One-step Purification of Poly-His Tagged Penicillin G Acylase Expressed in E. coli

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Abstract The inexpensive large-scale production of pure PGA (Penicillin G Acylase) has been a commercial goal. PGA has been used as a model enzyme in the development of simple one-step purification methods. In this study, the purification of poly-His tagged PGA protein secreted into the periplasmic space was carried out by using immobilized metal-ion affinity chromatography (IMAC). The PGA gene was obtained from E. coli ATCC 11105. Codons encoding histidines were fused at the C-terminus of the PGA gene by PCR. E. coli JM109 harboring pPGA-HIS6 vector produced active his-tagged acylases in the presence of lac promoter during cultivation at 26°C. The maximum specific activity of the acylase purified by using one-step chromatography after osmotic shock was 38.5 U/mg and was recovered with the yield of 70%. Both 23 kDa (α) and 62 kDa (β) subunits were recovered by using IMAC with just C-terminus tagging of the β subunit. The purification of the periplasmic fraction by osmotic shock and that of purified acylase was increased by 2.6-fold and 19-fold, respectively, compared to the crude extract.

Key words: Penicillin G acylase, E. coli ATCC 11105, IMAC, one-step purification, osmotic shock

Penicillin G acylase (PGA) is an industrially important enzyme, which converts penicillin G to 6-aminopenicillanic acid (6-APA), a key intermediate of semisynthetic penicillin derivatives. The production cost of 6-APA partially depends on the production of acylase enzymes, and it is important to separate large quantities of penicillin acylase at a low cost. Recently, isolation of PGA has been reported by using physical and chemical treatment [15], and aqueous two-phase [11] and multi-step procedures [22, 25]. In addition, some

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authors have described separations on the basis of natural affinity of penicillin G acylase to metal ions [8] and under pseudo-affinity conditions with a variety of chromatographic supports [9]. However, the unspecificity of the interactions and contamination by host proteins caused complex purification procedures and low purity [7, 22, 25].

Immobilized metal-ion affinity chromatography (IMAC) is being increasingly used to purify proteins fused with the poly-His tag and as a tool for the inexpensive and simple purification of large quantities of industrial enzymes. Some attractive features of IMAC include the low cost of metals and the ready capability of regeneration of the stationary phase. Generally, a small hexa-histidine residue is fused into the C- or N-terminal of the target protein. This fusion rarely affects the activity or stability of the fused protein. When PGA fused poly-His tag is expressed, the proteins secreted into the periplasm may be separated by affinity chromatography in a one-step process after osmotic shock. IMAC may improve the purity of PGA with osmotic shock. Both sonication and application of lysozyme are useful for obtaining soluble proteins from cytoplasm. However, the sonication method is not acceptable for handling cells on a large-scale. Also, lysozyme is expensive for industrial applications. Even in the case of affinity resin, unspecific bonding of small amounts of residues remains a problem [1]. Therefore, the proteins secreted into the periplasmic space may be selectively extracted by osmotic shock, and the contamination by host proteins is relatively reduced.

A variety of mutant strains have been developed for the high expression of PGA proteins [20] but the purification of acylase remains a core issue. In addition, the pac gene of E. coli ATCC 11105 was cloned into different expression vectors. However, these vectors suffer from a drawback, namely that they show instability of the secretary pathway [19, 25]. It should be borne in mind that penicillin G acylase is heterodimeric, if one is to obtain active enzymes.

An expressed single precursor (94 kDa) protein is secreted into the periplasm after removing signal sequences and becomes two subunits, α subunit (23 kDa) and β subunit (62 kDa). The spacer peptide is removed during processing [4, 19]. The signal sequences are essential for processing and maturation of the acylase. Processing of the precursor protein requires translocation through the plasma membrane [18, 24]. Therefore, the position of the fused histidine tag and the promoter of expression vectors are important for the effective secretion and processing of PGA.

Effective secretion and high expression were examined as well as potential usefulness as a purification method. After harvesting cells, a simple and time-saving purification step may protect target proteins from degradation. In this study, recombinant strains were synthesized for the expression of His_6-tagged PGA using genetic engineering techniques, and one-step purification was achieved using IMAC after osmotic shock.

MATERIALS AND METHODS

Materials

E. coli ATCC 11105 was from KCTC (Korean Collection for Type Cultures, Daejeon Korea). pUC19 vector, E. coli JM109, E. coli ER2420 harboring pACYC184 plasmids, and all restriction enzymes were prepared by New England BioLabs, Inc. (Beverly, U.S.A.). pGEM®-T Easy vector and pfu polymerase, as an enzyme for PCR and protein marker in SDS-PAGE, were purchased from Promega Co. (Madison, U.S.A.). Taq polymerase was from Takara Co. (Shiga, Japan). The markers used in agarose gel were of 1 kb plus a DNA ladder from Invitrogen Co. (Carlsbad, U.S.A.). Imidazole, $NiSO_4$, HiTrap™ Chelating HP columns were from Pharmacia Biotech (Buckinghamshire, England). PCR primers were synthesized by Bioneer Co. (Daejeon, Korea) (Table 1). All reagents for electrophoresis, penicillin G (sodium salt), as a substrate for the activity assay, 6-APA, bovine serum albumin and Bradford reagent used to measure protein concentrations were purchased from Sigma Co. (St. Louis, U.S.A.).

Genetic Techniques

DNA manipulation was essentially performed according to previously described protocols [21]. GeneAmp PCR

System 2400 purchased from Applied Biosystems (Foster City, U.S.A.) was used for the PCR procedures. E. coli ATCC 11105 was cultivated in media containing yeast extract 0.2%, casein hydrolysate 0.2%, K_2HPO_4 0.7%, KH_2PO_4 0.3%, Na-Citrate·3H_2O 0.05%, $MgSO_4$·7H_2O 0.01%, $(NH_4)_2SO_4$ 0.1%, and glucose 0.2%. When the OD_{600} reached approximately 0.3, chromosomes from E. coli ATCC 11105 were extracted as previously described [21]. Penicillin G acylase (PGA) gene containing a flanking region was amplified by PCR with PGA1 and PGA2 primers. The PGA gene was cut with HindIII and EcoRI and inserted into pUC19 vectors (pPGA-1) and E. coli JM109 was transformed by pPGA-1. Six oligonucleotides were synthesized by Bioneer Co., and full sequencing results were obtained from Macrogen Co. (Seoul, Korea).

Poly-His placed at the C-terminal of the PGA gene was obtained by PCR with PGA1 and HIS2 primers. PCR products were inserted into pUC19 cut with HindIII and XbaI (pPGAH-1). This vector was cloned in E. coli JM109 and the completed gene sequences were sequenced.

PCR products of the 1.1 kb chloramphenicol resistance gene were obtained from pACYC184 by PCR using CM1 and CM2 primers and subcloned into pGEM®-T Easy vector after A-tailing with dATP and Taq polymerase. After the transformation, E. coli JM109 harboring the Cm gene was prepared by blue/white colony selection in LB media containing X-gal, IPTG, and ampicillin 50 μg/ml. The plasmids were digested with restriction enzymes HindIII and XbaI and were inserted into pUC19 (pUC19-Cm). The pUC19-Cm vector was cloned in E. coli JM109 by using in media containing chloramphenicol, and sequenced. Overlaps between internal restriction enzyme sites of the PGA and Cm genes were considered. XmnI and BsrFI were used for inserting the Cm gene into pPGAH-1. The Ap gene fragment of pPGAH-1 was deleted by digesting it with XmnI and BsrFI. The Cm gene was inserted between XmnI and BsrFI of pPGAH-1 (Fig. 1). Consequently, the pPGA-HIS6 vector carrying His_6-tagged PGA was synthesized and harbored in E. coli JM109 for an expression study.

Expression and Osmotic Shock

The cells carrying pPGA-HIS6 were cultured in casein medium (0.4% casein hydrolysate, 0.8% yeast extract,

Table 1. List of primers used for PCR.

Primer	Sequences (5'->3')
PGA1	GACCTGAGCTGTCTCTCT (18mer)
PGA2	AGCCGGAATTCGATTGTGGTGGAACTGG (28mer)
HIS2	GGGCGTCTAGAATTAATGATGATGATGATGGTGTCTCTGAACGTGCAACAC (51mer)
CM1	TCACCGAACGTTTTCGTCAGTAGCTGAACAGGAG (34mer)
CM2	CTGACACCGGCGGGTCGAATTTGCTTTCGA (30mer)

HindIII

CRP binding   -35   -10   RBS   α   Poly-His$_6$ XbaI   Termination signal   β

pac promoter site   Secretion Signal   Spacer   Stop codon

(a)

disrupted lacZ
XbaI
XmnI
Cm
pPGA-HIS6
5900 bp
BsrFI
His6-tagged PGA gene
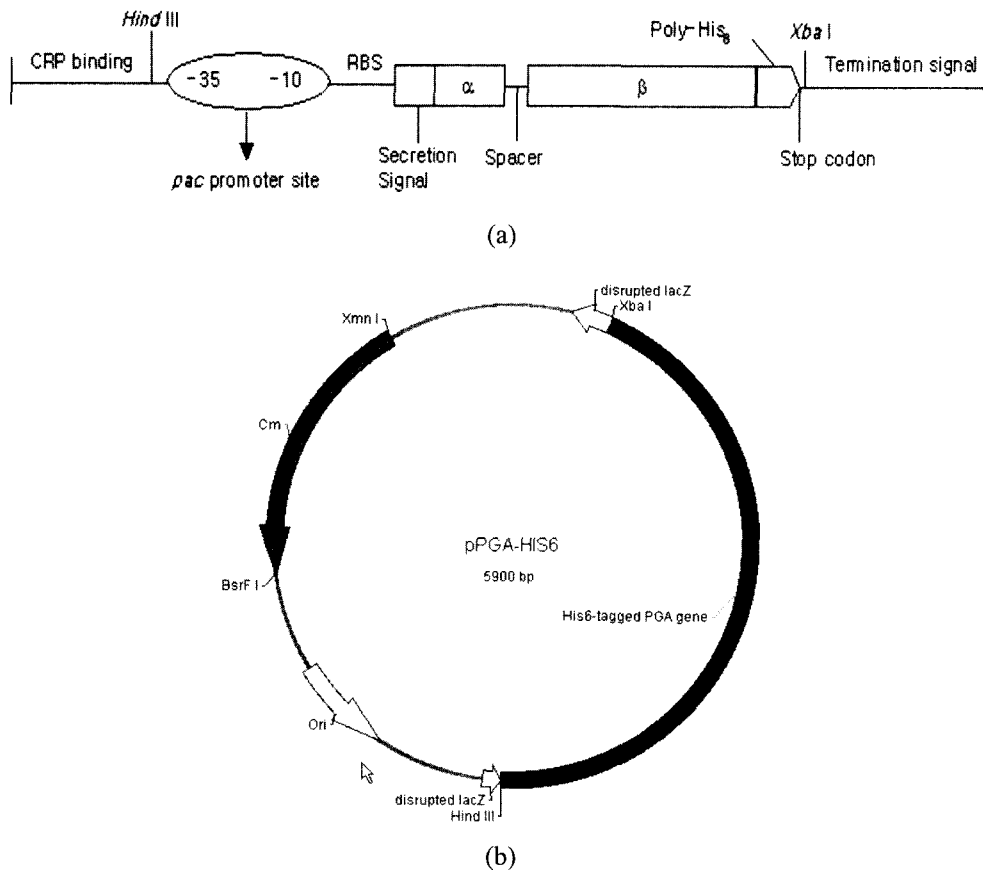Ori
disrupted lacZ
HindIII

(b)

Fig. 1. Structure of the cloned poly-His tagged PGA gene.
(a) α and β represent the small- and large-subunits coding regions, respectively. The poly-His tagged PGA fragment was inserted into pUC19 with HindIII and XbaI. (b) Schematic diagram of plasmid pPGA-HIS6. Expression of the acylase gene is regulated by the lac promoter. lacZ: β-galactosidase gene.

0.42% K_2HPO_4, 0.3% KH_2PO_4, pH 6.9) [12] containing 50 μg/ml of chloramphenicol. The cell pellet was resuspended in 30 mM Tris-Cl, 20% sucrose, 1 mM EDTA (pH 8) for osmotic shock. Then, the cells were mildly stirred using a magnetic stirring bar at room temperature for 10 min and collected by centrifugation at 10,000 ×g for 10 min at 4°C. The supernatant was discarded, the shocked pellet was resuspended thoroughly in ice-cold 5 mM $MgSO_4$ [5] and the cell suspension was stirred slowly for 10 min on ice. During this step, the periplasmic proteins were released into the buffer. The periplasmic fraction was obtained after centrifugation at 10,000 ×g for 10 min at 4°C. Also, the cell pellet was resuspended in 50 mM of phosphate buffer (pH 8) and disrupted by ultrasonication (5 s/5 s burst) to prepare the soluble and insoluble fractions.

The periplasmic fraction was concentrated by using the 10% TCA precipitation method [21] before SDS-PAGE analysis. Concentrated periplasmic proteins were added to equal volumes of 2× SDS-gel loading buffer and heated for 3 min at 70°C. The expression of recombinant PGA proteins was analyzed by 12% SDS-PAGE gel according to established protocols [21].

Preparation of Supports

A HiTrap™ HP column packed with 1 ml of Chelating Sepharose was washed with distilled water, filled with 0.5 ml of 0.1 M $NiSO_4$, and washed with distilled water. Periplasmic samples were dissolved in a start buffer [10 mM sodium phosphate (pH 7.4), 0.5 M NaCl]. In order to avoid clogging of the column, the sample was filtrated through a 0.45 μm filter. This step may not be essential because the periplasmic fraction was clear enough for the further purification step.

Activity Assay

The PDAB (p-dimethylaminobenzaldehyde) method [3, 6, 13, 14, 16, 17] by Balasingham et al. [2] was used for the activity analysis. A 0.25 ml of periplasmic fraction or eluted fraction was added to 0.25 ml of 15 mM penicillin G (sodium salt) and incubated for 3 min at 40°C. One unit of enzyme activity was defined as the amount of enzyme required to produce one μmole of 6-APA from 15 mM of penicillin G at 40°C.

Measurement of Protein Concentration

The protein concentration was estimated by using 0.1 ml of 0.1–1.4 mg/ml protein samples. Pre-mixed Bradford

reagent (Sigma Co.), using Coomassie blue [3, 10, 23], was prepared and bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Sequence Analysis of Penicillin G Acylase

The PGA gene was amplified from the chromosomal DNA of *E. coli* ATCC 11105. Consequently, *E. coli* JM109 cells were transformed with plasmids pPGA-1. The coding region for PGA was fully sequenced. Complete sequences were obtained by the dideoxy chain termination method using six oligonucleotides. Compared with the nucleotide sequence numbers reported by Schumacher *et al.* [24], the sequences between positions 1-2,593 were identical and the amino acid sequences for PGA are the same. The complete nucleotide sequence contained the PGA gene and flanking regions.

Construction of His₆-tagged PGA

The enzyme activity of PGA in the periplasmic fraction is inhibited under β-lactamase since this causes destruction of the β-lactam rings of penicillin G. Therefore, the chloramphenicol resistance gene (Cm) was inserted, which contains putative Shine Dalgano sequences, into an expression vector for PGA. It was substituted for the ampicillin resistance gene (Ap) between *Xmn*I and *Bsr*FI to test PGA activity immediately after the expression of PGA. The resistances of the pUC19-Cm vectors were estimated by the growth of *E. coli* JM109 transformed with pUC19-Cm on LB agar media containing 35 μg/ml of chloramphenicol. The findings indicate that the recombinant strain had high plasmid stability, since almost all cells harboring pUC-Cm were grown on agar plates in the presence of chloramphenicol. Putative regulation sites plus the ORF of the Cm gene have a size of 1.1 kb. The PGA gene was amplified from the chromosomal DNA of *E. coli* ATCC 11105. Codons encoding histidines, whose codon usage has been considered, were fused at the C-terminus of the PGA gene by PCR. The total poly-His tagged acylase gene had a size of 2.6 kb. Vectors harboring the poly-His tagged acylase gene (pPGAH-1) did not have a CRP binding site and the termination signal was from *lacZ*. Consequently, pPGA-HIS6 vector (5.9 kb) carrying both the Cm gene and his-tagged PGA gene was completed. Figure 2 shows fragment patterns of cloned plasmids cut with *Xmn*I and *Bsr*FI for construction of pPGA-HIS6 and those of the constructed vector obtained by digestion with restriction enzymes *Hind*III and *Xba*I.

Expression and Activity

E. coli ATCC 11105 strain needs phenylacetic acid (PAA) as an inducer and transcriptional effects with PAA need
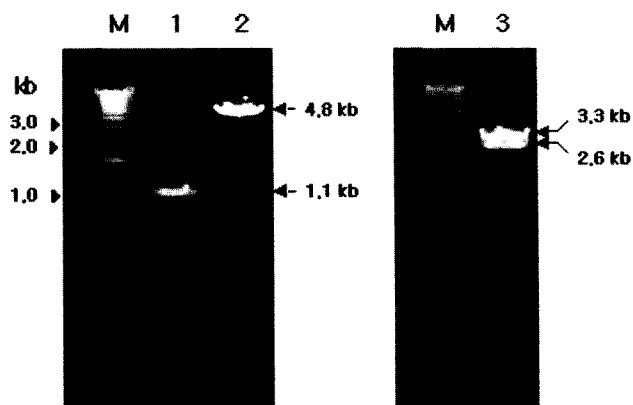

Fig. 2. Agarose gel electrophoresis patterns of cloned plasmids. Lane M, DNA size marker; lane 1, fragments removed Ap gene after digestion of pPGAH-1 with *Xmn*I and *Bsr*FI; lane 2, fragments coding Cm gene purified from pUC19-Cm cut with *Xmn*I and *Bsr*FI; lane 3, Fragments of pPGA-HIS6 (5.9 kb) cut with *Hind*III and *Xba*I. The gels contained 0.9% agarose.

nucleotide sequences upstream of the *Hind*III site in the PGA gene [10]. The poly-his tagged PGA fragment of 2.6 kb in the pPGA-HIS6 vector contains from *Hind*III to histidine hexamer followed by a stop codon at the C-terminal (Fig. 1). The protein expression in *E. coli* JM109 harboring pPGA-HIS6 vectors was optimized at 26°C by using casein medium (pH 6.9) containing 50 μg/ml of chloramphenicol and controlled by IPTG without PAA. The recombinant host carrying the PGA gene under control of the *lac* promoter produced active His-tagged acylases. The induction effect of IPTG was estimated by adding 0-1 mM IPTG as a final concentration. One fresh colony of transformed cells was cultured in 2 ml of medium containing 50 μg/ml chloramphenicol. The 1% of the cells grown overnight was inoculated into fresh medium. When OD₆₀₀ reached 0.6-0.9, the cells were induced by IPTG, and grown for 48 h. The final concentration of the cells was adjusted with the same optical density. After harvesting, the cell pellet was resuspended in 50 mM phosphate buffer (pH 8) and sonicated. The supernatant containing recombinant histidine-tagged acylase was then obtained. Without IPTG, an enzyme activity of 0.343 U/ml with a specific activity of 0.92 U/mg was detected, whereas with 1 mM IPTG that of 0.672 U/ml with a specific activity of 2.02 U/mg was detected in broth. Therefore, the specific activity with 1 mM IPTG was as high as 2.2-fold than that without IPTG. However, there were few differences of expression levels among the range of 0.1 mM-1 mM IPTG from the vector containing 2.6 kb poly-His tagged acylase. The fact that the acylase was fully expressed with 0.1 mM IPTG indicates that constitutively expressed acylase can be used for one-step purification. To obtain the periplasmic fraction, the cells were grown for 48-70 h. and induced with 1 mM IPTG when the OD₆₀₀

M 1 2 3 4 5 6

225 ▶
150 ▶
100 ▶
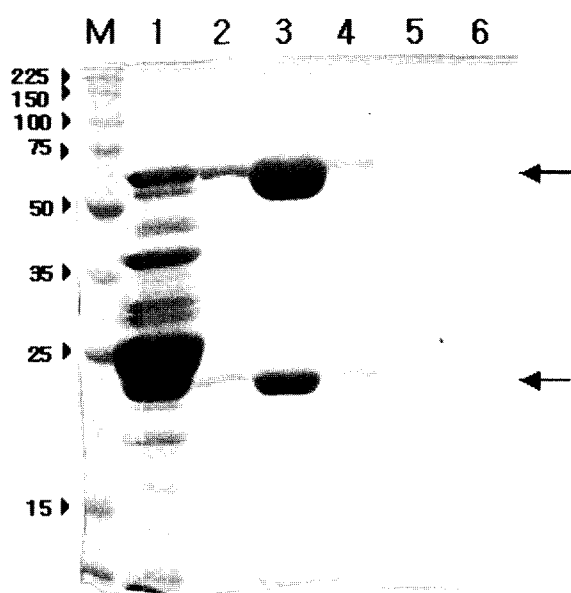75 ▶

50 ▶

35 ▶

25 ▶

15 ▶

Fig. 3. SDS-PAGE of poly-His tagged penicillin G acylase on 12% gel.
Lane M, standard protein marker (in kilodaltons); lane 1, periplasmic fraction after osmotic shock; lanes 2-6, eluted poly-His tagged PGA fraction #1-5. Gel was stained with Coomassie brilliant blue R-250. The upper and lower arrows indicate β (62 kDa) and α (23 kDa) subunits, respectively.

reached approximately 0.8. A 10 ml of the periplasmic fraction was obtained from 200 ml of the cell broth after osmotic shock. Recombinant acylase in the periplasmic fraction was analyzed using 12% SDS-PAGE (Fig. 3). Processing of the acylase with tagging was stable and mature acylase consisted of the two processed subunits.

The specific activity of the recombinant acylase from the periplasmic space was 5.29 U/mg in the presence of 0.1 mM IPTG. Its specific activity was 2.6-fold higher compared with the 2.02 U/mg of the crude extract (Table 2). In large-scale production, the recovery of acylase with the periplasmic fraction may require a small amount of resin. The acquisition of the periplasmic fraction by osmotic shock with inexpensive sucrose removed the major host proteins, which would result in cost savings.

Enzyme Purification

A chelating column charged by 0.1 M nickel salt solution was equilibrated with a start buffer (10 mM sodium phosphate (pH 7.4), 0.5 M NaCl). The enzyme solution was loaded using a syringe. After osmotic shock, the periplasmic fraction was dissolved in the start buffer plus 5 mM of $MgSO_4$, which did not affect resin affinity. Optimization of the washing and elution buffers was performed by stepwise gradient elution with 10 mM sodium phosphate (pH 7.4), 0.5 M NaCl containing 10 mM- 500 mM imidazole. The collected fractions were precipitated with TCA and analyzed by using SDS-PAGE. The phosphate buffer containing 40 mM imidazole was adequate as a washing buffer for the elution of almost all host proteins. Also, poly-His tagged PGA of high purity was eluted with 200 mM imidazole as an elution buffer. Thus, 10 mM sodium phosphate (pH 7.4) buffer containing 0.5 M NaCl and 40 mM imidazole was used as a washing buffer and 10 mM sodium phosphate (pH 7.4) buffer containing 0.5 M NaCl and 200 mM imidazole was used as an elution buffer for the purification of poly-His tagged PGA as determined by the optimization results. Equilibration with start buffer was followed by loading with periplasmic solution (18.28 U). The column was then washed with washing buffer and eluted with elution buffer. Eluted samples were analyzed by 12% SDS-PAGE after TCA precipitation. Figure 3 shows the concentration patterns of the eluted fractions. Histidine-tagged PGA proteins were identified to be 23 kDa (α subunit) and 62 kDa (β subunit). Major proteins were collected in fraction number 3. Table 2 indicates that the specific activity of purified acylase was 38.5 U/mg by activity assay. It was purified 7.3-fold with respect to the inlet periplasmic solution (5.29 U/mg). Adsorbed portion of recombinant acylase on resins charged with Ni^{2+} exceeded 95%. It was found from SDS-PAGE analysis that this one-step purification method by using histidine could produce pure PGA of high activity. In addition, host protein contamination was negligible and the recovery yield, based on activity, was about 70%. The purification of eluted fraction was 19-fold compared with the supernatant fraction (2.02 U/mg). Periplasmic fractions contain few components of fermentation broth and few salts. These properties have some merits on the readily changable buffer conditions and high efficiency of the resin. In industry, one-step purification may be performed in a simple batch reactor using affinity resin [1]. Consequently, pure acylase was recovered simply by using osmotic shock without any pre-treatment, and separated in an effective and rapid one-step manner by using IMAC.

Table 2. Purification profile of poly-His tagged penicillin G acylase.

Sample	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Activity recovery (%)
Periplasmic fraction	18.28	3.455	5.29	2.6	-*
Eluted total fraction (#1- 5)	12.68	-	-	-	70%
Eluted fraction (#2)	10.9	0.283	38.5	19	-

* Not measured.

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