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Cloning, expression, and characterization of pyrrolidone carboxyl peptidase from the archaeon *Thermococcus litoralis*

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Abstract The gene encoding pyrrolidone carboxyl peptidase (Pcp) has been cloned from the hyperthermophilic archaeon *Thermococcus litoralis*. The recombinant enzyme has been expressed in *Escherichia coli*, purified, and characterized. The *T. litoralis* Pcp demonstrates strong sequence homology to previously characterized bacterial Pcps. Some investigations have been carried out on enzyme substrate specificity and stability.

Key words Peptidase · Biotransformations · Thermostability · *Thermococcus* · Catalytic triad

Introduction

The pyrrolidone carboxyl peptidases (Pcps) are a group of exopeptidases responsible for the removal of amino-terminus pyroglutamate from a variety of peptides and proteins (Doolittle and Armentrout 1968) (Fig. 1). The enzyme has been found to be widely distributed in both eukaryotes and prokaryotes (Szewczuk and Kwiatkowska 1970). The bacterial enzymes characterized to date are dimers or tetramers of ~24-kDa subunits and appear to have a somewhat sporadic distribution; the enzyme is not consistently found within different strains of the same species, for example. The eukaryotic enzymes thus far studied are primarily mammalian and have been divided into two classes. The type I Pcp is a soluble enzyme of similar size to the bacterial Pcps, while the type II enzyme appears to be a much larger membrane-associated enzyme (Mantle et al. 1991). As yet, no sequence data are available for the eukaryotic enzymes. Biochemical studies on all three types of Pcp have revealed two mechanisms of catalysis. The bacterial and type I eukaryotic enzymes appear to be cysteine-protease-type enzymes with an essential catalytic sulfhydryl group (Yoshimoto et al. 1993). The type II enzyme by contrast is strongly inhibited by chelating agents and so has been classed as a metallopeptidase (Wilk and Wilk 1989).

The physiological function of Pcps has only been unambiguously determined in some eukaryotic systems. The type II mammalian enzyme is heavily concentrated in the central nervous system, associated with synaptosomal membranes (O'Connor and O'Cuinn 1984). Its main function appears to be modulation of bioactive peptides (such as thyrotropin-releasing hormone, TRH) by removal of the pyroglutamic acid (pGlu) terminal group (Griffiths et al. 1980). A so-called ectoenzyme that degrades TRH has been cloned from a mammalian source and identified as a zinc metallopeptidase (Schauder et al. 1994). The type I mammalian enzyme is also highly specific for a variety of neuropeptides, but has yet to be implicated in a specific biological process (Mantle et al. 1990). Little is known about the cellular function of the bacterial and archaeal enzymes. It has been proposed that Pcp is important in the final stages of peptide catabolism, regulating the level of free pGlu in the cell. Other workers have suggested that high concentrations of pGlu-blocked peptides are cytotoxic and thus Pcp plays a detoxification role (Awadé et al. 1994). This idea is rather speculative, however, and further studies are required to delineate the enzyme's purpose. Commercially, Pcps have been exploited for the deblocking of pGlu from polypeptides to enable sequencing by Edman degradation (Edman 1950). There is also interest in using Pcps for diagnostic purposes, because highly specific fluorogenic substrates are available that allow the identification of

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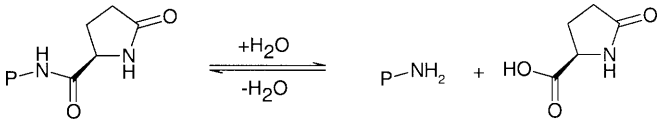

Fig. 1. The enzymatic reaction catalyzed by the Pcp enzyme. *P* denotes continuation of polypeptide chain

bacteria based on the presence or absence of Pcp activity (Mulczyk and Szuwczuk 1970).

The nucleotide sequences of more than ten Pcps have now been determined and demonstrate a high degree of sequence similarity; in particular, a cysteine- and histidine-containing motif is entirely conserved in all sequences. These residues were postulated to comprise a papain-type catalytic triad. Site-directed mutagenesis experiments have established the critical role of these residues (Lesaux et al. 1996). The recent elucidation of the structure of the Pcp from *Thermococcus litoralis* (Singleton et al. 1999) supports a mechanism involving a catalytic triad – Cys, His, and Glu. It also demonstrated that Pcps form a distinct class of cysteine proteases, structurally unrelated to any other. Here we describe the cloning, overexpression, and preliminary biochemical characterization of this enzyme together with some studies on its substrate specificity.

Materials and methods

Thermophile cell paste was obtained from CAMR (Porton Down, Wiltshire). Organic reagents were from Sigma (St. Louis, MO, USA). Hydrolase substrates were supplied by Chiroscience (Cambridge, UK). Media were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). All other reagents were of AnalaR quality. Restriction endonucleases were from Boehringer (Mannheim, UK), and T4 ligase was from Promega (UK Ltd., Southampton, UK). All other enzymes and antibiotics were from Sigma.

Cloning of the *pcp* gene

Genomic DNA was isolated from *T. litoralis* strain NS-C by grinding cell paste under liquid nitrogen, followed by treatment with 0.5% (v/v) Tween-20, 0.5% (v/v) Triton X-100, and RNaseA (200 μg/ml) at 37°C, pH 8. After 1 h, proteinase K was added to 2 mg/ml and incubation continued for a further hour. Released genomic DNA was purified on a Qiagen Tip 500/G in accordance with the manufacturer's instructions. The DNA was partially digested using *Sau*3A, and fragments of approximately 4 kb were isolated by sucrose density-gradient centrifugation. The purified fragments were ligated into *Bam*HI-cut, dephosphorylated pTrc-99 vector and transformed into *E. coli* XL1-Blue. Transformants were plated out on LB agar plates containing ampicillin at 0.1 mg/ml and spread with 50 μl of 0.25 M isopropyl-thio-β D-galactoside (IPTG). Colonies expressing esterase or peptidase activity were screened by overlaying the plates with 0.5% (w/v) agarose in 100 mM potassium phosphate buffer containing 0.5 mg/ml β-naphthyl acetate and 0.5 mg/ml Fast Blue BB salt. Positives were identified by rapid formation of a deep red color. These colonies were removed from the plates, grown in overnight minicultures, and plasmids prepared. The plasmid inserts were sequenced by the dideoxy chain termination method on an Applied Biosystems ABI377 automated sequencer from the *trc*I primer site on the vector (5′-CAATTAATCATCCGGCTC-3′) with a series of nested deletions constructed in the plasmid using the exonuclease III/nuclease SI method (Henikoff 1984). Once the coding sequence of the *pcp* gene had been identified, the gene was amplified from genomic DNA using PCR and ligated into the vector pKK223-3 under the control of the *tac* promoter.

Pcp activity assay

Protein concentration was determined by the method of Bradford (1976) with the BioRad assay kit (BioRad, Hercules, CA, USA), using bovine serum albumin as a standard. The concentration of pure Pcp was determined using the method of Warburg and Christian (Warburg and Christian 1941) with an extinction coefficient of $E_{280}^{1\%} = 0.63$, as determined from primary sequence analysis.

Pcp activity was followed spectrophotometrically by adding the enzyme to a solution of L-pyroglutamyl-β-naphthylamide (10 mM in potassium phosphate buffer, 50 mM pH 7.5) and following the release of β-naphthylamine at 340 nm. An extinction coefficient of $1.78 \times 10^3 M^{-1} cm^{-1}$ was used for the β-naphthylamine. For experiments at elevated temperatures, the reaction mixture was prewarmed to the appropriate temperature before addition of the enzyme.

Overexpression and purification of Pcp

Plasmids containing the *pcp* gene were transformed into *E. coli* strain MC1061 and grown to an A_{600} value of 1.0. Expression was induced by addition of IPTG to a final concentration of 1 mM. Growth was continued for 4 h and the cells harvested by centrifugation. Cell lysis was achieved by sonication in buffer A (50 mM potassium phosphate, pH 7.5; 10 mM dithiothretitol, DTT), and the clarified supernatant was heat treated at 70°C for 20 min. After centrifugation to remove precipitated proteins, an ammonium sulfate cut of 40%–60% saturation was taken and the precipitated protein resuspended in buffer A. The protein was desalted and purified by application to a Superdex 200 HiLoad 16/60 gel filtration column (Pharmacia) followed by passage over a BioScale Q2 anion-exchange column (Bio-Rad). Pcp eluted in the wash, with all other impurities retained on the column. The resulting protein was pure as judged by SDS-PAGE carried out as described by Laemmli (1970).

Thermostability and solvent stability assays

The temperature resistance of Pcp was assayed by incubating the enzyme in prewarmed, pH-adjusted buffer A at a range of temperatures from 60° to 100°C. Samples of the enzyme were taken at appropriate time points from 0 to 300 min, chilled on ice, then assayed as described. The data was plotted as the percent of residual activity remaining after time of incubation at the relevant temperatures.

Resistance of the Pcp enzyme to organic solvents was tested by incubating 100 µl of a 2 mg/ml solution of protein in solvents at concentrations ranging from 10%–100% (v/v) for 1 h at room temperature. Any precipitated protein was removed by centrifugation. The residual activity in 50 µl of enzyme was then assayed in the standard way.

Molecular mass determination

Native molecular mass was determined by gel filtration on a Superdex 200 16/60 column (Pharmacia) in buffer A with the addition of 10 mM NaCl. The column was calibrated with standards of 13.7–440 kDa.

Biotransformation assays

The activity of Pcp against a variety of organic substrates was assessed by incubating the substrate (10 mg/ml) in 0.1 M potassium phosphate, pH 7.5, with 5% v/v of Pcp-purified cell-free extract. Methanol to a total of 10% (v/v) was occasionally added to increase the solubility of the substrate. The reactions were incubated at 50°C with shaking. After 1–4 h, reaction products were extracted from the solution into ethyl acetate, dried with anhydrous $MgSO_4$, and concentrated by rotary evaporation before analysis by gas chromatography. Reaction products were analyzed on a Chirasil Dex CB column at 100°–160°C using helium (14 psi) as a carrier.

Results

Nucleotide and protein sequence of *T. litoralis pcp* gene

The *pcp* gene was found to be composed of a single 663-bp reading frame coding for a polypeptide of 220 amino acids with a molecular mass of 24747 Da. The ATG methionine initiation codon is preceded by a putative archaeal ribosome binding site, GGAGG, and the G+C molar content is typical of *T. litoralis* (38% according to the sequences deposited in Genbank). The translated amino acid sequence was consistent with a previously determined peptide sequence (Singleton and Littlechild, unpublished results). A multiple sequence alignment of the *T. litoralis* Pcp is shown against previously determined Pcps from the bacteria *Staphylococcus aureus* (Patti et al. 1995), *Streptococcus pyrogenes* (Cleuziat et al. 1992), *Bacillus subtilis* (Awadé et al. 1992), *Bacillus amyloliquefaciens* (Yoshimoto et al. 1993), *Pseudomonas fluorescens* (Gonzales and Robert-Baudouy 1994), *Mycobacterium bovis* (Kim and Choe, unpublished data), *Deinococcus radiodurans* R1 (White et al. 1999), and the archaea *Pyrococcus furiosus* (Tsunasawa et al. 1998) and *Pyrococcus horikoshii* (Kawarabayashi et al. 1998) (Fig. 2).

As expected from previous biochemical and crystallographic studies, the active site catalytic triad of Glu-82 Cys-145, and His-169 are totally conserved, and only highly conservative substitutions are seen in the adjacent residues. Structural data suggest that the highly conserved hydrophobic motif LxTGFxPF from residues 5 to 13 forms one side of the active site pocket of the enzyme, while it seems likely that the totally conserved Gly-71 is part of the oxyanion "hole" stabilizing the tetrahedral catalytic intermediate. The greatest sequence variety is seen around positions 181 to 186. The *T. litoralis* sequence is unique in having a highly hydrophobic FFLL insertion here, which has been shown to form the core of the tetrameric enzyme, with the insertion from each subunit meeting at this point (Singleton et al. 1999). This sequence is absent from the other Pcps and indeed in the structure of the enzyme from *B. amyloliquefaciens* (Odagaki et al. 1999), which shows no equivalent central core. This bacterial enzyme is reported to be a dimer in solution and a tetramer in the crystal. It is tempting to speculate that the insertion is a thermostabilizing feature because it is present in the *T. litoralis* enzyme but not the mesophilic bacterial enzymes. However, the two *Pyrococcus* sequences lack this feature, suggesting that the structural integrity of this enzyme at elevated temperatures is maintained in other ways. Interestingly, Cys-192, which in the *Thermococcus* Pcp structure forms a disulfide bridge between adjacent subunits, is also present in the *P. furiosus* enzyme, where it is probable that it fulfils a similar role.

Overexpression and properties of recombinant Pcp

The *T. litoralis* Pcp enzyme was readily overexpressed in *E. coli* under the control of an inducible *tac* promoter with yields of 24 mg/l culture. Details of the purification are shown in Table 1. Purification of the enzyme was facilitated by heat treatment of the cell extract, and the enzyme was be subsequently purified to homogeneity as judged by SDS-PAGE (Fig. 3). Gel filtration studies of the native process showed a molecular mass between 84 and 108 kDa, suggesting that the native enzyme is tetrameric. This oligomeric state was maintained under strong (15 mM DTT) reducing conditions, suggesting the intramolecular disulfide bridge is either highly resistant to reduction, even at elevated temperature, or not essential to maintain the tetrameric structure.

Experiments with inhibitors showed the enzyme to be highly sensitive to the thiol-blocking agents iodoacetimide and *N*-ethylmaleimide, with complete inhibition observed at micromolar inhibitor concentrations. The serine protease inhibitors benzamidine and phenylmethylsulfonyl fluoride had no effect on activity.

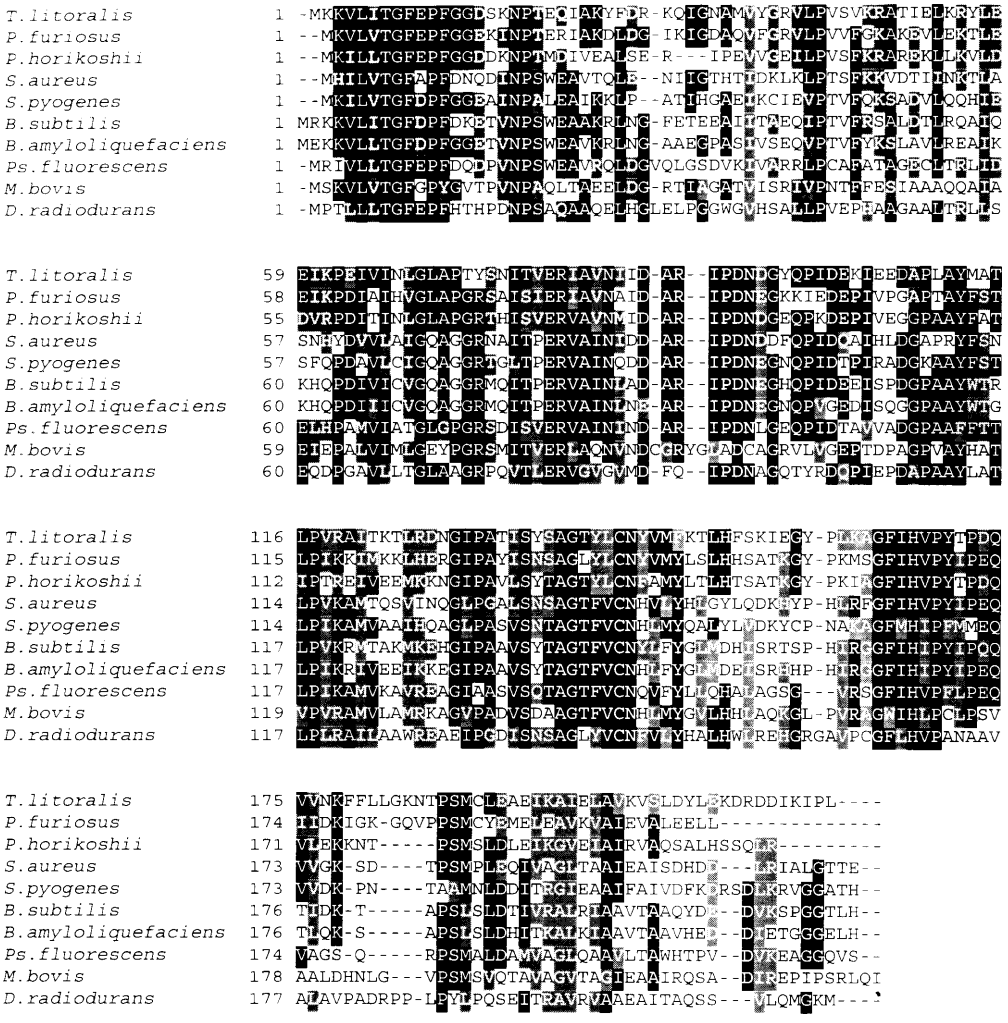

Fig. 2. Multiple sequence alignment of Pcp enzyme sequences. Conservative residue changes are shown in *gray* and totally conserved residues in *black*

Table 1. Steps in the pyrrolidone carboxyl peptidase (Pcp) purification procedure and associated yields in *Thermococcus litoralis*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	870	148	0.17	0	100
Heat treatment	152	139	0.9	5.4	94
AS fractionation	110	125	1.1	6.5	85
Superdex 200	56	88	1.6	9.4	59
Q2 anion exchange	24	84	3.5	20.6	57

The temperature dependence of Pcp activity is shown in Fig. 4. An activity "optimum" is observed at 70°C, which is below the reported growth temperature of 85°C for *T. litoralis*. Rates of thermal inactivation of the enzyme are shown in Fig. 5. The half-life of the enzyme at its optimum activity temperature, 70°C, is approximately 1 h. Solvent stability tests (Fig. 6) show that the enzyme is able to retain its stability at concentrations up to 90% (v/v) ethanol and 75% (v/v) methanol but is less stable in isopropanol and acetonitrile. However, at 50% (v/v) concentrations of these latter two solvents, more than 50% of the initial activity is retained.

Substrate specificity

The enzyme was tested against a variety of esters, but the only nonpyroglutamyl ester showing any activity was *p*-nitrophenyl acetate. The enzyme was active against ethyl

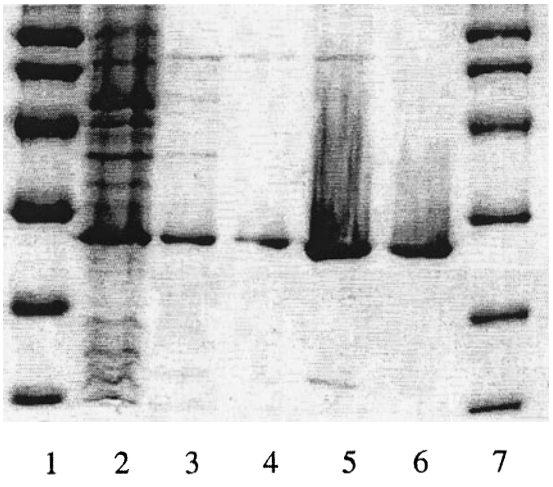

Fig. 3. SDS-PAGE gel electrophoresis showing the purification of recombinant *Thermococcus litoralis* Pcp enzyme. The gel was run from top to bottom. *Lanes: 1*, MW markers [phosphorylase B (97400), bovine serum albumin (66200), ovalbumin (45000), carbonic anydrase (31000), trypsin inhibitor (21500), lysozyme (14400)]; *2*, control *E. coli* cell extract; *3*, induced *E. coli* carrying esterase-coding plasmid cell extract; *4*, heat-treated cell extract; *5* and *6*, ion-exchange fraction; *7*, MW markers as in *lane 1*

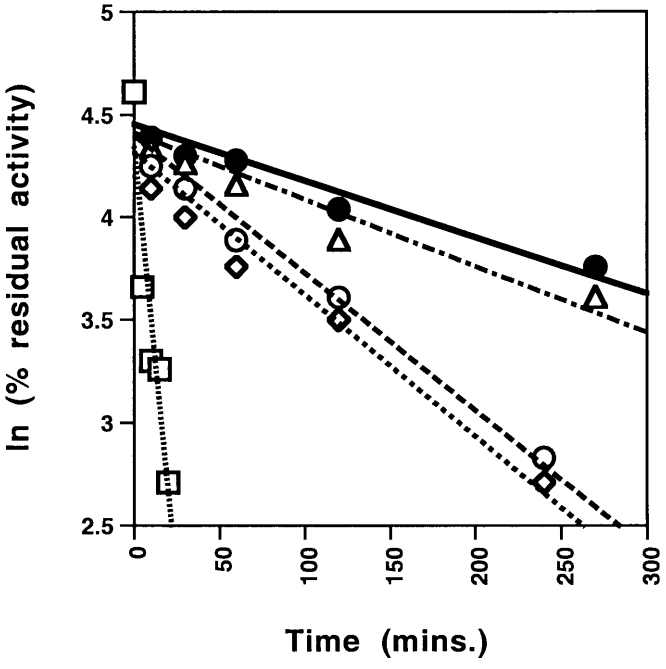

Fig. 5. Stability of the *T. litoralis* Pcp enzyme at different temperatures: □, 100°C; ◇, 90°C; ○, 80°C; △, 70°C; ●, 60°C

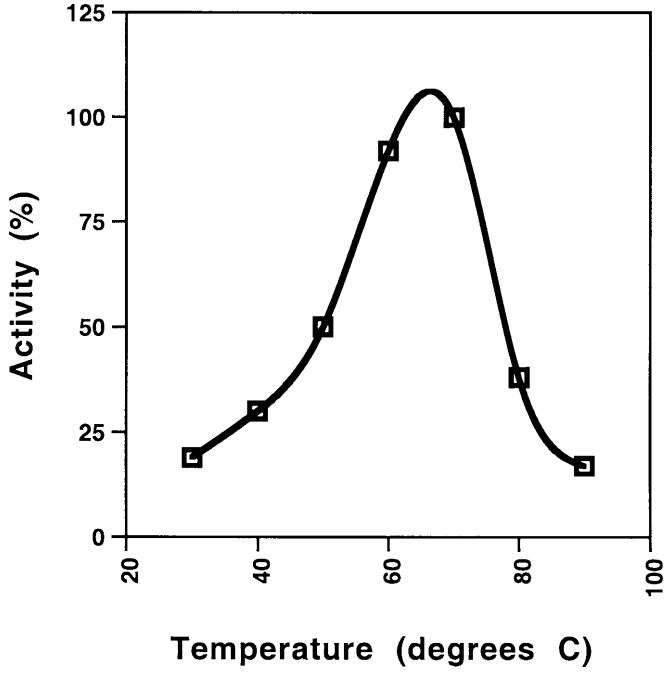

Fig. 4. Temperature "optimum" for enzymatic activity of the *T. litoralis* Pcp enzyme

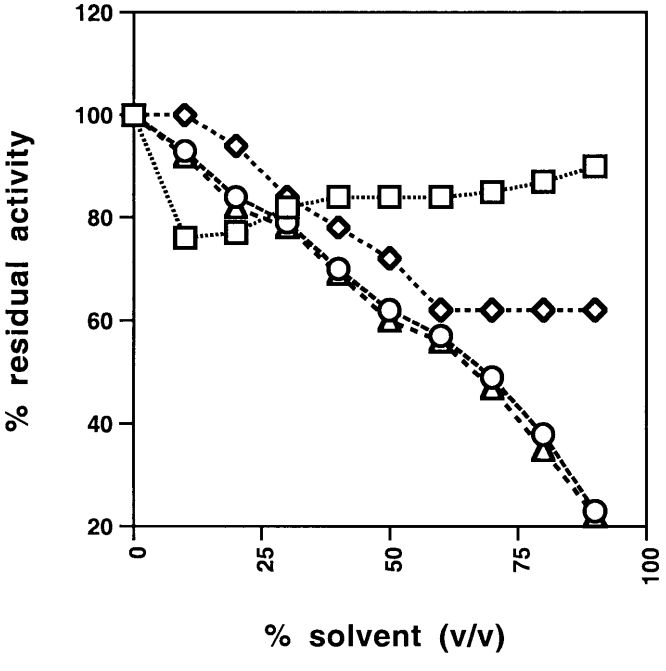

Fig. 6. Stability of the *T. litoralis* Pcp enzyme in different solvents: □, methanol; ◇, ethanol; ○, isopropanol; △, acetonitrile

pyroglutamate (45% conversion in 4h). The stereospecificity of the enzyme against the latter substrate is demonstrated by conversion to pyroglutamic acid with an E value (defined as the ratio of specificity constants for conversion of each enantiomer) of 450 for L-pyroglutamate. The enzyme also efficiently removes L-pyroglutamate from L-pyroglutamyl esters of racemic 2-phenylethanol (10% conversion in 1h) and amides of racemic 2-phenylethylamine (40% conversion in 1h), but without stereoselectivity.

Discussion

We have described the cloning and some properties of pyrrolidone carboxyl peptidase from the hyperthermophilic archaeon *T. litoralis*. The gene and the derived amino acid sequence described is the third Pcp enzyme from the archaeal kingdom and is similar to both other archaeal Pcps and the bacterial enzymes. However, several important differences exist, such as the insertion of hydrophobic residues that make up a central core of the *T. litoralis* enzyme as determined from the three-dimensional structure (Singleton et al. 1999). No study has been made to date of the wider distribution of this enzyme in Archaea; however it is interesting that it appears in at least three of the archaeal genome sequences so far studied, suggesting that its occurrence may be greater in the thermophilic Archaea than in the Bacteria. No definitive function has yet been assigned to the Pcps, although some suggestions indicate its use in nutrient metabolism and detoxification of cellular constituents. The fact that the enzyme appears to be prevalent in organisms whose major mode of metabolism is peptide fermentation suggests that the enzyme may well be involved in some aspect of the catabolism of these nutrient sources. Nevertheless, it is surprising that an enzyme with such high specificity for a pyroglutamyl-containing substrate, and which has a cysteine residue that is easily oxidized as part of its active site, is involved in general peptide metabolism. Further functional studies are required to clarify the role of Pcp enzymes. It is also of interest that the type I vertebrate Pcps, which are involved in modulation of bioactive peptide activity, may be similar to the bacterial and archaeal enzymes (Cummins and Connor 1998).

Study of the mammalian Pcp I enzymes is important because they are thought to be involved in the propensity of amyloid precursors to form insoluble plaques, resulting in Alzheimer's type diseases (He and Barrow 1999). The major commercial application of Pcp enzymes to date has been their use for deblocking proteins to be sequenced by the Edman degradation method (Mozdzanoski et al. 1998). Pyroglutamate may be used a protecting group for small peptides used as prodrugs (Bundgaard and Moss 1989) and in protection of drugs such as methotrexate (Smal et al. 1995). The stability of the *T. litoralis* Pcp enzyme to both elevated temperature and organic solvents makes it a robust catalyst for use in organic synthesis and for other commercial applications.

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