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Purification and characterization of recombinant pyrrolidone carboxyl peptidase of *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis pyrrolidone carboxyl peptidase (Pcp) overexpressed in *Escherichia coli* was purified to homogeneity in less than 12 h using ammonium sulphate precipitation and hydrophobic interaction chromatography. The enzyme, which removes amino-terminal L-pyroglutamic acid from peptides, appears to be a tetramer of 25 200 molecular mass subunits. The protein cross-reacted with polyclonal antibodies raised against Pcp from *Streptococcus pyogenes*. The overexpressed enzyme exhibits an absolute substrate specificity towards N-terminal pyroglutamyl residues with a Michaelis constant of 1.04 m*M* for L-pyroglutamyl-β-naphthylamide. The enzyme could be used for the removal of pyroglutamyl residues that block amino termini of proteins and peptides before performing Edman sequential degradation.

INTRODUCTION

Pcp (L-pyroglutamyl peptide hydrolase, EC 3.4.11.8), an enzyme that selectively removes amino-terminal L-pyroglutamic acid from peptides and proteins, is present in many bacteria [1]. It is also found in plant, animal and human tissues [2]. The enzyme is used in protein sequencing to unblock proteins and polypeptides with an N-terminal pyroglutamyl residue [3]. Many purification procedures for these enzymes have been described, but all of them are long and time-consuming. Moreover, these procedures involve several chromatographic steps and the yields are relatively low [4–9].

From *Bacillus subtilis*, in which it was first studied, the enzyme has been only partially purified [1]. Recently, the structural genes encoding Pcp from this bacteria and from *Streptococcus pyogenes* have been cloned and characterized in our laboratory [10,11]. This has allowed us to overexpress the enzymes in *Escherichia coli*.

The present work describes an improved scheme for the purification of *B. subtilis* Pcp after its over expression in *E. coli*. In addition, some

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biochemical, enzymatic and immunological properties of the enzyme are reported.

EXPERIMENTAL

Materials

The Progel-TSK phenyl-5PW column (75 mm × 7.5 mm I.D.) was purchased from Supelco.

L-Pyroglutamyl-β-naphthylamide, fast red B salt, alanine-containing dipeptides, Pcp from *Bacillus amyloliquefaciens*, anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugates, 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium were obained from Sigma. Ammonium sulphate was purchased from Prolabo. All other reagents used were of analytical grade.

Pcp production

Plasmid pTG1 [10] is a derivative of pT7-5 [12] containing the entire *pcp* gene of *B. subtilis* 168. This plasmid was introduced into *E. coli* K38 [13] harbouring plasmid pGP1-2, which includes the DNA encoding the bacteriophage T7 RNA polymerase [12]. Cells were grown at 30°C in a Luria broth (LB) medium [14] containing ampicillin and kanamycin (50 μg/ml) until the absorbance 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. Cells were cultured for an additional 2 h and harvested.

Enzyme purification

All the steps of the purification were conducted at 4°C in the presence of 10 m*M* 2-mercaptoethanol and 10 m*M* EDTA.

After growth in 250 ml of medium for Pcp over expression, cells were centrifuged at 5000 *g* for 15 min and resuspended in 10 ml of extraction buffer (50 m*M* potassium phosphate, pH 8). The bacteria were disrupted in a French pressure cell (Aminco) at 7 MPa, and cell debris was removed by centrifugation at 15 000 *g* for 15 min. In order to precipitate nucleic acids, 1% protamine sulphate was added dropwise to the supernatant to a final concentration of 0.15%. After centrifugation, the proteins were precipitated by addition of solid ammonium sulphate. Proteins precipitating between 40 and 55% saturation were collected by centrifugation at 18 000 *g* for 15 min and then the pellet was resuspended in 1 ml of extraction buffer containing 1 *M* ammonium sulphate. All the Pcp activity was in this precipitated fraction. A 0.25-ml aliquot of this preparation was applied to a Progel-TSK phenyl-5PW column previously equilibrated with the extraction buffer containing 1.2 *M* ammonium sulphate. The column was washed with the same buffer, and the proteins were eluted at 0.8 ml/min with a decreasing linear gradient of ammonium sulphate (1.2 to 0.0 *M*) in a high-performance liquid chromatographic (HPLC) system (Waters). Fractions (0.4 ml) were collected with an automatic fraction collector.

Assay of Pcp activity

Qualitative detection of Pcp activity was based on the method previously described by Mulczyk and Szewczuk [15]. Quantitative detection of activity was based on that described by Lee *et al.* [16] using L-pyroglutamyl-β-naphthylamide as substrate. Enzyme activity was expressed in units. One unit was the number of micromoles of β-naphthylamine liberated per minute.

Hydrolysis of dipeptides

The rates of hydrolysis of alanine-containing dipeptides were determined at 30°C using a conductimetric method as previously described by Ballot *et al.* [17] and Manika-Saizonou *et al.* [18]. A 4.5-ml aliquot of the 1 m*M* substrate solution in 5 m*M* Tris–HCl (pH 8.5) was added to a 5-ml conductimetric temperature-regulated cell (type MCCD, Solea-Tacussel, Villeurbanne, France). After thermal equilibrium, enzyme was added and the increase in conductance due to hydrolysis of the substrates was measured between 5 and 40 min after enzyme addition (temperature = 30 ± 0.01°C) with a B-640 Wayne Kerr bridge.

Analytical methods

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [19]. Molecular masses in native conditions were determined on non-denaturing polyacrylamide gels (7.5, 9, 11 and 13% acrylamide) according to Hedrick and Smith [20]. Coomassie blue was used for staining protein.

Protein determination. Protein concentrations were determined using the Bio-Rad protein assay with bovine gammaglobulin as standard.

Other methods

Preparation of antisera. Polyclonal antibodies against *S. pyogenes* Pcp were obtained in rabbits by a series of four weekly injections of the purified protein emulsified with complete Freund's adjuvant. Antibodies used in our experiments were collected seven days after the last inoculation.

Western blot analysis. Immunoblots were prepared essentially according to the procedure of Towbin *et al.* [21]. After the proteins had been electrophoretically transferred, the nitrocellulose membranes were blocked for 2 h at 37°C by immersion in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (20 m*M* phosphate, 150 m*M* sodium chloride, pH 7.2). They were then incubated overnight at room temperature with rabbit *S. pyogenes* Pcp antiserum diluted in PBS containing 0.05% Tween and 0.5% BSA. After subsequent washings the sheets were incubated for 4 h in the same buffer containing alkaline phosphatase-conjugated goat anti-rabbit IgG at 1 μg/ml. Serological reactions were detected by the colorimetric method described by Blake *et al.* [22].

N-Terminal sequence determination. The N-terminal sequence of the Pcp from *B. subtilis* was determined by automated Edman degradation [23] of the purified protein (240 pmol) on a gas-phase protein sequencer coupled to a phenylthiohydantoin analyser.

Thin-layer chromatography. Thin-layer chromatography was carried out on silica gel plates (Kieselgel 60 F_{254}, Merck). Elution was effected with butanol–acetic acid–water (4:2:2, v/v) and spots revealed by ninhydrin (1% in methanol).

RESULTS AND DISCUSSION

Purification and biochemical characterization of the overexpressed Pcp

B. subtilis 168 recombinant Pcp was purified to homogeneity from *E. coli* with the procedure described. The protein elution profile from the Progel-TSK phenyl-5PW column shows a major peak that corresponds to Pcp activity (Fig. 1). The enzyme was eluted from the column at approximately 0.24 *M* ammonium sulphate, which implies a relatively high degree of hydrophobicity. The step allowed a total separation from endogenous proteins.

The results of each stage in a typical purification are shown in Table I and the protein profiles on SDS-PAGE of the corresponding steps are shown in Fig. 2. The purified protein preparation

TABLE I

PURIFICATION OF *B. SUBTILIS* Pcp FROM *E. COLI*

Activity was determined using 1 m*M* L-pyroglutamyl-β-naphthylamide as substrate. One unit corresponds to the amount of enzyme required to hydrolyse 1 μmol of substrate per min at 30°C and pH 7.0.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification factor
Cell-free extract	130	330	2.5	100	1
Ammonium sulphate (55%)	23.4	235	10	71	3.95
TSK phenyl-5PW	4.5	96	21.2	29	8.38

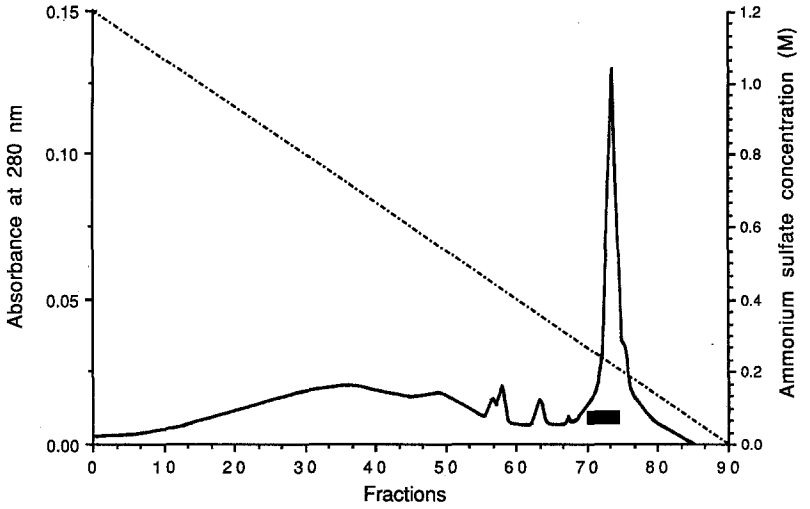

Fig. 1. Protein elution profile detected at 280 nm from Progel-TSK phenyl-5PW (solid line). The column was eluted with a linear decreasing gradient from 1.2 to 0 *M* ammonium sulphate in 0.05 *M* phosphate buffer pH 8 (dashed line). The major peak corresponds to Pcp activity. Pooled fractions are indicated by the solid bar.

gives a single band on SDS-PAGE corresponding to an M_r of 25 200 (Fig. 2, lanes 4 and 5). This result is in agreement with the M_r of 23 777 predicted from the nucleotide sequence of the gene [10]. The M_r of the native enzyme determined as described by non-denaturing PAGE is approximately 91 000. These results suggest that the active form of the enzyme is a tetramer. The purification scheme we used allowed us to obtain more than 4 mg of pure Pcp from a 250-ml culture (Table I). The yield of the purified enzyme was about 30% and was higher than those obtained by other published procedures [5–9].

The purification procedure presented in this paper provides a simple and rapid (less than 12 h) method for the purification of Pcp overexpressed in *E. coli*. The same procedure has been used to purify to homogeneity the *S. pyogenes* JRS4 Pcp overexpressed in *E. coli* (in preparation).

Immunological studies

Fig. 3 shows Western blot analyses obtained with different protein extracts in denaturing conditions, using polyclonal antibodies raised against *S. pyogenes* JRS4 Pcp.

S. pyogenes Pcp antiserum specifically reacts with Pcp in crude extracts from *S. pyogenes* and from *E. coli* containing the *S. pyogenes* overexpressed protein. Crude extracts from *S. pyogenes*

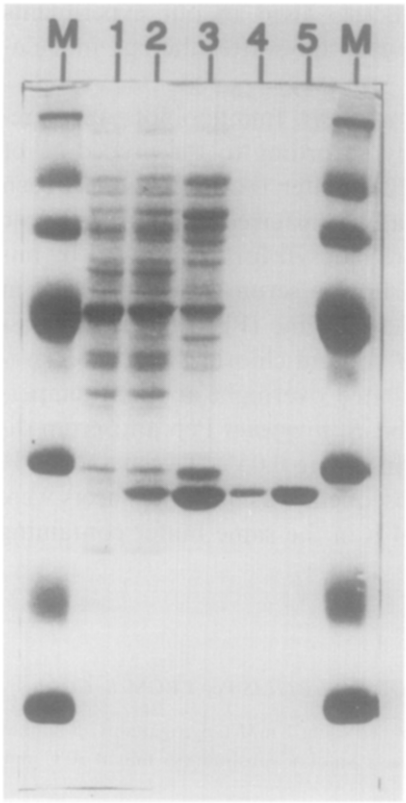

Fig. 2. SDS-PAGE of the different stages in the purification procedure. Lanes: M = molecular mass markers. From bottom to the top: 15.1 kDa, 17.85 kDa, 28.25 kDa, 41.8 kDa, 67.45 kDa, 103.15 kDa and 204.9 kDa; 1 = cell-free extract from *E. coli* K38 harbouring pT7-5 plasmid (reference, 40 μg); 2 = cell-free extract from *E. coli* K38 harbouring pTG1 plasmid (containing the *pcp* gene from *B. subtilis*, 40 μg); 3 = proteins precipitated at 55% ammonium sulphate (40 μg); 4 and 5 = purified Pcp (5 and 15 μg, respectively).

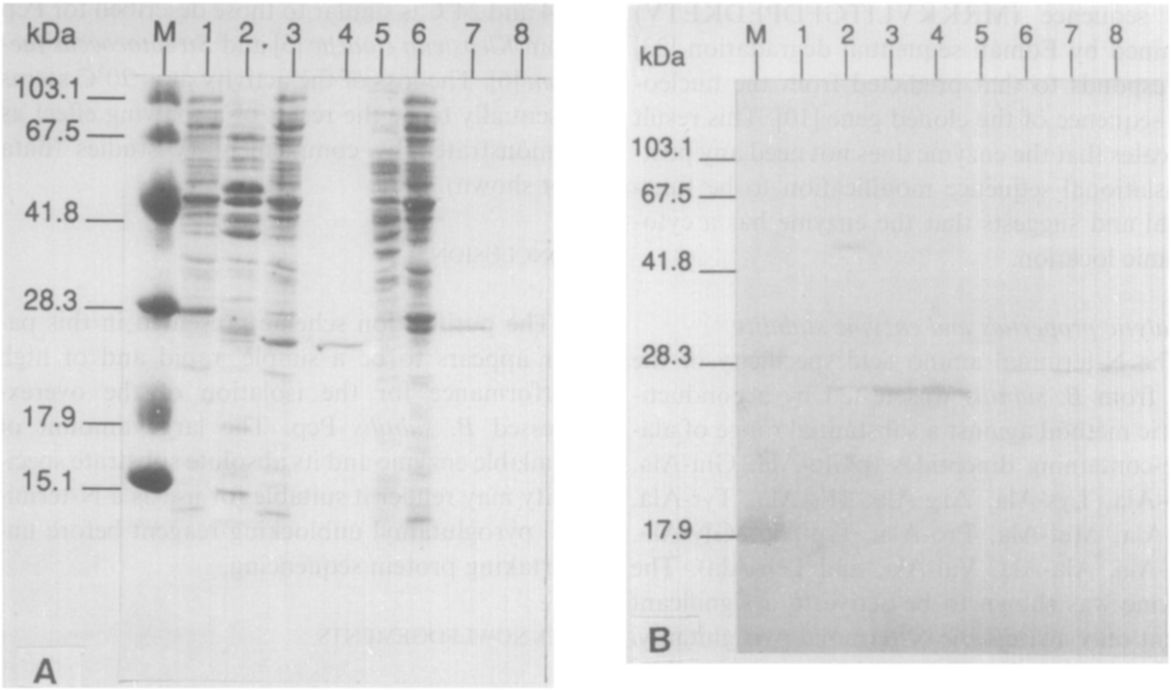

Fig. 3. SDS-PAGE (A) and Western blotting (B) of diverse sample. Lanes: M = precoloured molecular mass markers; 1 = cell-free extract from *E. coli* K38 harbouring pT7-5 plasmid (60 and 1 μg, respectively); 2 = cell-free extract from *S. pyogenes* JRS4 (60 and 10 μg); 3 = cell-free extract from *E. coli* K38 harbouring pT7-5 with the *pcp* gene from *S. pyogenes* as insert (60 and 1 μg); 4 = purified Pcp from *S. pyogenes* (5 and 0.3 μg); 5 = cell-free extract from *B. subtilis* 168 (60 and 10 μg); 6 = cell-free extract from *E. coli* K38 harbouring pT7-5 with the *pcp* gene from *B. subtilis* as insert (60 and 1 μg); 7 = purified Pcp from *B. subtilis* (5 and 0.3 μg); 8 = commercial purified Pcp from *Bacillus amyloliquefaciens* (5 and 0.3 μg).

also exhibit an antigenic polypeptide with an approximate molecular mass of 45 000 (Fig. 3B, lane 2). It is worth noting that in *Streptococcus faecium* a Pcp with a subunit molecular mass of 42 000 has been demonstrated [6]. Thus, the 45 000 antigenic polypeptide detected in *S. pyogenes* crude extract could be a subunit of another Pcp that is different from the enzyme encoded by the cloned *pcp* gene [11]. This suggests that, even though it has never been reported previously in bacteria, *S. pyogenes* possesses at least two different Pcps.

B. subtilis recombinant Pcp and Pcp from *Bacillus amyloliquefaciens* cross-reacted with the antiserum (Fig. 3B, lanes 6, 7 and 8). However, the signal was stronger with Pcp from *B. amyloliquefaciens* than with that of *B. subtilis* (Fig. 3B, lanes 7 and 8). This indicates that, immunologically, Pcp from *B. amyloliquefaciens* presents more similarities with *S. pyogenes* Pcp than the enzyme of *B. subtilis*. Comparison of the deduced amino acid sequence of *B. subtilis* and *S. pyogenes* Pcp revealed that they share 47.5% identity [11]; the homology between *S. pyogenes* and *B. amyloliquefaciens* Pcps should be higher. No cross-reaction was detected in *B. subtilis* crude extract (Fig. 3B, lane 5); this suggests that this protein may be produced at a very low level in *B. subtilis*.

Immunological studies encourage us to envisage purification of Pcp by an immunoaffinity column. This should facilitate the purification of Pcps from crude extracts of other bacterial species that demonstrate a Pcp cross-reaction with the antiserum.

N-Terminal amino acids sequence analysis

Purification of the *B. subtilis* Pcp allowed the analysis of its N-terminal amino acid sequence.

The sequence (MRKKVLITGFDPFDKETV) obtained by Edman sequential degradation [23] corresponds to that predicted from the nucleotide sequence of the cloned gene [10]. This result indicates that the enzyme does not need any post-translational sequence modification to be functional and suggests that the enzyme has a cytoplasmic location.

Catalytic properties and enzyme stability

The N-terminal amino acid specificity of the Pcp from *B. subtilis* was tested by a conductimetric method against a substantial range of alanine-containing dipeptides (pGlu-Ala, Glu-Ala, Asp-Ala, Lys-Ala, Arg-Ala, His-Ala, Tyr-Ala, Ser-Ala, Met-Ala, Pro-Ala, Trp-Ala, Gly-Ala, Phe-Ala, Ala-Ala, Val-Ala, and Leu-Ala). The enzyme was shown to be active to a significant extent only against the N-terminal pyroglutamyl residue (pGlu-Ala). This result was confirmed by thin-layer chromatography. Of the sixteen dipeptides tested at 1 m*M* concentration, only pGlu-Ala was hydrolysed after 24 h incubation with 20 n*M* purified Pcp (data not shown). The absolute substrate specificity of this enzyme is encouraging for its potential utilization in protein sequencing to unblock polypeptides with N-terminal pyroglutamyl residues before undergoing Edman sequential degradation.

The effect of pH on the rate of 1 m*M* L-pyroglutamyl-β-naphthylamide hydrolysis by *B. subtilis* Pcp was studied in the range between pH 5.9 and 9.3 using appropriate buffers. The maximal activity was obtained between pH 6.8 and 7.5. The Michaelis constant (K_M) of the enzyme for L-pyroglutamyl-β-naphthylamide as substrate was found to be 1.04 m*M* at pH 7.0. These results are slightly different from those found by Szewczuk and Mulczyk [1] (optimum pH 8, K_M = 1.7 m*M*), but are consistent with those found for Pcps from other bacteria [5–7].

The stability of the enzyme was studied at 24, 4 and −20°C. After a one-month storage at these temperatures in 50 m*M* phosphate buffer pH 7 containing 10 m*M* 2-mercaptoethanol, the purified enzyme retains 72, 87 and 30% of its initial activity, respectively. This relatively high stability at 4 and 24°C is similar to those described for Pcp from *Klebsiella cloacae* [5] and *Streptococcus faecium* [6]. The loss of the activity at −20°C seems essentially to be the result of a thawing effect as demonstrated by complementary studies (data not shown).

CONCLUSION

The purification scheme presented in this paper appears to be a simple, rapid and of high performance for the isolation of the overexpressed *B. subtilis* Pcp. The large amount of available enzyme and its absolute substrate specificity may render it suitable for use as a N-terminal pyroglutamyl unblocking reagent before undertaking protein sequencing.

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