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One step purification and characterization of the pyrrolidone carboxyl peptidase of *Streptococcus pyogenes* over-expressed in *Escherichia coli*

A. Awadé, Th. Gonzalès, Ph. Cleuziat* and J. Robert-Baudouy

Laboratoire de Génétique Moléculaire des Microorganismes, Bâtiment 406, Institut National des Sciences Appliquées, 20 avenue Albert Einstein, 69621 Villeurbanne cedex, France

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Pyrrolidone carboxyl peptidase (EC 3.4.11.8) (Pcp), an enzyme which selectively removes pyrrolidone carboxylic acid (PCA) from some PCA-peptides and -proteins, was demonstrated in bacteria and in plant, animal and human tissues. In this paper we describe the purification to homogeneity of the enzyme of *Streptococcus pyogenes*, over-expressed in *Escherichia coli*. This was achieved, for the first time in one step, by hydrophobic interaction chromatography. Analysis under non-denaturing conditions revealed a molecular mass of 85 kDa and in the presence of sodium dodecyl sulfate gave a molecular mass of 23.5 kDa. Investigations on enzymatic properties showed that the Pcp over-expressed in *E. coli* disclosed properties similar to those found for the enzyme extracted from *S. pyogenes* or for some other Pcps studied previously. Thus the over-expressed enzyme should serve as a suitable source for N-terminal unblocking prior to some PCA protein sequencing.

Peptidase; Pcp protein; Over-expression; Purification; *Streptococcus pyogenes*

1. INTRODUCTION

Pyrrolidone carboxyl peptidase (EC 3.4.11.8) (Pcp) activity has been demonstrated in a variety of bacteria, in plant, animal and human tissues [1–]. This oligomeric enzyme [7] is able to catalyze the hydrolysis of N-terminal pyrrolidone carboxylic acid (PCA) from PCA proteins or -peptides. This property has been used in protein sequencing to unblock such polypeptides [8] before undertaking Edman [9] sequential degradation.

Since Pcp was discovered in *Bacillus subtilis* [1], attempts have been made to purify the enzyme from different organisms [4,5,7,10]. In many cases, the purification was partial and needed numerous steps. Moreover the yields were relatively low because of the low abundance and the instability of this enzyme.

Recently, the molecular characterization of the structural gene encoding Pcp from *Streptococcus pyogenes* has been achieved [11]. In this paper, we describe the one-step purification to homogeneity of Pcp of *S. pyogenes* over-expressed in *E. coli*, using a hydrophobic interaction column. This work also explores the N-terminal sequence and the biochemical and enzymatic characteristics of this enzyme.

Correspondence address: A. Awadé, E.N.S.B.A.N.A., Laboratoire de Biotechnologie, Campus universitaire Montmuzard, 21100 Dijon, France. Fax: (33) 80 39 66 11.

Abbreviations: Pcp, pyrrolidone carboxyl peptidase; L-pyr-β-NA, L-pyroglutamyl-β-naphthylamide; L-pyr-*p*-NI, L-pyroglutamyl-para-nitroanilide; PCA, pyrrolidone carboxylic acid; OD, optical density.

Present address: Laboratoire des Sondes Nucléiques, bioMérieux SA, ENS de Lyon, 46, allée d'Italie, 69634 Lyon cedex 07, France.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

The optical densities (OD) were measured on a UVIKON spectrophotometer (KONTRON). A Progel-TSK Phenyl-5PW column (7.5 cm × 7.5 mm) was bought from Supelco.

Ammonium sulfate was purchased from Prolabo (Rhône-Poulenc). L-pyroglutamyl-β-naphthylamide (L-pyr-β-NA), L-pyroglutamyl-para-nitroanilide (L-pyr-*p*-NI), Fast red B salt, iodoacetamide and iodoacetate were obtained from Sigma. All other chemicals were analytical grade reagents.

2.2. *Over-expression of the Pcp*

Plasmid pPC39 (Fig. 1), which is a derivative of pT7-5 [12] including the *S. pyogenes* [13] *pcp* gene, was introduced in *E. coli* K38 [14] harboring plasmid pGP1-2 which includes the DNA encoding bacteriophage T7 RNA polymerase [12]. Cells were grown at 30°C in LB medium [15] containing ampicillin and kanamycin (50 μg/ml) until the OD at 595 nm reached 1.5. At this stage, the synthesis of bacteriophage T7 RNA polymerase from pGP1-2 was induced by shifting the culture temperature to 42°C. After 25 min growth, rifampicin (200 μg/ml) was added to the medium to shut off transcription by *E. coli* RNA polymerase and cells were cultured for 2 h at 37°C.

2.3. *Enzyme assays*

Qualitative detection of Pcp activity was based on the method previously described by Mulczyk and Szewczuk [16]. For 250 μl assays, an aliquot of the enzyme sample was mixed to a final volume of 225 μl with 0.05 M potassium phosphate buffer, pH 7, including 0.01 M EDTA. After 3 min incubation at 37°C, 25 μl of 10 mM L-pyr-β-NA in methanol was added to the reaction mixture. This mixture was then incubated for 30 min at 37°C and a drop of Fast red B salt solution (3 mg/ml in ethylene glycol monomethyl ether) was added to the assays. Samples containing Pcp activity showed a red-coloured complex of Fast red B and β-naphthylamine.

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Quantitative analysis of Pcp activity was performed by two methods, one with L-pyr-β-NA and the other with L-pyr-*p*-NI as substrates. Both methods are based on procedures previously described by Lee et al. [17] and Fujiwara and Tsuru [18]. An aliquot of the enzyme was mixed to a final volume of 0.9 ml with 0.05 M potassium phosphate buffer, pH 7, containing 0.01 M EDTA. After 3 min incubation at 30°C, the reaction was initiated by adding 0.1 ml of a 10 mM solution of L-pyr-β-NA in methanol or L-pyr-*p*-NI in *N,N'*-dimethylformamide. Pcp activity was then directly followed for 1.5 min by absorbance of the released β-naphthylamine at 340 nm or *p*-nitroaniline at 410 nm. The enzyme activity was expressed in units as the number of micromoles of product liberated per min.

2.4. *Analytical methods*

Protein concentration was determined using the Bio-Rad protein assay, with bovine gamma globin as a standard.

SDS-PAGE was performed on slab gels according to the method of Laemmli [19] using a 15% resolving gel (1 × 100 mm) and 5% stacking gel (1 × 20 mm). A protein marker kit from BRL was used for molecular weight calibration.

Molecular masses in native conditions were determined on non-denaturing polyacrylamide gels according to Hedrick and Smith [20] using, as standard proteins from Sigma, α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and β-galactosidase (116 kDa). Protein bands on polyacrylamide gels were revealed by Coomassie blue staining.

2.5. *N-Terminal sequence determination*

The N-terminal sequence of Pcp was determined by automated Edman degradation [9] of the purified protein on a gas-phase protein sequencer including on line identification of the phenylthiohydantoin derivatives.

3. RESULTS AND DISCUSSION

3.1. *Purification of the enzyme*

The purification steps of the over-expressed Pcp of *S. pyogenes* are summarized in Table 1. Cells were grown for over-expression in 250 ml medium as described above. All the steps of the purification were performed between 0 and 4°C. After 15 min centrifugation at 5,000 × *g*, the cell pellet was washed with the extraction buffer (potassium phosphate 0.05 M, pH 7, containing 0.01 M EDTA and 0.01 M β-mercaptoethanol), and resuspended in 10 ml of the same buffer. Bacteria were lysed by passage through a French pressure cell (AMINCO) at 7 MPa. After centrifugation of the lysate at 15,000 × *g* for 15 min, the supernatant was recovered and nucleic acids were precipitated by adding 1 vol. of 1% sulfate protamine to 6 vols. of supernatant. This step reduces the viscosity of the sample and therefore facilitates further chromatography. After 20 min centrifugation at 10,000 × *g*, the pellet was discarded and the supernatant was subjected to ammonium sulfate fractionation (40 and 55% ammonium sulfate saturation); precipitated proteins were recovered by centrifugation at 18,000 × *g* for 15 min. The 55% fraction containing the bulk of the Pcp protein was suspended in 1 ml extraction buffer containing 1 M ammonium sulfate. An aliquot of this preparation, generally 200–300 μl was applied to a Progel-TSK Phenyl-5PW column previously equilibrated with the extraction buffer containing 1.2 M ammonium sulfate. After thoroughly washing the column (20 min) with the same buffer, proteins were eluted with a decreasing gradient (extraction buffer containing 1.2 to 0 M ammonium sulfate) in an HPLC system lasting 45 min. 400 μl fractions were collected at a flow rate of 0.8 ml/min.

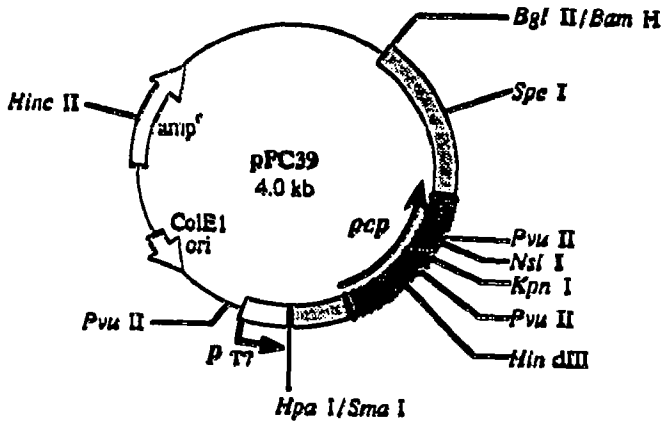

Fig. 1. Physical map of plasmid pPC39. The plasmid was constructed by insertion of a 1.6 kb *BamHI–HpaI* fragment containing the *pcp* gene of *Streptococcus pyogenes* (shaded area) into the *BglII–SmaI* sites of the expression vector pT7-5 [11]. In this plasmid the gene encoding pyrrolidone carboxyl peptidase (blackened arrow) is placed under the control of the T7 bacteriophage promoter (p_{T7}) for selective expression. The main restriction sites are indicated.

Table 1

Purification of *S. pyogenes* Pcp from an over-expression source

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (- fold)
Cell-free extract	137	845	6.2	100	1
Ammonium sulfate (55%)	58.6	556	9.5	65.7	1.5
TSK Phenyl-5PW	10.9	227	20.8	26.8	3.4

As described in section 2, after fractionated precipitation of proteins extracted from *E. coli* with ammonium sulfate, purification to homogeneity was carried out by TSK Phenyl-5PW chromatography. The Pcp activity was quantitatively tested using L-pyr-β-NA as substrate. One unit corresponds to the number of micromoles of β-naphthylamine released per min.

As shown in Fig. 2, two peaks (1 and 2) containing Pcp activity were eluted from the Progel-TSK Phenyl-5PW column. An identical protein band was present in the two peaks as shown by SDS-PAGE analysis (Fig. 3, lanes 3 and 4) and by non-denaturing PAGE (data not shown). The recovery of the enzyme was estimated to be approximately 30%.

In spite of the variety of Pcp enzymes which have been purified, no purification scheme capable of yielding a homogenous enzyme in one step has been developed to date. This is the first report of a single-step procedure involving hydrophobic interaction chromatography which leads to homogeneous Pcp by starting with an overproducing bacterial source. This chromatography has already been used to purify Pcp from guinea-pig brain which may exhibit hydrophobic character, but it was associated with other chromatographies such as gel filtration on Sephadex and calcium phosphate-cellulose [21]. The use of an hydrophobic interaction column to separate soluble Pcp from endogeneous proteins indicates that this protein has a relatively high degree of hydrophobicity, even if it is not a membrane-bound form such as that of *Klebsiella cloacae* [4].

One interest in our work lies in the fact that the enzyme from *S. pyogenes* has never been purified and characterized. Another important point is the speed of the purification (less than one day) and the relatively high yields, since the over-expressed enzyme represented approximately 30% of the total proteins in bacteria. Indeed the production of enzyme from a 250 ml culture medium was at least 10 mg whereas for Pcp's extracted from the original organisms the production was lower. For instance only 2.2 mg of Pcp was purified from *K. cloacae* cell-free extracts containing 2.2 g of total protein [4], whereas in our study we have purified approximately 11 mg protein from a cell-free extract containing 137 mg of protein.

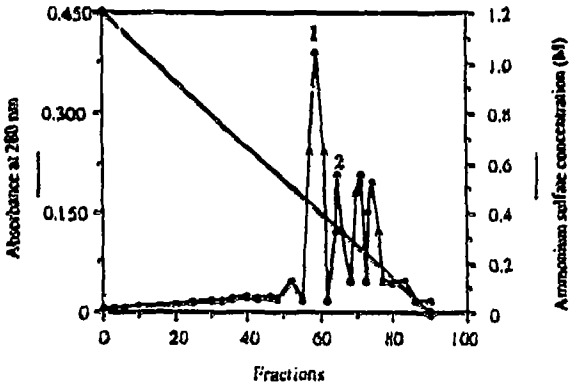

Fig. 2. HPLC purification of *S. pyogenes* Pcp from the 55% ammonium sulfate fraction of proteins extracted from *E. coli* containing the over-expressed enzyme. Chromatography was performed on a Progel-TSK Phenyl-5PW column. The absorbance at 280 nm was detected and fractions were qualitatively assayed for Pcp activity as described in section 2. Two peaks contained Pcp activity: (1) fractions 55–62 and (2) fractions 63–68.

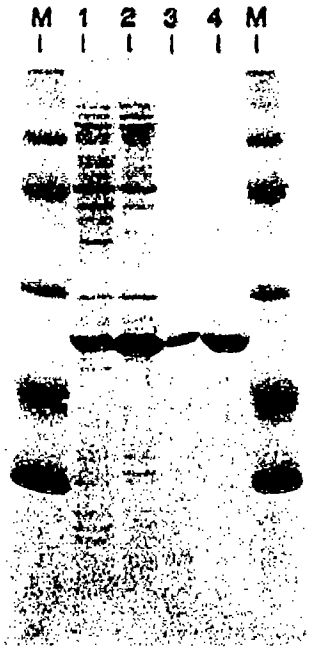

Fig. 3. Purification of pyrrolidone carboxyl peptidase. SDS-PAGE of the different steps of the purification procedure. Lane 1, 30 μg of soluble cell free protein fraction; lane 2, proteins precipitated at 55% ammonium sulfate saturation; lanes 3 and 4, 3 and 10 μg, respectively, of proteins contained in any of the two peaks displaying Pcp activity after chromatography on Progel-TSK Phenyl-5PW. Lane M, molecular mass standards; from bottom to top (kDa): 15.1, 17.8, 29, 41.8, 66, 97.4 and 204.9.

3.2. *Biochemical properties and N-terminal sequence analysis of the* S. pyogenes *Pcp*

The isoelectric point of the Pcp of *S. pyogenes* was estimated by isoelectric focusing to be 5.3; this allowed the use of non-danaturing polyacrylamide gels at pH 8.8 to determine the native protein molecular mass. Purified Pcp protein was run in 7.5, 9, 11, and 13% non-danaturing polyacrylamide gels. The relative migration of each protein was determined in three repeat experiments and the molecular mass of the Pcp protein was determined to be 85 kDa, according to Hedrick and Smith [20]. The molecular weight of the protein as estimated by gel filtration was approximately 80 kDa [11]. The molecular weight of the purified protein was also determined by 15% SDS-PAGE and the mean molecular mass from three experiments was 23.5 kDa. These results indicated that this enzyme should be a tetramer. These propeties are similar to those found for the *B. subtilis* Pcp [22]. It is interesting to mention that in another species of *Streptococcus*, *S. faecium*, the molecular mass of Pcp was estimated to 42 kDa by SDS-PAGE [5]; thus twice the molecular mass determined by the same method for the enzyme of *S. pyogenes*. This divergence in the same

genus shows the structural complexity of this class of enzyme. Moreover, it has been shown that the enzyme extracted from human skeletal muscle [10] has a molecular weight of 22 kDa determined by gel filtration; is this enzyme a monomer?

The N-terminal amino acid sequence obtained by sequential degradation of the protein: MKILVTGFDPFGGEAINP corresponds to that deduced from the nucleotide sequence of the *pcp* gene [11]. This is evidence that we have in fact over-expressed the cloned *pcp* and that this protein does not need any post-translational signal sequence cleavage to be functional [23]. This means that the enzyme has a cytoplasmic location, as is the case for Pcp's from *B. amyloliquefaciens* and different *B. subtilis* strains [7,22]. Although the role of this class of peptidases has not been investigated thoroughly enough in microorganisms, one might think that Pcp should be involved in intracellular protein metabolism. Pcp has been characterized as one of the enzymes catabolizing thyrotropin-releasing hormone in rat brain [24], thus this enzyme should be involved in hormonal metabolism in animals.

3.3. *Enzymatic properties of Pcp of* S. pyogenes

Enzymatic properties were studied by using L-pyr-β-NA and L-pyr-*p*-NI as substrates. No spontaneous hydrolysis was observed during our experiments. For all the investigations, controls without enzyme were carried out.

The K_m values were determined at pH 7. The concentrations used ranged from 0.1 to 2 mM for L-pyr-β-NA and from 1 to 15 mM for L-pyr-*p*-NI. Substrates were dissolved in 0.1 ml of methanol or N,N'-dimethylformamide, respectively, so that the final concentration of these solvents in the reaction mixture was always 10%. The K_m values of the enzyme were determined, according to Lineweaver–Burk plots, to be approximately 1.79 mM for L-pyr-β-NA and 20 mM for L-pyr-*p*-NI. The purified enzyme contained in the two peaks (see section 3.1.) presented almost the same catalytic properties. Similar values were also obtained for the enzyme extracted from *S. pyogenes*. The K_m value of *S. pyogenes* Pcp for L-Pyr-β-NA is also similar to that of the Pcp of *B. subtilis* for the same substrate [1]. Even if the K_m values seem to be high, that of the *S. pyogenes* enzyme for L-pyr-β-NA is consistent with those found for Pcp's from other bacteria [4,5,7].

The pH dependence of Pcp activity was studied in 0.05 M sodium citrate buffers (pH 5.0–7.0), 0.05 M potassium phosphate buffers (pH 5.5–8) and 0.05 M Tris-HCl buffers (pH 7–9.3) containing 0.01 M EDTA, with 1 mM L-pyr-β-NA as substrate. The absorbance of β-naphthylamine at 340 nm is maximal and constant in the pH ranges used [17]. The enzyme exhibited an optimal activity at pH 7. This optimum pH is similar to that of Pcp of *B. subtilis*.

The influence of some metal ions was investigated using 1 mM L-pyr-β-NA as substrate (data not shown). These ions were used as chloride salts and the activities were determined in a 0.05 M Tris-HCl buffer, pH 7, after 3 min incubation at 30°C. The enzyme was inhibited by Hg^{2+} and, to a lesser magnitude, by Zn^{2+} and Co^{2+}. Other ions such as Mg^{2+}, Ca^{2+} and Mn^{2+} had almost no effect on the enzyme activity. The effects of divalent ions were similar on *B. subtilis* and *S. pyogenes* Pcp activity. The enzyme was also inhibited by sulphydryl-blocking reagents such as iodoacetamide and iodoacetate (data not shown), suggesting that Pcp from *S. pyogenes* should be an SH enzyme. The amino acid sequence of the *S. pyogenes* Pcp, deduced from the nucleotide sequence of the the gene, showed that this protein contains four cysteine residues [11]; a cysteine residue should form a part of the catalytic site of *S. pyogenes* Pcp. This enzyme is also inhibited by 2-pyrrolidone, which was shown to be a competitive inhibitor of Pcp of *S. faecium* [5].

Regarding enzymatic properties, Pcp's from *S. pyogenes* and *B. subtilis* [1] are very similar. This is in agreement with the relatively high sequence homology between these two proteins [11].

3.4. *Stability of the purified Pcp*

The effects of subsequent freezing and thawing were studied by freezing the purified enzyme sample in 0.05 M phosphate potassium buffer containing 0.01 M EDTA and 0.01 M β-mercaptoethanol. Samples were frozen for 1 h at −20°C and slowly heated on ice. These studies revealed that only 67% of the activity is recovered after one cycle; after 5 series of freezing–thawing, the remaining activity represented 30% of the initial activity.

The study of the enzyme stability (in the same buffer) for a relatively long period at −20°C, 4°C and 24°C revealed that almost 60% of the initial activity was recovered after 1 month storage at 4°C, but only 43% of the initial activity was recovered after the same time at room temperature. Approximately 75% of the activity was recovered after 15 days at 4°C and 60% after the same time at room temperature. At −20°C, besides the effect described previously (67% recovery following one freezing-thawing cycle) the initial activity was almost unaltered after two months storage.

3.5. *Conclusion*

The pyrrolidone carboxyl peptidase produced by over-expression of *S. pyogenes pcp* gene possesses biochemically and enzymatically similar properties to those known for Pcp purified from different organisms. Thus this enzyme purified to high quantities should serve as a suitable source for N-terminal unblocking prior to protein sequencing. Site-directed mutagenesis on the gene, especially in the area highly conserved between *S. pyogenes* and *B. subtilis pcp* genes, and expression of the resulting proteins should allow the determination of the

role of some amino acids in the catalytic activity and in the enzyme stability. Moreover, since high quantities of the purified protein could be produced, cristallization of the enzyme should be undertaken to study its structure.

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