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GLYCOSYLATION AND PH STABILITY OF PENICILLIN G ACYLASE FROM *PROVIDENCIA RETTGERI* PRODUCED IN *PICHIA PASTORIS*

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Abstract — Penicillin G acylase (PAC) is one of the most widely used enzymes in industrial synthesis of semi-synthetic antibiotics. The *Providencia rettgeri* pac gene was expressed to a level of 2.7 U/ml using the *Pichia pastoris* expression system. The recombinant enzyme was purified and its glycosylation status was determined. It was found that both subunits (α and β) of the enzyme were N-glycosylated, while the β-subunit also contained O-glycans. It was also observed that rPACP.rett. was stable in a wide range of pH, which, in addition to the previously proved high thermostability, makes it an attractive biocatalyst from an industrial point of view.

Key words: Penicillin acylase, glycosylation, pH stability, *Pichia pastoris*

UDC 577.18:577.2

INTRODUCTION

Biochemically active penicillin G acylase (E.C. 3.5.1.11; PAC) from *Providencia rettgeri* is a heterodimer which consists of an α-subunit (24.5 kDa) and a β-subunit (65 kDa) held together by noncovalent forces (Daumy et al., 1985). The given enzyme catalyzes the conversion of benzylpenicillin, via hydrolysis of the acyl group in the benzylpenicillin side chain, to release phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA), which is an important precursor utilized in industrial synthesis of the semi-synthetic antibiotics. The deacetylation reaction occurs at slightly alkaline pH (7.5-8.5). At lower to neutral pH values (4.0-7.0), the enzyme catalyzes the N-acylation of 6-APA with the analogs of PAA to produce semi-synthetic penicillins such as ampicillin, amoxycillin, and oxacillin (Shewale et al., 1990). It was found that the native enzyme's poor stability towards pH changes is an important problem for its application in the synthesis of semi-synthetic penicillins (Kazan et al., 1996).

In the search for an optimal expression system for $rPAC_{P.rett.}$ production on an industrial scale, the *pac* gene has been expressed in *E. coli* (Daumy et al., 1986; Chou et al., 2000), *Saccharomyces cerevisiae* (Ljubijankić et al., 1999), and *Pichia pastoris* (Ševo et al., 2002; Šenerović et al., 2006) expression systems. The efficiency of $rPAC_{P.rett.}$ production varied in these expression systems, as did enzyme characteristics such as specific activity, kinetic parameters, and stability.

The aim of this study was to analyze production of secreted biochemically active $rPAC_{P.rett.}$ using *P. pastoris* strain LN5.5 with four copies of the *pac* gene. In so doing, we found that the enzyme produced in this expression system has high thermo and pH stability, which is probably a consequence of the specific glycosylation pattern we observed.

MATERIALS AND METHODS

Strains and media

Providencia pastoris strain LN5.5 with four copies of the *pac* gene integrated into the genome was used for the production of *Providencia rettgeri* PAC (Šenerović et al., 2006). The yeast strain was cultured on YPD medium [1% (w/v) yeast extract, 2% bactopeptone, 2% (w/v) glucose], BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate,

pH 6.0, 0.13% yeast nitrogen base, 0.04% biotin, and 1% glycerol), and BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.3% yeast nitrogen base, 0.04% biotin, and 0.5% methanol). Depending on growth duration, 10 μl/ml and 100 μl/ml of the antibiotic tetracycline was added in order to prevent culture contamination.

Recombinant PAC production

The $rPAC_{P.\ rett.}$-producing strain was grown and induced in BMGY and BMMY, respectively. A measured volume (150 ml) of BMGY medium was inoculated with 0.15 ml of fresh overnight culture and incubated at 30˚C. After two days of growth, when optical density at 600 nm reached 18, the culture was centrifuged (4000xg, 15 min at room temperature) and the pellet resuspended in the same volume of BMMY medium. The culture was then incubated for 6 days under 1% methanol induction at 30°C with vigorous shaking (200 rpm).

Determination of PAC activity

Activity of PAC was measured in the cell-free medium by standard colorimetric procedure (Kutzbach et al., 1974). One unit (1 U) of enzyme activity is defined as the quantity of PAC catalyzing the hydrolysis of 1 μmol of 6-nitro-3-(phenylacetamido)-benzoic acid (NIPAB) per minute at 25°C.

Protein purification procedure

After six days of induction, cells of *P. pastoris* $rPAC_{P.\ rett.}$-producing culture were removed by centrifugation (4000xg, 30 min. at +4˚C). The supernatant (150 ml) was desalted and concentrated approximately 15-fold using an Amicon ultrafiltration unit, model 8400, equipped with a type membrane YM10 (Amicon Inc.). The sample was precipitated with ammonium sulfate to a final concentration of 85%. After precipitation, the sample was centrifuged for 20min in a Sorwall SS34 rotor at 11,500 rpm and a temperature of +4˚C.

The $rPAC_{P.\ rett.}$ was purified at room temperature with a low-pressure liquid chromatography system (GradiFrac, Pharmacia Biotech) using a slightly modified version of a previously published procedure (Ljubijankić et al., 2002). The process of $rPAC_{P.rett.}$ purification was monitored by the standard colorimetric assay based on hydrolysis of the synthetic substrate NIPAB.

After ammonium sulfate precipitation, the sample was dialyzed against 100 mM sodium phosphate buffer, pH 7, containing 1.7 M ammonium sulfate. The sample was applied to a HiLoad 16/10 phenyl Sepharose high-performance column (Pharmacia Biotech) and eluted with a linear gradient of from 0 to 1.7 ammonium sulfate and 100 mM sodium phosphate, pH 7.0.

All fractions containing PAC activity were pooled, dyalized against 20 mM Bis-Tris, pH 6.5, and loaded onto a HiLoad 16/10 Q Sepharose Fast Flow column (Pharmacia Biotech). Activity of PAC was eluted with a linear gradient of from 0 to 1 M NaCl in equilibration buffer (20 mM Bis-Tris, pH 6.5). After PAC activity assay, the relevant fractions were pooled and the sample was analyzed on 12.5% SDS/PAGE.

Electrophoretic analysis and immunoblotting

The SDS/PAGE procedure was performed using 12.5% - polyacrylamide gels as described by Hames and Rickwood (Hames and Rickwood, 1990). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

Western-blotting analysis was carried out as described by Burnette (1981) using polyclonal antibody against *P. rettgeri* PAC raised in rabbit (Ševo et al., 2002). The desalted and concentrated cell-free medium was loaded on SDS/PAGE (12.5%), and the separated proteins were transferred to a nitrocellulose membrane using the Semi-Dry Multiphor II system (Pharmacia) for 1 h at 0.8 mA per cm^2 of membrane. The membrane was blocked with 2% non-fat dried milk in washing buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20) and then subjected to immunoreaction with polyclonal anti–$rPAC_{P.\ rett.}$ antibodies. The secondary antibody (goat anti-rabbit immunoglobulin G) conjugated with alkaline phosphatase (Sigma) was used at 1:8000 dilution. Immunoblots were developed with nitro blue tetrazolium [5-bromo-4-chloro-3-indolyl phosphate

(BCIP/NBT)] as a color substrate according to instructions of the manufacturer (Promega).

Protein characterization

Mobility-shift experiments were performed with the peptide N-glycosidase F (PNGase F) (Roche) according to the instructions given by the supplier. The glycosylation status of secreted $rPAC_{P.\ rett.}$ was determined using the Immun Blot kit for glycoprotein detection (Bio-Rad, Hercules, CA, USA).

To determine pH stability, purified $rPAC_{P.rett.}$ was incubated in buffers with different pH values [50 mM sodium acetate (pH 3.0-5.5), sodium phosphate (pH 6.0- 7.0), Tris-HCl (pH 7.5-9.5)] at +4°C for 2 hours. The remaining PAC activity was assayed under the standard conditions described above. The experiment was repeated three times independently, and the data presented are mean values (± SD) of the triplicate determinations.

RESULTS AND DISCUSSION

Production and purification of $rPAC_{P.\ rett.}$

Pichia pastoris strain LN5.5 was cultured for six days under 1% methanol induction at 30°C, yielding 2.7 U/ml of recombinant protein. Such $rPAC_{P.rett}$ production is significantly higher than any yield obtained on a small-scale in *E. coli, S. cerevisiae* or *P. pastoris* hosts (Ljubijankić et al., 1999; Chou et al., 2000; Ševo et al., 2002).

The results of SDS/PAGE of total proteins secreted in extracellular medium of *P. pastoris*-induced culture is shown in Fig. 1a. Western blot analysis using anti-PAC antibodies of the culture supernatant from induced culture is shown in Fig 1b. Immuno blot analysis of $rPAC_{P.rett.}$ revealed the presence of two isoforms of the mature β-subunit and several smaller peptides interacting with $rPAC_{P.\ rett.}$ antibodies, probably products of unspecific $rPAC_{P.rett.}$ proteolysis. A band corresponding to the mature α-subunit of 24.5 kDa could not be detected.

In order to characterize the $rPAC_{P.\ rett.}$ secreted into the medium, the enzyme was purified using hydrophobic interactions and ion-exchange chromatography as described in Materials and Methods. Western blot analysis of the purified enzyme is shown in Fig. 2. Western blot confirmed the presence of two bands in the range of around 6 kDa that correspond to the size of the mature β-subunit, and two bands of 30 and 35-kDa that could be matched to glycosylated forms of the α-subunit (Fig. 2, lane 2). We previously showed that presence of two isoforms of the α-subunit is a consequence of the difference of six amino acids between them (Ljubijankić et al., 1999). Other bands that we supposed to be $rPAC_{P.\ rett.}$ proteolytic products were removed by purification (compare Fig. 1b, lane 2 and Fig. 2, lane 2).

Following Western blot, we further investigated the glycosylation profile of purified $rPAC_{P.\ rett.}$

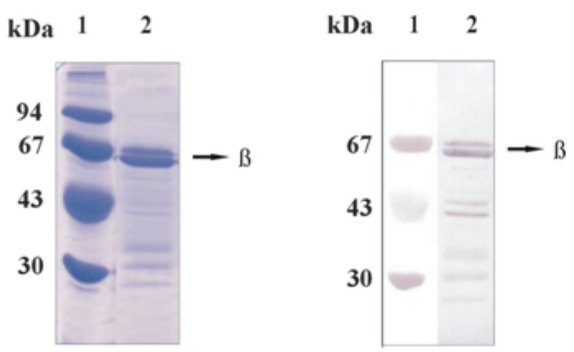

Fig. 1. Results of SDS/PAGE (a) and Western-blot analysis (b) of culture supernatant from *P. pastoris* strain LN5.5 expressing $rPAC_{P.\ rett.}$. Lanes: 1- LMW marker (Amersham, Pharmacia), 2- LN5.5 cell-free medium after 6 days of 1% methanol induction.

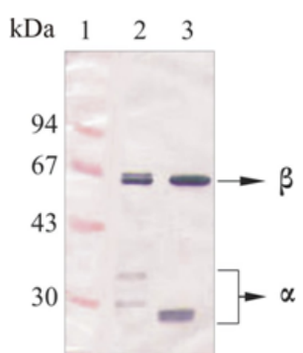

Fig. 2. Characterization of purified $rPAC_{P.\ rett.}$ by Western blot. Lanes: 1- LMW marker (Amersham, Pharmacia), 2- purified rPACP.rett. without PNG-ase F treatment, 3 - purified $rPAC_{P.rett.}$ with PNG-ase F treatment.

Characterization of rPAC$_{P. rett.}$ secreted by P. pastoris

For determination of the glycosylation status of rPAC$_{P.rett.}$ produced in *P. pastoris*, the enzyme was enzimatically deglycosylated with PNGase F, which cleaves the bond between asparagine and N-acetyl glucosamine. In order to remove sugars completely, rPAC was denatured beforehand by 10-min incubation at 95°C in a denaturating buffer.

In a mobility shift experiment performed with purified rPAC$_{P. rett.}$ treated with PNGase F, the larger fraction of the β-subunit disappeared, while the two α-subunits [which differ in six amino acids (Ljubijankić et al., 1999)] migrated faster. This indicated that the entire α-subunit and one part of the β-subunit were N-glycosylated (Fig. 2, lane 3).

Yeast cells utilize the same type of N-glycosylation recognition sequence (Asn-X-Ser/Thr) as do higher eukaryotic cells, but as Montesino et al. (1998) showed, neither the potential site for N-glycosylation nor the degree of carbohydrate addition can be predicted. From such low increase in molecular mass between different isoforms of both subunits (by not more than 5 kDa, Fig. 2), we can conclude that hyperglycosylation did not occur during the secretion of rPAC$_{P. rett.}$. This is in accordance with findings that the most common oligosaccharides of *P. pastoris*-secreted proteins are $Man_{8-14}GlcNAc_2$ (Montesino et al., 1998).

In order to detect possible presence of O-linked sugars, we performed immunostaining of sugars using the periodate oxidation (biotin hydrazide/avidin horseradish peroxidase) method. After immunostaining of a purified N-deglycosylated sample, a reaction with the β-subunit was observed, indicating the possible presence of O-glycans in this subunit (Fig. 3). Although the presence of N-glycosylation is well documented and is common for proteins expressed in *P. pastoris*, there is little information concerning the particular amino acid consensus sequence or structural features of the protein that directs or specifies the O-glycosylation site (Tsujikawa et al., 1996; Duman et al., 1998; Montesino et al., 1998). The obtained rPAC$_{P.rett}$ may be one of the few examples of recombinant proteins produced in *P. pastoris* where O-sugars were documented (Juge et al., 1996; Tsujikawa et al., 1996; Heimo et al., 1997; Brierley, 1998). It is also interesting that the profile of glycosylation of rPAC$_{P. rett.}$ secreted by *P. pastoris* completely differs from the rPAC$_{P. rett.}$ produced in *S. cerevisiae* (Ljubijankić et al., 1999). The α-subunits of rPAC$_{P. rett.}$ secreted by baker's yeast were O-glycosylated, while a significant number of β-subunit molecules were nonglycosylated.

Since the long-term stability of an enzyme is a major concern in its industrial application, we further examined the pH stability of purified rPAC$_{P. rett.}$ produced in *P. pastoris*. This analysis demonstrated that rPAC$_{P. rett.}$ produced in *P. pastoris* was stable in a wide range of pH, exhibiting almost 100% of total activity between pH 5.5 and 10 (Fig. 4). This high pH stability, together with the previously shown significantly higher thermostability (Ševo et al., 2002)

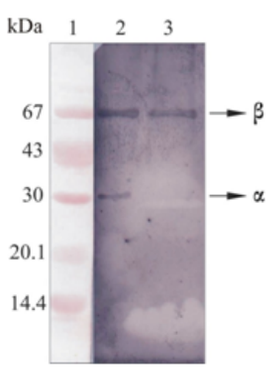

Fig. 3. Characterization of purified rPAC$_{P. rett.}$ by glycoprotein immunoassay. Lanes: 1- LMW marker (Amersham, Pharmacia), 2 - purified rPAC$_{P. rett.}$ without PNG-ase F treatment, 3- purified rPAC$_{P.rett.}$ with PNG-ase F treatment.

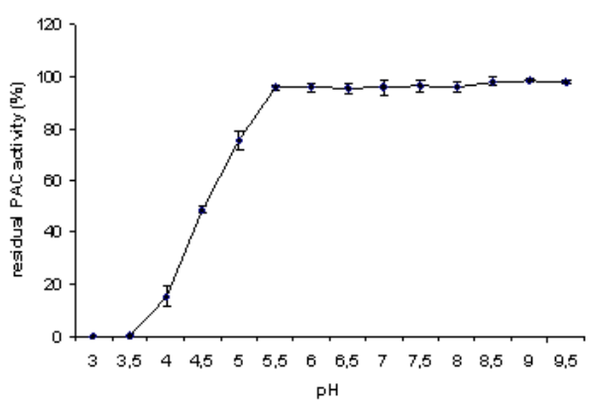

Fig. 4. The pH stability of purified rPAC$_{P. rett.}$

of this form of recombinant enzyme compared with *E. coli*- and *S. cerevisiae*-produced enzyme, make $rPAC_{P.rett.}$ secreted by *P. pastoris* a more attractive biocatalyst than its non-glycosylated counterparts produced in bacteria or in baker's yeast. It is a well-known fact that enzyme stability is influenced by factors such as hydration effects (Srivastava, 1991) and intramolecular and intermolecular cross-linking (Yamagata et al., 1994). There are many studies where a significant increase of enzyme stability towards heat, proteolysis, and storage was obtained using carbohydrate chains as stabilizing agents (Srivastava, 1991; Yamagata et al., 1994; Masárová et al., 2001). In view of these results, we presume that the observed variations of $rPAC_{P. rett.}$ stability are a consequence of different carbohydrate contents obtained in the two yeast hosts. Studying expression of penicillin G acylase from *Providencia rettgeri* in *Pichia pastoris* strain LN5.5, we demonstrated advantages of this expression system over previously used *E. coli* and *S. cerevisiae*. Besides the greater amount of bioactive $rPAC_{P. rett.}$ secreted by *P. pastoris*, we showed that the recombinant enzyme exhibited a completely different glycosylation profile, as well as significantly higher stability, which make it an attractive biocatalyst for the production of semi-synthetic antibiotics on an industrial scale. Further studies are required to determine the exact carbohydrate structure of the oligosaccharide of $rPAC_{P.rett.}$ from *S. cerevisiae* and *P. pastoris*.

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ГЛИКОЗИЛАЦИЈА И РН СТАБИЛНОСТ ПЕНИЦИЛИН Г АЦИЛАЗЕ ИЗ *PROVIDENCIA RETTGERI* ПРОИЗВЕДЕНЕ У *PICHIA PASTORIS*

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Пеницилин Г ацилаза (PAC) је један од најшире коришћених ензима у индустријској синтези полусинтетских антибиотика. У овом раду добијени ниво експресије PAC гена из *Providencia rettgeri* у експресионом систему *Pichia pastoris* износио је 2.7 U/ml. Рекомбинантни ензим је пречишћен и одређен је његов гликозилациони статус. Нађено је да осим што су обе субјединице ензима (α и β) N-гликозиловане, β субјединица садржи још и O-гликане. Такође је установљено да је рекомбинантна $PAC_{P.\ rett.}$ стабилна у широком pH опсегу што ју је, заједно са предходно установљеном високом термостабилношћу, учинило изузетно привлачним биокатализатором са индустријске тачке гледишта.