World J Microbiol Biotechnol (2011) 27:713–718
DOI 10.1007/s11274-010-0484-6

SHORT COMMUNICATION

The BapF protein from *Pseudomonas aeruginosa* is a β-peptidyl aminopeptidase

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Received: 16 March 2010 / Accepted: 14 June 2010 / Published online: 22 June 2010

Abstract A gene encoding a so far uncharacterized β-peptidyl aminopeptidase from the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1 was cloned and actively expressed in the heterologue host *Escherichia coli*. The gene was identified in the genome sequence by its homology to the S58 family of peptidases. The sequence revealed an open reading frame of 1,101 bp with a deduced amino acid sequence of 366 amino acids. The gene was amplified by PCR, ligated into pET22b(+) and was successfully expressed in *E. coli* BL21 (DE3). It was shown that the enzyme consists of two polypeptides (α- and β-subunit), which are processed from the precursor. The enzyme is specific for N-terminal β-alanyl dipeptides (β-Ala-Xaa). BapF hydrolyses efficiently β-alanine at the N-terminal position, including H-β^3hAla-pNA, H–D-β^3hAla-pNA and β-Ala-L-His (L-carnosine). D- and L-alaninamide were also hydrolysed by the enzyme.

Keywords *Pseudomonas aeruginosa* · β-peptidyl aminopeptidase · BapF

Introduction

Aminopeptidases are enzymes that catalyze the cleavage of amino acid residues at the N-terminal position of peptides and proteins and they are widely distributed in eukaryotic and prokaryotic organisms (Gonzales and Robert-Baudouy 1996). So called β-peptidyl aminopeptidases are the first family of enzymes which hydrolyze a variety of short β-peptides and peptides containing β-amino acids. Therefore, these enzymes were classified into the new peptidase family S58 according to the MEROPS database (Rawlings et al. 2010). Until now, only four β-peptidyl aminopeptidases from *Proteobacteria* have been isolated and biochemically characterized. The enzymes are synthesized as proproteins which are processed during their maturation into two polypeptides (α- and β-subunit). The mature β-peptidyl aminopeptidases are composed of four α- and four β-polypeptide chains (Geueke and Kohler 2007). β-peptidyl aminopeptidases of this type are predicted in archaea, bacteria, fungi and plants, but not in viruses or animals (Rawlings et al. 2010). However, the physiological role of these enzymes is still unknown and at present it is not clear whether the capability to hydrolyse β-peptides *per se* is their major physiological function.

The opportunistic human pathogen *Pseudomonas aeruginosa* possesses a variety of different proteases most of which have been described to contribute to virulence and play certain roles in establishment of *P. aeruginosa* infections (Lyczak et al. 2000). By homology comparison with known β-peptidyl aminopeptidases, we have identified the open reading frame *PA1486* in the genome sequence of *P. aeruginosa* PAO1 (Stover et al. 2000) encoding a so far hypothetical protein as a putative member of the peptidase family S58. In this article we address this protein to be a β-peptidyl aminopeptidase and the identification, cloning and expression of the gene we now name *bapF* as well as biochemical properties of the protein are described.

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Materials and methods

Bacterial strains, media and growth conditions

Escherichia coli DH5α (Table 1) was used as host for cloning of the β-peptidyl aminopeptidase *bapF*. For expression experiments *E. coli* BL21 (DE3) (Table 1) was used. All strains were grown in Luria–Bertani (LB) medium. Precultures for all experiments were prepared overnight in 10 ml LB medium in flasks at 30 or 37°C and were used to inoculate main cultures to initial optical densities of 0.05 ($O.D._{580\ nm}$) in LB medium. Main cultures were incubated at 30 or 37°C in a rotating shaker at 150 rpm. Plasmid-carrying *E. coli* cells were selected with 100 $\mu g \cdot ml^{-1}$ of ampicillin.

Cloning of the β-peptidyl aminopeptidase-encoding gene

Recombinant DNA techniques were performed as described by Sambrook et al. (1989). DNA fragments were amplified by PCR standard methods. DNA modifying enzymes (Fermentas) were used according to manufacturer's instructions. Plasmid DNA was prepared as described by Birnboim and Doly (1979) and by using the HiSpeed plasmid purification midi kit or, for genomic DNA from *P. aeruginosa*, the DNeasyTissue-Kit (Qiagen, Hilden, Germany).

The gene *bapF (PA1486)* (www.pseudomonas.com) was amplified by PCR from chromosomal DNA of *P. aeruginosa* PAO1 (Table 1) using primers *PA1486up* 5′-AAAAAG AGTCCATATGCGCGCTCGA-3′ and the reverse primer *PA1486dwn* 5′-AAAAAAAATTCTTAAGGCGTCCCGG CC-3′ introducing *Nde*I and *Eco*RI sites for the construction of the expression plasmid pEF. The gene was amplified within 30 cycles (2 min 95°C, 30 s denaturation at 95°C, 30 s annealing at 59°C, and 1:30 min extension at 72°C) and 10 min final extension at 72°C. The PCR product was digested and ligated into plasmid pET22b(+) (Table 1), resulting in the expression plasmid pEF (Table 1). After the cloning the host *E. coli* DH5α was transformed with the resulting plasmid. Finally, the insert was sequenced to verify the expression plasmid (Sequiserve, Vatterstetten, Germany).

Expression of recombinant *bapF* and preparation of cell extracts

E. coli BL21 (DE3) was transformed with the plasmid pEF carrying *bapF* under the control of the T7 promoter. Recombinant *E. coli* BL21 (DE3) harbouring pEF was subcultured at 30°C for 16 h in a flask containing 10 ml of LB medium supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin. To induce gene expression, isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.4 mM. The expression culture was inoculated with the overnight culture to an $O.D._{580\ nm}$ 0.05. The culture was grown at 30°C with rotary shaking until the $O.D._{580\ nm}$ reached 0.4–0.7. The culture was induced with 0.4 mM IPTG and further grown for 4 h using the same conditions. After this time the cells were harvested by centrifugation at 9,173*g* for 30 min at 4°C. Bacterial cells were suspended in 0.05 M sodium phosphate buffer (pH 6) and disrupted by sonification (Branson Sonifier, power 50%, 30 W). The soluble fraction was filtered through a 30 kDa cut off cell to eliminate proteins which are smaller than 30 kDa. The filtered soluble fraction was used for enzyme activity assays.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype/Phenotype	Reference or source
Strain		
E. coli		
DH5α	*supE44* Δ(*lacZYA-argF*)*U196* (Δ *80* Δ *lacZM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Woodcock et al. 1989
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (Δ *cIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*)	Studier and Moffatt 1986
P. aeruginosa		
PAO1	Wild-type	Holloway et al. 1979
Plasmids		
pET22b(+)	$P_{T7\Phi10}$ Ap^r ColE1 pelB $lacI^q$	NOVAGEN, Madison, USA
pEF	Ap^r; pET22b containing the 1.1 kb *Nde*I/*Eco*RI PCR fragment bearing the *bapF* gene	This work

[α] Abbreviations: Ap^r Ampicillin resistance

Enzyme activity assay

The enzyme assay for BapF was modified from Geueke et al. 2006. Enzyme activity was assayed routinely at 37°C by measuring hydrolysis of H-βhGly-pNA (Bachem, Weil am Rhein). The formation of *p*-nitroaniline was measured spectrophotometrically at 405 nm. The reaction mixture contained 5 mM H-βhGly-pNA, 50 mM Tris/HCl (pH 8), 10% (v/v) dimethylsulfoxide, and enzyme in limiting amounts. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 µmol *p*-nitroaniline per minute from H-βhGly-pNA under the above conditions. The hydrolysis of carnosine was measured at 37°C by quantification of the released amino acids β-Ala and L-His, respectively, with HPLC. Protein concentration was determined by the method of Bradford (1976). All other substrates were measured using the same method for the standard substrate. To determine the pH-optimum of BapF a universal buffer system was used (Teorell and Stenhagen 1938). The remaining enzyme activity after incubation of the enzyme for one hour at several temperatures was measured with the routine spectrophotometric assay to determine the temperature optimum of BapF.

Results and discussion

In homology searches using the BLAST algorithm, we found that the predicted product of gene *PA1486* from *P. aeruginosa* shows sequence homology to known β-peptidyl

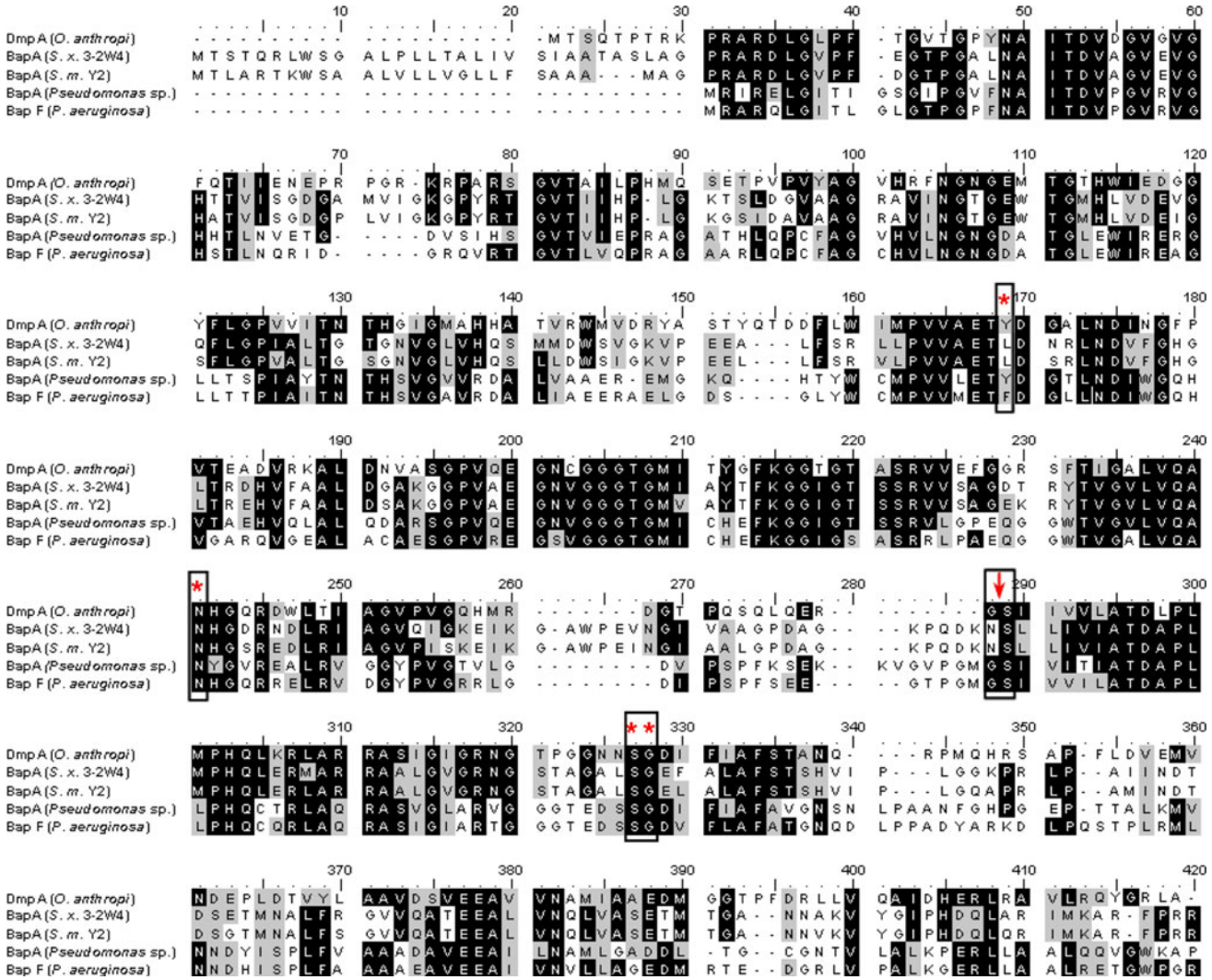


Fig. 1 Alignment of the amino acid sequence of BapF from *Pseudomonas aeruginosa* PAO1 and the known β-Peptidyl-aminopeptidase amino acid sequences from *Ochrobactrum anthropi* (DmpA), *Sphingosinicella* sp. strains 3–2W4 and Y2, and *Pseudomonas* sp. strain. Identical amino acids are marked in *black* and similar amino acids are marked in *gray*. The cleavage sites of the proteins are marked with an *arrow* and residues which are involved in the catalytic mechanism are marked with a *black box*. The sequences have the following accession numbers: DmpA from *O. anthropi* LMG7991, CAA66259; BapA from *Sphingosinicella xenopeptidylictica* 3-2W4, AAX93858; BapA from *Sphingosinicella microcystinivorans* Y2, ABC59253.1; BapA from *Pseudomonas* sp. MCI3434, BAE02664

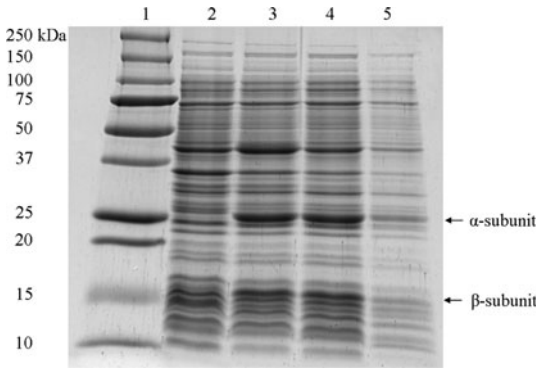


Fig. 2 SDS/polyacrylamide gel electrophoresis of BapF. *Lane 1* molecular-mass standard (BioRad, München); *Lane 2* crude cell extract from *E. coli* BL21 (DE3) harbouring the empty vector; *Lane 3* crude cell extract from *E. coli* BL21 (DE3) harbouring the expression vector; *Lane 4* soluble fraction; *Lane 5*: soluble fraction filtered through 30 kDa cut-off cell. According to the gel, polypeptides with a molecular mass of 24.7 and 13.8 kDa were designated as α- and β-peptide, respectively

aminopeptidases (Komeda and Asano 2005) (Fig. 1), but in the *Pseudomonas* database (www.pseudomonas.com) it was listed as a hypothetical protein. The protein we now name BapF showed highest sequence identity (69%) to a β-peptidyl aminopeptidase from another *Pseudomonas* sp. (Komeda and Asano 2005). The sequence identities for the other described β-peptidyl aminopeptidases are 45% (DmpA from *Ochrobactrum anthropi*) (Fanuel et al. 1999), 39 and 38% for the β-peptidyl aminopeptidases from *Sphingosinicella* sp. (Geueke et al. 2006).

Like the first described β-peptidyl aminopeptidase DmpA from *O. anthropi* (Fanuel et al. 1999), BapF has to be synthesised as a single polypeptide precursor. When *bapF* was expressed, two distinct bands with an apparent molecular mass of 24 and 14 kDa were observed in SDS–PAGE analysis (Fig. 2). This result perfectly agrees with the predicted molecular masses of the resulting subunits (24.7 and 13.8 kDa), when the preprotein is processed at the putative cleavage site between the Gly236-Ser237 peptide bond of the precursor, indicating that BapF is processed like other N-terminal nucleophile aminohydrolases (Ntn) of this type (Fanuel et al. 1999, Komeda and Asano 2005; Geueke et al. 2006). It presents functionally equivalent residues in the catalytic centre when compared with other Ntn hydrolases, and is therefore likely to use the same catalytic mechanism.

BapF is a β-Peptidyl aminopeptidase

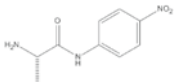
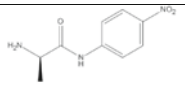
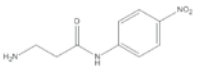
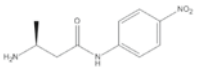
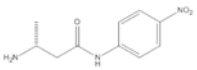
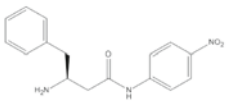
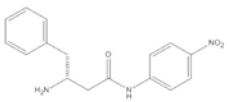
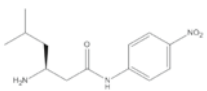
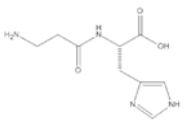
The *bapF* gene was cloned into the T7-based expression vector pET22b(+) and expressed in *E. coli* BL21 (DE3). The soluble fractions of the cells carrying the BapF expression plasmid or the empty vector as a control were used to determine peptidase activity of BapF. As expected, BapF failed to hydrolyse substrates of general proteases, e. g. no activity was detected with skim milk as a substrate (data not shown).

Table 2 Activity of BapF for the amino acid *p*-nitroanilides and the ▶ β/α-mixed dipeptide carnosine

The substrate specifity of BapF as a putative β-peptidyl aminopeptidase and its activity towards several substrates for this class of enzymes was measured under standard assay conditions at 37°C (Table 2). Hydrolysis activity was observed on substrates with an Alanine at the N-terminal side without any obvious preference for one of the enantiomers. In contrast, peptides harboring the aromatic amino acid phenylalanine were not cleaved. Interestingly, the naturally occurring β-peptide carnosine was a substrate for BapF. The catalytic mechanism that was postulated for DmpA of *O. anthropi* is similar to the mechanism of the other Ntn-hydrolases (Bompard-Gilles et al. 2000) and could be similar also for *P. aeruginosa* BapF, since the relevant amino acids are conserved in BapF (Fig. 1). According to this the free α-amino group of the catalytic active nucleophile Ser237 may act as a base and enhances the nucleophilic character of the hydroxyl group. The carbonyl carbon of the substrate would then be attacked by the Ser237 hydroxyl group and a covalent enzyme-substrate complex is formed. This oxyanion intermediate is stabilized by the backbone-NH-group of Phe130 and the side chain-NH_2 of Asn202. Ser275 and Gly276 are interacting with the Ser237 and could be indirectly involved in the catalytic mechanism. Glu128 is the only acidic amino acid close to the active site; it seems to bind the N-terminal α-amino group of the substrate molecule (Bompard-Gilles et al. 2000; Geueke and Kohler 2007).

Effects of pH and temperature on activity of BapF

BapF was further characterized and the pH- and the temperature optima were determined. The tests were done with the soluble fraction of extracts from cells of the expression cultures, harbouring the vector pEF. The pH-optimum of BapF was determined under standard assay conditions with a universal buffer adjustable to broad pH-range (Teorell and Stenhagen 1938). BapF showed a pH-optimum at pH 5.5, but was also found to be active at pH 6 to 7 and at a high alkaline pH 12. The enzyme is completely inactive at pH 4 and exhibited only 10 to 20% activity at a pH range from 7.5 to 11 (Fig. 3). The optimum under moderate acidic conditions is unique for BapF since all other known β-peptidyl aminopeptidases have optima under neutral or alkaline conditions. For example, DmpA from *O. anthropi* showed its pH-optimum in a neutral pH-range from pH 7.5 to 8.5, Bap A from *Pseudomonas* sp. at pH 9–10, 3–2W4

Substrate	Structure	Activity BapF
H-L-Ala·pNA		+
H-D-Ala·pNA		+
H-βhGly-pNa		+
H-L-β^{3}hAla·pNA		+
H-D-β^{3}hAla·pNA		+
H-L-β^{3}hPhe·pNA		–
H-D-β^{3}hPhe·pNA		–
H-L-β^{3}hLeu·pNA		–
Carnosine		+

Bap A from *Sphingosinicella xenopeptidilytica* between 8 and 9 and Y2 BapA from *Sphingosinicella microcystinivorans* at an alkaline pH of 10 (Geueke and Kohler 2007).

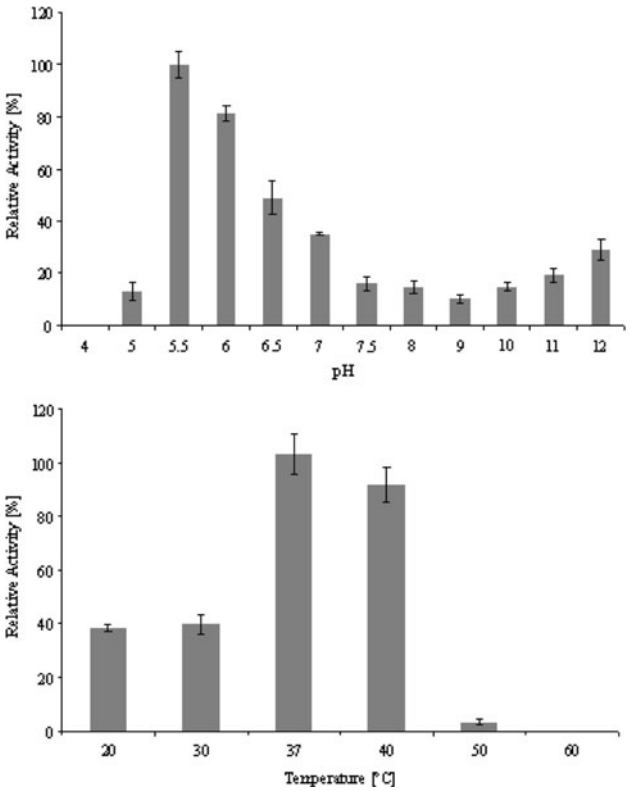


Fig. 3 Effects of temperature and pH on BapF. The maximal protease activities observed were set as 100% relative activity. The results are mean values of triplicate assays ∓SD

BapF shows significant substrate conversion from 20°C up to 40°C (38 and 91% of its maximum activity) (Fig. 3). The temperature optimum was at 37°C (Fig. 3) and the enzyme was fully inactivated when heated above 50°C which is comparable to the most homologous *β*-peptidyl aminopeptidase BapA from *Pseudomonas* sp. which lost its activity above 55°C (Komeda and Asano 2005).

The physiological function of *β*-Peptidyl aminopeptidases is currently not known. However, they have been suggested to have a certain potential in biotechnological applications. The design of unnatural *β*-peptides has attracted increasing attention over the last years. Peptides containing *β*-amino acids showed enhanced resistance towards proteolytic enzymes and therefore they show a high pharmaceutical potential. Increasing interest for compounds with *β*-peptide structures causes in turn an increasing demand for enantiopure *β*-amino acids as building blocks. Besides well-established chemical methods for the synthesis of enantiopure *β*-amino acids, enzyme-catalyzed reactions based on *β*-peptidyl aminopeptidases can be regarded as an interesting alternative (Heck et al. 2009). Thus BapF from *P. aeruginosa* as a novel member of this enzyme family may be a valuable tool in the repertoire of biocatalysts for these applications.

Acknowledgment Conflict of interests statement None.

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