a greater specificity constant in the reaction of hydrolysis of a colorimetric substrate; however, they are inferior to the wild type in the synthesis of ampicillin by acyl transfer.

DOI: 10.3103/S0027131408020120

Penicillin acylase (PA) is one of the most well-studied representatives of the recently discovered superfamily of enzymes with N-terminal nucleophile (Ntn hydrolase), which are activated by means of a unique autocatalytic cleavage of an inactive polypeptide precursor [1, 2]. PA from different sources is widely used in the pharmaceutical industry for production of 6-aminopenicillanic (6-APA) and 7-aminodesacetoxycephalosporanic acids (7-ADCA)—key compounds in the synthesis of new penicillins and cephalosporins [3]. The broad substrate specificity and stereoselectivity make it possible to use PA in fine organic synthesis to obtain enantiomers of α-, β-, and γ-amino acids [4, 5]; for highly efficient chemo- and stereoselective acylation of amino compounds in an aqueous medium; and for obtaining of enantiomers of amines, amino alcohols [6, 7], aminonitriles [8], and chiral sulfhydryl compounds [9, 10].

The biocatalytic potential of PA makes possible its use in bioengineering for creation of biocatalysts with improved properties. It has been shown that it is possible to change the specificity and synthetic properties of PA [11–13] using genetic engineering methods. In the last study, in particular, as a result of multiple point substitutions, the binding domain of the acyl group of the substrate in the active site was substantially changed, as a result of which hydrolysis of cephalosporin C became possible [13]. Molecular modeling, allowing the role of amino acid residues of the active site in the mechanism of action of the enzyme to be established [14], provides new possibilities in the development of methods of rational use of PA. Another important achievement for bioengineering in recent years is the construction of permuted single-chain PA [15], whose expression does not depend on autocatalytic cleavage.

The goal of this study is the cloning of the PA gene from *E. coli* strain that is a PA producer and the creation of an enzyme expression system, as well as the obtaining of PA mutants and the study of their catalytic properties.

EXPERIMENTAL

Cloning of the PA gene from E. coli. For cloning of the wild-type PA gene, a strain of *E. coli* (PA producer) from the collection of strains of the State Research Center for Antibiotics was used. The wild-type PA gene was obtained using polymerase chain reaction (PCR). For isolation of the gene, the following primers were used:

1. PAC_For: 5'-cttccagaggat**catatg**aaaaatagaaatcgtatgatc-3';

2. PAC_Rev: 5'-tgcc**gaattc**aagcttatctctgaacgatagatcc-3'.

For convenience of cloning, primers contained restriction sites: primer 1, *Nde*I (ensures insertion at the ATG codon), and primer 2, *Eco*RI. Restriction sites in

primer sequences are boldfaced. To a mixture of oligonucleotides (20 pmol of each), 2.5 μL of 10× PCR buffer supplied by the manufacturer together with the enzyme, 2 μL $MgCl_2$ solution (25 mM), 2 μL dNTP solution (2.5 mM of each), 1 μL of plasmid DNA solution (50 ng/μL), 0.5 μL *Taq* DNA polymerase (5 U/μL), and 17 μL of deionized water were added to a total volume of 25 μL. For PCR, SibEnzyme reagents were used. PCR was carried out on a Tertsik apparatus (DNK-Tekhnologiya, Russia) under the following conditions: 25 cycles (1 min at 94°C, 1 min at 50°C, and 2 min at 72°C) with subsequent 5-min incubation at 72°C. PCR products were purified by electrophoresis in 1% agarose gel. For this, PCR products were run in the gel and the piece of the gel containing the band of the required size was cut out. DNA from the gel was isolated using a DiatomTM DNA Elution kit (Laboratoriya Izogen, Russia). The isolated fragment was processed with restriction endonucleases *Nde*I and *Eco*RI. As a vector for cloning, we chose pET24a (Novagen). This vector contains a gene ensuring resistance to kanamycin. The presence of the restriction site *Nde*I enables the gene to be inserted exactly at the ATG codon. The vector was processed with the same restriction endonucleases: *Nde*I and *Eco*RI. The restriction products were also purified by electrophoresis in 1% agarose gel. Restriction fragments of the PCR product and the vector for cloning were ligated using T4 phage DNA ligase (SibEnzyme). *E. coli* TG1 cells were transformed with the ligation mixture. Plasmid DNA was isolated from individual clones according to a standard method [16]. DNA sequencing was performed using an ABI PRISM BigDye Terminator v. 3.1 reagent kit with subsequent analysis of the reaction products on an ABI PRISM 3100 Avant automatic DNA sequencer at the Genom Collective Use Center.

Mutant forms with point mutations were obtained using two-stage PCR. Two primers were involved in each reaction: a mutagenic primer (forward or reverse) and a primer ensuring cloning (with *Bcl*I or *Pvu*II restriction sites). The products obtained were purified and added to a third PCR. The primers at the mutation site were planned in such a way as to ensure sufficient overlap of products. For the third PCR, the products of the two previous PCRs were annealed with each other with the formation of a final product of the required size with a mutation. The PCR product obtained at the second stage was purified and processed with restriction endonucleases *Bcl*I and *Pvu*I. Since restriction enzyme *Bcl*I is sensitive to DNA dam methylation, plasmids containing the wild-type PA gene were transformed into *E. coli* strain JM110, defective for two methylases, dcm and dam. An unmethylated pEPAC plasmid containing the wild-type PA gene was processed with the same restriction endonucleases, *Bcl*I and *Pvu*I. The purified restriction products were ligated and *E. coli* TG1 cells were transformed with the ligation mixture. Plasmid DNA was isolated from individual clones according to a standard method [16]. DNA sequencing was performed using an ABI PRISM BigDye Terminator v. 3.1 reagent kit with subsequent analysis of the reaction products on an ABI PRISM 3100 Avant automatic DNA sequencer at the Genom Collective Use Center.

Cultivation. The growth of *E. coli* cells with recombinant PA was carried out in 100-mL or 1-L shake flasks containing two or four baffles on a Multitron shaker (Infors, Germany). The working volumes of medium were 40 and 100 mL, respectively. The inoculum was grown overnight at 37°C and 180 rpm. The medium contained 30 μg/mL kanamycin. As an inducer of protein biosynthesis, isopropyl-β-*D*-thiogalactoside (IPTG; the final concentration was 0.1 mM) was used, which was added after the cell suspension reached an optical density of 0.6–0.8 at 600 nm (A_{600}). Then cells were cultured for another 12–40 h at 15–17°C. Cells were precipitated by centrifuging on a Beckman J-21 centrifuge (United States) for 20 min at 7500 rpm and 4°C. As additives in selection of culture conditions, glycerol was used.

Enzyme isolation and purification. The procedure of isolation and purification of recombinant PAs included extraction by osmotic shock, hydrophobic chromatography, and desalting. Cell suspensions were centrifuged for 20 min at 3500 rpm and 4°C (a Beckman centrifuge, Germany). After removal of the supernatant, the precipitate was resuspended in 1/10 the volume of osmotic shock buffer **A** cooled to 0°C (20% sucrose, 100 mM Tris–HCl, 10 mM EDTA, pH 8.0) and centrifuged for 10 min at 5000 rpm and 4°C. The supernatant was removed and the precipitate was resuspended in 1/10 the volume of osmotic shock buffer **B** cooled to 0°C (1 mM EDTA, pH 8.0) and centrifuged for 15 min at 7000 rpm and 4°C. To the obtained supernatant, we added, with stirring, 1 M KH_2PO_4 solution, pH 7.0, to a final phosphate concentration of 50 mM and then ammonium sulfate to a final concentration of 1.5 M sulfate. An aliquot (30–60 mL) of the obtained PA solution was transferred to a Butyl Toyopearl 650 M column equilibrated in buffer 1 (50 mM KH_2PO_4, 1.5 M $(NH_4)_2SO_4$, 0.1 M KCl, 0.02% NaN_3, pH 7.5) and eluted in a linear gradient of 1.5–0.0 M $(NH_4)_2SO_4$ with buffer 2 (50 mM KH_2PO_4, 0.02% NaN_3, pH 7.5). Fractions having PA activity were combined taking into account the distribution of components of the initial solution, desalted on a HiTrap Desalting column with Sephadex G-25 (Amersham Biosciences, Sweden), and concentrated in an Amicon M-3 ultrafiltration cell (Amicon Corp., United States) using a Diaflo membrane (Amicon). Glycerol (up to 10%) was added to the obtained enzyme preparation, and it was stored at −20°C. The purity was monitored using analytical electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (a Bio-Rad electrophoresis apparatus). The purity of the obtained enzyme preparations was not less than 95%.

Determination of enzyme activity. The activity of mutant forms of PA was determined potentiometrically and spectrophotometrically. In the first case, the enzyme activity was determined by hydrolysis of a freshly prepared 2 mM solution of benzylpenicillin by titration of phenylacetic acid (PAA) forming in the course of the reaction with a solution of 0.02 N KOH with a Titrino 719 titrator (Metrohm, Switzerland) at 25°C and pH 7.5 in the presence of 0.1 M KCl. In the second case, the PA activity was determined according to accumulation of a chromophore in the process of enzymatic hydrolysis of a 1 mM solution of a *m*-carboxy-*p*-nitro-anilide of PAA (NIPAB) at 400 nm on a Shimadzu UV-1601 spectrophotometer (Japan). The reaction was carried out at 25°C in 0.01 M phosphate buffer, pH 7.5, 0.1 M KCl.

Determination of the concentration of active sites. The absolute concentration of active sites of each of the mutant forms of PA was determined by titration of active sites of the enzyme with the irreversible inhibitor phenylmethylsulfonyl fluoride (PMSF) according to the traditional method [17]. The remaining enzymatic activity was determined spectrophotometrically according to the hydrolysis of a colorimetric substrate and potentiometrically according to the hydrolysis of the natural substrate as described above.

Study of the influence of metal ions and complexing agents on enzymatic activity. The influence of complexing agents and metal ions Me^{2+} on the catalytic activity of native and recombinant PAs was studied upon determination of enzymatic activity in the absence and in the presence of complexing agents, ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), as well as the salts $CaCl_2$, $MgCl_2$, $MnCl_2$, and $CoCl_2$, at pH 7.5 and 25°C in 0.1 M Tris–HCl and 0.1 M KCl. The concentration of added effector in the reaction mixture was 1 mM.

Determination of kinetic parameters. The kinetic parameters of enzymatic transformation (K_M and k_{cat}) were determined by analysis of the dependence of the initial rates of hydrolysis on the substrate concentration. Reactions were conducted within the framework of the Michaelis–Menten scheme ($S_0 \gg E_0$) in 0.01 M phosphate buffer (pH 7.5, 0.1 M KCl) at 25°C.

HPLC analysis. The quantitative determination of components of the reaction mixture was performed by reverse phase HPLC on a Perkin-Elmer Series 200 chromatographic system (United States): a Luna C18 column (Phenomenex, United States), 250 × 4.6 mm, particle size 5 μm; mobile phase CH_3CN–water (40 : 60), 0.68 g/L KH_2PO_4, 0.5 g/L sodium dodecyl sulfate, pH 3.0; flow rate 0.8 mL/min; injection volume 10 μL; UV detection at 210 nm.

Study of the synthesis/hydrolysis (S/H) ratio of the reaction of enzymatic synthesis of ampicillin. The initial rates of accumulation of products of synthesis (S) and hydrolysis (H) in the reaction of ampicillin synthesis by acyl transfer from phenylglycinamide (*D*-PGA) to 6-APA catalyzed by PA were studied at pH 6.3 in buffer-free medium at 25°C. The initial concentrations of reagents were 500 mM *D*-PGA and 300 mM 6-APA. The reaction was carried out in a thermostated cell of a Titrino 719 titrator at 25°C, pH 7.5, and 0.1 M KCl with constant stirring. The duration of the experiment was from 1 to 4 h depending on the catalytic activity and concentration of the enzyme preparation. During the reaction, aliquots were taken from the reaction mixture, diluted with the mobile phase, and analyzed by HPLC.

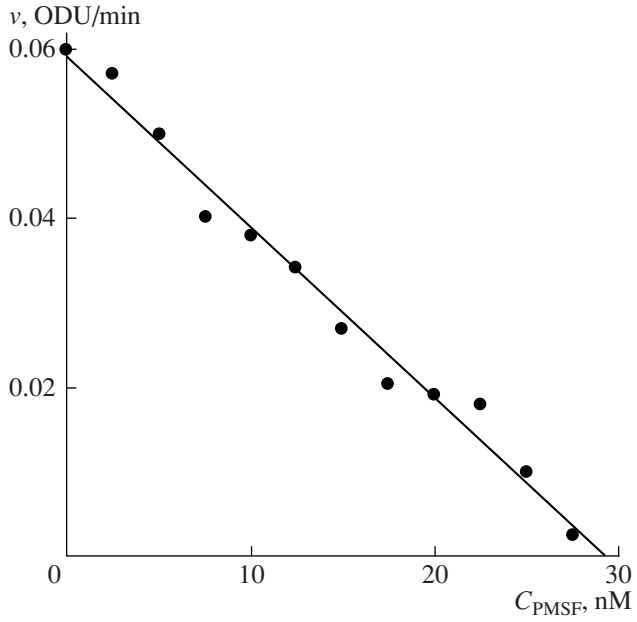
Table 1. Cultivation of *E. coli* cells with recombinant PA at 17°C

Mutant	Enzyme yield		
	Units per liter of medium, U/L	Medium, mg/L	Wet cells, mg/g
Wild type	153	17.9	2.3
M1	150	18.1	2.4
M11	5	0.6	0.1
M12	80	10.2	2.1
M101	138	16.5	2.4

RESULTS AND DISCUSSION

For cloning of PA, the earlier described system based on pET vectors was used [18, 19]. As a result, clones were obtained containing the wild-type PA gene. Test culture of recombinant PA showed the presence of the enzyme in active and soluble forms. Cultivation at a temperature of 30–37°C leads to the predominant expression of the enzyme as inclusion bodies. A decrease in the temperature often makes it possible to obtain the enzyme in active and soluble forms [20], but at the same time leads to a slowing of the growth of cells and an increase in the cultivation time. In connection with the above, the inoculum was grown at 37°C and the temperature was decreased immediately before the addition of the inducer (IPTG). The optimal culture temperature (17°C) was chosen experimentally. A further decrease in the temperature in the case of wild-type PA causes a decrease in the enzyme yield. Optimization of the culture conditions allowed us to obtain approximately 20 mg of active enzyme from a liter of culture medium. Thus, we created a construction for obtaining recombinant PA in active and soluble forms.

The construction created was subsequently used for obtaining of single, double, and triple mutants. In study of the influence of culture conditions on the yield of mutant forms, it was shown that, with a decrease in the culture temperature from 17 to 15°C for the double mutant M11, the yield of the enzyme increases from 10.2 to 72.2 mg/L, i.e., sevenfold (Table 1). In the case



Titration of a homogeneous preparation of penicillin acylase from *E. coli* by phenylmethylsulfonyl fluoride. Incubation conditions: 0.002 M KH_2PO_4, pH 6.5, 25°C. Determination of residual activity: 0.01 M KH_2PO_4, pH 7.5, 25°C.

of the double mutant M12, a decrease in the temperature leads to a decrease in the yield of active enzyme. The addition to the system of glycerol to a final concentration of 5 g/L leads to an analogous result: the yield of the enzyme additionally doubles and amounts to 136 mg/L in the first case, while in the second it decreases twofold. The reasons for the decrease in the yield of mutant M12 with a decrease in culture temperature and with the addition of glycerol have not been identified and require further study. The obtained experimental data indicate the need to optimize the culture conditions of every PA mutant.

Taking into account the specific features of cultivation of each of the preparations, biomass was accumulated for preparatively obtaining wild-type PA, as well as mutant forms. After the corresponding isolation and purification procedures, homogeneous preparations of recombinant enzymes were obtained for further studies. According to X-ray structure (XRSA) data [21], in the structure of PA from *E. coli*, a Ca^{2+} ion is present, which coordinates six amino acid residues of the protein globule with the formation of an EF motif binding the two α-helices of both polypeptide chains of the enzyme. Kinetic studies showed that the presence in the reaction mixture of the complexing agents EDTA and EGTA and/or *s* and *d* metal cations does not influence the activity of the studied mutant forms of PA.

For quantitative characterization and comparison of the catalytic properties of PA mutants, it is necessary to determine the absolute concentration of active sites of enzyme preparations. It is known that enzymes of this family have a high affinity to benzyl radical, and PMSF is a highly efficient titrant of PA active sites [22]. To estimate the possibility of use of PMSF for titration of the obtained recombinant PAs, the inactivation of mutants on incubation with this reagent was studied. The studies showed that PMSF is a strong irreversible inhibitor of all PA mutants: the reaction of formation of the inactive covalent complex phenylmethylsulfonyl–PA, as in the case of the wild-type enzyme, is completed within several minutes at an equimolar concentration of the titrating agent, and the complex under the given conditions is very stable. Independent monitoring of the residual activity with regard to natural benzylpenicillin and its analogue, the colorimetric substrate NIPAB, in the process of titration of active sites of wild-type PA and mutant forms showed that modification of the serine of the PA active site in all cases leads to an identical loss of enzymatic activity with regard to the studied substrates. Thus, it can be stated that the enzymatic transformation of substrates occurs at the same segment of the active site, which is in agreement with the data of [23]. It should be noted that the concentration of each of the recombinant enzymes determined by titration of active sites (figure) was equal to the concentration of the protein determined according to the known molar absorption coefficient of native PA from *E. coli* at 280 nm (μ = 2.22 mL/(mg cm)). This fact indicates that the obtained recombinant PAs have the correctly folded structure.

The determination of the concentration of protein active sites makes it possible to quantitatively characterize and compare the catalytic properties of the obtained PA preparations on the basis of absolute values of kinetic parameters of enzymatic reactions. The kinetic parameters of enzymatic hydrolysis of NIPAB (Table 2) for wild-type PA found in this study fully cor-

Table 2. Kinetic parameters of enzymatic hydrolysis of NIPAB (pH 7.5, 25°C, 0.1 M KCl)

Enzyme preparation	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $\mu M^{-1}\ s^{-1}$	$(k_{cat}/K_M)/(k_{cat}/K_M)$
Wild type	26	30	0.87	1
M1	53	9.0	5.9	6.8
M11	38	45	0.84	0.97
M12	40	15	2.7	3.1
M101	35	15	2.3	2.7

Table 3. Initial rates of accumulation of products of synthesis (S) and hydrolysis (H) of the reaction of enzymatic synthesis of ampicillin by acyl transfer

PA preparation from *E. coli*	Initial concentrations, mM		Initial rates	
	D-PGA	6-APA	$v(S/E_0)$, s^{-1}	S/H
Wild type	15	25	–	1.9
	500	300	20	8.3
M1	500	300	1.2	3.7
M11	500	300	4.8	7.2
M12	500	300	1.3	0.8
M101	500	300	1.2	1.1

respond to the results obtained by us earlier for PA from *E. coli* strain ATCC 9637. The experimental data show that the obtained mutants are characterized by a greater value of catalytic parameters in comparison with the wild-type enzyme (Table 2).

Of particular interest is the use of PA as a biocatalyst for obtaining antibiotics, in particular, ampicillin. The determination of the initial kinetic parameters of the reaction of ampicillin synthesis by the method of acyl transfer from an acyl donor (*D*-PGA) to the "antibiotic nucleus" (6-APA) showed (Table 3) that, under conditions close to optimal, for preparatively obtaining ampicillin, mutant M11 most efficiently catalyzes acyl transfer and is only slightly inferior in this reaction to the wild-type enzyme.

Thus, in this study, the wild-type PA gene was isolated and cloned, a system for expression of recombinant PA in active and soluble forms in *E. coli* cells was created, PA mutants were obtained, culture conditions were optimized, recombinant PAs were isolated and purified, a procedure of characterizing PA preparations was developed, and initial kinetic studies of the corresponding enzymatic transformations were carried out. DNA sequencing was performed at the Genom Collective Use Center of the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences (http://www.genome-centre.narod.ru/).

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 07-08-00696).

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