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A thermostable L-aminoacylase from *Thermococcus litoralis*: cloning, overexpression, characterization, and applications in biotransformations

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Abstract A thermostable L-aminoacylase from *Thermococcus litoralis* was cloned, sequenced, and overexpressed in *Escherichia coli*. The enzyme is a homotetramer of 43 kDa monomers and has an 82% sequence identity to an aminoacylase from *Pyrococcus horikoshii* and 45% sequence identity to a carboxypeptidase from *Sulfolobus solfataricus*. It contains one cysteine residue that is highly conserved among aminoacylases. Cell-free extracts of the recombinant enzyme were characterized and were found to have optimal activity at 85°C in Tris-HCl at pH 8.0. The recombinant enzyme is thermostable, with a half-life of 25 h at 70°C. Aminoacylase inhibitors, such as mono-*tert*-butyl malonate, had only a slight effect on activity. The enzyme was partially inhibited by EDTA and *p*-hydroxymercuribenzoate, suggesting that the cysteine residue and a metal ion are important, but not essential, for activity. Addition of Zn^{2+} and Co^{2+} to the apoenzyme increased the enzyme activity, whereas Sn^{4+} and Cu^{2+} almost completely abolished enzyme activity. The enzyme was most specific for substrates containing *N*-benzoyl- or *N*-chloroacetyl-amino acids, preferring substrates containing hydrophobic, uncharged, or weakly charged amino acids such as phenylalanine, methionine, and cysteine.

Key words Thermostable · L-Aminoacylase · Carboxypeptidase · Biotransformations · Substrate specificity · *Thermococcus litoralis*

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Introduction

The use of enzymes for industrial biotransformations is becoming increasingly important. Enzymes play important roles in the production of a variety of biotechnology products and in processes in the food and beverage industries. Biotransformations carried out by partially purified enzymes or whole cells have been used in the production of a wide variety of compounds, from bulk to fine chemicals. Enzymes are especially useful in the production of fine chemicals and pharmaceuticals because of their chemo-, regio-, and, more particularly, their enantioselectivity (as reviewed by Schulze and Wubbolts 1999).

Biocatalysis is frequently preferred over chemocatalysis because of high performance under mild conditions. This characteristic minimizes problems of isomerization, racemization, epimerization, and rearrangements (Patel 2000) and reduces the quantity of side products that can occur during chemical processes. Enzymes from thermophilic organisms may be more suitable in some biotransformations because they are more stable to temperature, organic solvents, and chaotrophic agents (Huber and Stetter 1998). Higher temperatures can be an advantage in some biotransformations as they can increase the solubility and rate of diffusion of reactants and decrease the viscosity of the solution. The diversity and high thermal stability of thermophiles and hyperthermophiles makes them attractive targets for the development of new biocatalysts operating at high temperatures (Huber and Stetter 1998).

Many pharmaceutically active structures are nitrogen-containing compounds that can be derived from either L- or D-amino acids (Drauz 1997). There is a large growth in the area of unnatural amino acids. For example, L-*tert*-leucine is a precursor to many pharmaceutically active compounds including antiviral compounds produced by Sandoz (Sandoz/Novartis, Basel, Switzerland), Biomega (Northfield, IL, USA), and Abbott (Abbott Park, IL, USA), as well as an antitumor compound produced by Zeneca (Zeneca/AstraZeneca, London, UK) (Bommarius et al. 1998). The aminoacylases from *Aspergillus, Penicillium, Bacillus stearothermophilus*, and *Escherichia coli* have been

shown to be useful tools for the resolution of L-amino acids from racemic *N*-acetyl-amino acids (Schulze and Wubbolts 1999; Dion et al. 1995). In addition, the *Aspergillus* aminoacylase has been reported to catalyze transesterifications using vinyl esters as acyl donors, making the enzyme useful in the kinetic resolution of secondary alcohols (Chibata 1996; Faraldos et al. 1997). Aminoacylases have been used industrially for the large-scale resolution of L-methionine and L-valine by Degussa (Kirschenallee, Darmstadt, Germany) at scales in excess of 100 tonnes per annum (Bommarius et al. 1998).

Thermococcus litoralis is a hyperthermophilic archaeon that produces a thermostable L-aminoacylase. Here we describe the cloning, sequencing, and overexpression of the aminoacylase in *Escherichia coli*. Cell-free extracts of the recombinant enzyme were characterized to determine its usefulness as a biocatalyst. The enzyme has been purified and crystallized. Preliminary X-ray diffraction studies have been described (Hollingsworth et al. 2002).

Materials and methods

Reagents and bacterial strains

All reagents were analytical grade or better. The water used was purified by the Purite (Oxon, UK) system. Enzyme substrates were obtained from Sigma Aldrich (Dorset, UK) and Bachem (Essex, UK). Microbiological media was obtained from Oxoid (Fisher, Leicestershire, UK). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Calbiochem (Darmstadt, Germany). *E. coli* strains XL1-Blue MRF' and DH5 and the plasmids pTrc99 and pUCcer11 (pUC19-derived expression vector) have been described by Amann et al. (1988) and Yanisch-Perron et al. (1985), respectively. All enzymes used in genetic manipulations were obtained from Promega (Madison, WI, USA). The enzymes chick lysozyme and benzonase were obtained from Sigma Aldrich and Merck (Leicestershire, UK), respectively. The Amano L-aminoacylase was obtained from Amano Pharmaceutical (Nagoya, Japan). All other chemicals were obtained from Sigma Aldrich and BDH (Leicestershire, UK).

Medium compositions

TSBA medium was composed of 30 g l^{-1} tryptone soya broth in double-distilled water (ddH_2O) containing 100 µgml^{-1} ampicillin. FG medium was composed of 8.0 g l^{-1} KH_2PO_4, 7.0 g l^{-1} K_2HPO_4, 1.0 g l^{-1} $(NH_4)_2SO_4$, 1.0 g l^{-1} $MgSO_4{\cdot}7H_2O$, 15.0 g l^{-1} yeast extract, 15.0 g l^{-1} hycase, 1.0 ml/l trace element solution, 3.0 ml/l polypropylene glycol, and 20.0 g l^{-1} glycerol. The trace element solution was composed of 3.6 g l^{-1} $CaCl_2{\cdot}2H_2O$, 2.4 g l^{-1} $CoCl_2{\cdot}6H_2O$, 0.85 g l^{-1} $CuCl_2{\cdot}2H_2O$, 5.4 g l^{-1} $FeCl_3{\cdot}6H_2O$, 0.3 g l^{-1} H_3BO_4, 250 ml concentrated HCl, 2 g l^{-1} $MnCl_2{\cdot}4H_2O$, 4.8 g l^{-1} $Na_2MoO_4{\cdot}2H_2O$, and 2.0 g l^{-1} ZnO.

Cloning of L-aminoacylase

The production and screening of a genomic library of *T. litoralis* in pTrc99 in *E. coli* strain *XL1-Blue MRF'* has been previously reported by Singleton et al. (2000). A positive clone containing the gene coding for a pyrrolidone carboxyl peptidase (Pcp) was isolated (Singleton et al. 2000). DNA sequencing of the 4.6-kb genomic fragment revealed several open reading frames (ORFs), upstream and downstream of the *pcp* gene. A putative aminoacylase was identified by homologous sequence alignment. The ORF encoding the aminoacylase was amplified by the polymerase chain reaction (PCR). Restriction endonuclease sites *Hin*DIII and *Bam*HI were incorporated within the 5′ and 3′-oligonucleotide primers, respectively. The PCR fragment was digested with *Hin*DIII and *Bam*HI, ligated into a pUCcer11, and transformed into *E. coli* strain DH5. Growth and induction profiling was carried out to optimize the expression of the recombinant aminoacylase (Hollingsworth et al. 2002).

Preparation of recombinant L-aminoacylase

Small-scale fermentation

An overnight starter culture of *E. coli* strain DH5 harboring the L-aminoacylase was grown in tryptone soya medium overnight at 37°C. A 10-ml inoculum was added to an ST Applikon (Schiedam, The Netherlands) bioreactor containing 9 l of growth (FG) medium with the addition of IPTG to a final concentration of 1 mM. The culture was incubated at pH 7.0 at 30°C for 24 h with aeration (1l min^{-1}) and agitation at 800 rpm. Primary harvesting of 80% of the culture was carried out, and a batch feed of 1.5 l of 1.3× FG medium plus inducer was added to the residual culture. Incubation was continued for a further 16 h before secondary harvesting and batch feeding was repeated. Two further harvests and feeds were carried out with 12- to 16-h incubations. The cells were harvested by centrifugation at 8,000 *g* for 30 min at 5°C, and the cell paste was retained at –20°C until further use.

Preparation of cell-free enzyme extracts

Cell-free enzyme extracts were prepared by two methods. The first method involved disrupting by sonication a 10% cell suspension in 100 mM Tris-HCl pH 8.0 at 2°C by 15 s sonication followed by 20 s resting for a total sonication time of 10 min. The extract was clarified by centrifugation at 11,000 *g* for 30 min at 4°C. The supernatant was heated at 70°C for 1 h to precipitate the majority of *E. coli* proteins, followed by centrifugation as before. The extract was filter-sterilized through a 0.22-µm filter (Whatman, Maidstone, UK) and stored at 4°C until further use. For the second preparation method, a 10% cell suspension containing 0.1% (w/v) Triton X-100, 1 mg ml^{-1} lyophilized lysozyme, 0.41 mg ml^{-1} $MgCl_2{\cdot}6H_2O$, and 3 U ml^{-1} benzonase was incubated for 16–24 h at 25°C. The mixture was centrifuged and the supernatant was filter-sterilized and stored as earlier.

Aminoacylase assays

The activity of the *T. litoralis* L-aminoacylase was determined by the detection of free amino acids cleaved from the substrate 50 mM *N*-benzoyl-L-phenylalanine (BLPhe) in 100 mM Tris-HCl, pH 8.0, for 10 min at 80°C. The reaction was stopped by withdrawing 0.02 ml of the reaction mixture and adding it to 1 ml of the stop reagent (45% acetonitrile in water containing 1 ml^{-1} H_3PO_4). The analysis was carried out by high performance liquid chromatography (HPLC) using a Hypersil (ThermoHypersil, Runcorn, UK) C18 BDS 5 µ column (150 × 4.6 mm dimensions). Separation was achieved by an isocratic flow of 2 ml min^{-1} of the stop reagent (mobile phase) within 3 min. The substrate and product peaks were detected by absorption at 210 nm. The percent conversion of the reaction was determined by comparing the peak area of phenylalanine with the sum of the phenylalanine and substrate peak areas, adjusting the values according to the different response factors of each compound (determined by standard curves of 0–1 mM phenylalanine and substrate standards). One unit of activity is defined as the cleavage of 1 µM phenylalanine per minute per milliliter of 10% cell extract from 50 mM BLPhe in 0.1 M Tris-HCl, pH 8.0, at 80°C. Activity toward substrates containing the amino acids phenylalanine, tyrosine, and tryptophan (e.g., 10 mM *N*-acetyl-DL-tryptophan) were performed as above, except that the wavelength of the UV detector was 225 nm for tryptophan and tyrosine substrates.

Activity toward the N-protected amino acids was also determined by a modification of the method using Cd/ninhydrin as described by Doi et al. (1981). The enzyme was added to 10 mM substrate in 100 mM Tris-HCl, pH 8.0, at 85°C with a total assay volume of 0.5 ml. The reaction was stopped and the color developed by the addition of 0.5 ml of Cd/ninhydrin reagent (0.8 g ninhydrin and 2.0 g $CdCl_2$ dissolved in a mixture of 80 ml 95% ethanol, 10 ml glacial acetic acid, and 20 ml water) and a further incubation of 10 min at 85°C. After the samples were cooled, the absorbance at 508 nm (400 nm for proline-containing substrates) was determined for each sample. Activity was calculated using extinction coefficients determined under the reaction conditions with 0–1 mM standards of each amino acid. One unit of activity is defined as the cleavage of 1 µM amino acid per minute per milliliter of 10% cell-free extract under the reaction conditions. This method was employed to determine the standard units of the Amano L-aminoacylase with 25 mM *N*-acetyl-L-phenylalanine (ALPhe) in 0.1 M Tris-HCl, pH 7.0, at 25°C.

Esterase assays

Activity toward *p*-nitrophenyl esters (e.g., *p*-nitrophenyl laurate) was determined by a discontinuous variation of the method of Janssen et al. (1994). The enzyme was added to 0.4 mM *p*-nitrophenyl-X (X = laurate, stearate, or palmitate) in 20 mM Tris-HCl, pH 8.0, at 85°C (final volume, 1 ml). The reaction was stopped and clarified by the addition of 0.5 ml of the copper phosphate reagent of Janssen et al. (1994), containing 10% (w/v) Triton X-100 and 6% (w/v) NaCl. The samples were centrifuged at 11,000 *g* for 2 min at 25°C, and absorbance was measured at 400 nm. Activity was determined using extinction coefficients determined under the reaction conditions with 0–1 mM *p*-nitrophenol standards. One unit of activity is defined as the cleavage of 1 µM *p*-nitrophenol per minute per milliliter of 10% cell-free extract under the reaction conditions.

Activity toward amino acid esters and N-protected amino acid-esters (e.g., *N*-acetyl-Phe-ethyl ester) was determined qualitatively by thin-layer chromatography (TLC). The enzyme was added to 1 mM substrate in 100 mM Tris-HCl, pH 8.0, at 85°C (final volume, 1 ml). The samples were cooled on ice and spotted onto Polygram SIL G/UV254 precoated plastic TLC plates (Alltech, Carnforth, UK). The mobile phase for TLC consisted of a biphasic mixture of 70% *n*-butanol, 2% acetic acid, and 28% water. Development of the spots was achieved by dipping the plates in the above Cd/ninhydrin reagent, allowing them to dry, and then heating the plates to develop the color.

Protease assays

General proteolytic activity was determined with 0.2% azocasein according to the method of Toogood et al. (2000), except the buffer used was 0.1 M Tris-HCl, pH 8.0, and the activity was determined at 85°C for up to 16 h.

Protein assay

The protein concentration was determined by a modification of the Bradford assay method (Peterson 1983) using a kit supplied by BioRad (Hertfordshire, UK). Bovine serum albumin (BSA) was used as the protein standard in the range of 0–100 µM.

Determination of molecular mass

Enzyme extract samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) to determine their degree of purity and molecular mass. Protein bands were stained using a Coomassie staining method (Read and Northcote 1981). Molecular weight standards in the range of 14.4–97 kDa were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Native molecular mass of the purified enzyme (Hollingsworth et al. 2002) was determined by gel filtration using a Superdex 200 Hiload 16/60 column (Pharmacia). The column was equilibrated with 10 mM Tris-HCl, pH 8.0, buffer at 0.3 ml/min and calibrated with the standards ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa). Then, 1 ml of 2 mg ml^{-1} purified aminoacylase (Hollingsworth et al. 2002) was run on the column at 0.3 ml/min. Sedimentation studies were carried out on the purified aminoacylase at 7.5 mg ml^{-1} in 10 mM Tris-HCl, pH 8.0, at the BBSRC

Hydrodynamic Service (Nottingham, UK). A sample from a 1 mg ml^{-1} solution of the purified aminoacylase was dialyzed against 5% acetic acid and underwent N-terminal amino acid sequence analysis at the Aberdeen Protein Sequencing facility (Scotland).

Optimization of reaction conditions

The optimal pH of the L-aminoacylase was determined with the substrate 10 mM *N*-acetyl-DL-tryptophan (ADLTrp) for 1 h at 70°C (85°C for Tris buffer) with a range of pH values and buffers. The buffers used were 0.1 M acetate (pH 4.5–5.5), 0.1 M Mes (pH 5.5–8.0), 0.1 M KH_2PO_4/K_2HPO_4 (pH 6.5–9.5), 0.1 M Tris-HCl (pH 7.0–8.5), and 0.1 M sodium borate (pH 8.5–9.5). The analysis was performed by HPLC, except the mobile phase was 45% methanol in water with 1 l^{-1} H_3PO_4. The optimal temperature of the L-aminoacylase was determined with 10 mM ADLTrp in 0.1 M Tris-HCl, pH 8.0, for 1 h in the temperature range 65°–95°C. The linearity of the reaction with respect to time and enzyme concentration were determined with 10 mM ADL-Trp in 0.1 M Tris-HCl, pH 8.0, at 85°C by varying the assay time periods and enzyme concentration, respectively.

Thermostability

The thermostability of the L-aminoacylase was determined by incubating the enzyme for 0–25 h at 60°–95°C. Samples were withdrawn at specific intervals and cooled rapidly on ice. The activity of the samples was determined by assaying with 10 mM *N*-benzoyl-DL-phenylalanine (BDLPhe) in 0.1 M Tris-HCl, pH 8.0, for 10 min at 85°C, using the Cd/ninhydrin analysis method.

Inhibitor studies

The enzyme was incubated with the inhibitors for 24 h at 4°C, then assayed for remaining activity using the substrate 10 mM BHDLPhe in 0.1 M Tris-HCl, pH 8.0, for 10 min at 85°C with the inhibitors in the assay at the same concentration as in the preincubation. Analysis was performed by HPLC.

Effect of metal ions on activity

Metal-free aminoacylase was prepared by adding *o*-phenanthroline to the enzyme to a final concentration of 10 mM. The extract was dialyzed against three changes of 10 mM Tris-HCl, pH 8.0, for 24 h at 4°C. To determine if any Zn^{2+} ions remained in the enzyme samples after this treatment, the samples were subjected to atomic absorption spectroscopy (Perkin-Elmer Analyst 100; Perkin-Elmer, Norwalk, CT, USA) calibrated with 0–1 ppm $Zn(NO_3)_2$ standards in ultrapure water.

Metal ions were added back to the apo-aminoacylase, to a final concentration of 10–100 µM, and incubated overnight at 4°C. Activity of the holoenzyme and apoenzyme were determined with 10 mM BDLPhe in 0.1 M Tris-HCl, pH 8.0, for 10 min at 85°C, with the metal ions in the assay at the same concentration as the enzyme samples. Analysis was performed by HPLC. To determine the optimal concentration of Zn^{2+} and Co^{2+} ions on activity, the L-aminoacylase was assayed with 10 mM ADLTrp in 0.1 M Tris-HCl, pH 8.0, containing 0–1 mM $ZnCl_2$ or $CoCl_2$, for 1 h at 70°C. Analysis of the results was carried out by HPLC analysis with 45% methanol in water containing 1 ml^{-1} H_3PO_4.

Substrate specificity

The *T. litoralis* and Amano aminoacylases were assayed for activity with 10 mM substrates in 100 mM Tris-HCl, pH 8.0, for 10–60 min at 85°C (25°C for the Amano enzyme). Analysis was performed by the Cd/ninhydrin method. The substrates were of the type N-X-amino acid/peptide, where X = acetyl-, CBZ-, BOC-, benzoyl- or chloroacetyl-, with a variety of L, D, and DL forms of natural and unnatural amino acids and peptides. Activity against the substrates *N*-benzoyl-pipcolate and *N*-benzoyl-Phe-Ala-Pro was analyzed by HPLC with 50% methanol (70% for the latter substrate) in 50 mM KH_2PO_4/K_2HPO_4, pH 7, at 1 ml min^{-1}.

The K_m of the L-aminoacylase for the substrate BLPhe was determined with 1–80 mM substrate in 0.1 M Tris-HCl, pH 8.0, for 20 min at 80°C. Analysis of the product formation was by HPLC (mobile phase: 45% acetonitrile in water containing 1 ml^{-1} H_3PO_4). Calculation of the K_m was by the direct linear plot method (Gul et al. 1998).

Stability and activity in organic solvents

The effect of organic solvents on activity was determined by assaying L-aminoacylase with the substrates 10 mM ALTrp and *N*-acetyl-D-tryptophan (ADTrp) for 24 h at 60°C in the presence of 0%–50% methanol, isopropanol, acetone, and acetonitrile. Samples were withdrawn at intervals and the activity was determined by HPLC (mobile phase, 45% methanol in water with 1 ml^{-1} H_3PO_4). To determine the effect of organic solvents on stability, L-aminoacylase was preincubated with 0%–50% of the foregoing organic solvents for 24 h at 60°C. Samples were withdrawn at intervals and the remaining activity was determined using the substrate 10 mM BDLPhe in 0.1 M Tris-HCl, pH 8.0, for 10 min at 85°C (HPLC, 45% acetonitrile in water with 1 ml/l H_3PO_4).

Scale-up biotransformations

Optimization of the biotransformation reaction with the substrate *N*-acetyl-DL-propargyl glycine (ADLPGly) was carried out in 10-ml volumes with constant shaking for 24–50 h at 70°C. To determine the effect of substrate concentration on activity, different concentrations of L-aminoacylase were assayed with 0.5–1.4 M ADLPGly in 0.1 M Tris-HCl, pH 8.0, for 50 h. In each case, the ratio of the

enzyme concentration to substrate concentration was kept constant (23 U ml^{-1} enzyme per 1 M substrate). The effect of enzyme concentration on activity was determined by assaying 0.002–0.08 U/ml L-aminoacylase with the substrate 0.7 M *N*-ADLPGly in 0.1 M Tris-HCl, pH 8.0, for 50 h. To determine the effect of temperature on activity, L-aminoacylase was assayed with the substrate 0.7 M ADLPGly in 0.1 M Tris-HCl, pH 8.0, for 50 h in the temperature range 50°–80°C. A scale-up biotransformation was carried out with 50 g of ADLPGly in 500 ml of 0.1 M Tris-HCl, pH 8.0, and with 213 U L-aminoacylase for 24 h at 75°C with continual mixing. The product formation in all cases was monitored during the time periods by withdrawing samples and analyzing the amino acid content by the Cd/ninhydrin method.

Results

Cloning, sequencing, and overexpression of L-aminoacylase

The gene encoding the L-aminoacylase was located directly upstream of the *pcp* gene (Fig. 1). The G+C content of the aminoacylase gene is 38%, consistent with other genes from *T. litoralis*. Figure 2 shows an alignment between the amino acid sequence of L-aminoacylase from *T. litoralis*, deduced from the DNA sequence, and other aminoacylase sequences (GenBank) from *Pyrococcus horikoshii* (accession number GI:325745; Kawarabayasi et al. 1998), *P. abyssi* (accession number GI:5458743), *Arabidopsis thaliana* (accession number GI:8655992), *Synechocystis* sp. (accession number GI:1653860), *Bacillus subtilis* (accession number GI:7448705), a carboxypeptidase from *Sulfolobus solfataricus* (accession number GI:1136220; Colombo et al. 1995), and carboxypeptidase G_2 from *Pseudomonas* sp. strain RS-16 (accession number GI:151184; Minton et al. 1984). The *T. litoralis* aminoacylase shows an 82% amino acid sequence identity with a carboxypeptidase/aminoacylase from *P. horikoshii* (Kawarabayasi et al. 1998) and a 45% identity with a carboxypeptidase from *S. solfataricus* (Colombo et al. 1995); it contains one cysteine residue (Cys_{106}) in a position that is highly conserved in these aminoacylases (see Fig. 2). L-Aminoacylase also contains the highly conserved residues Glu77, Glu79, and Glu197–Glu198 (native *T. litoralis* L-aminoacylase numbering), which are known to bind Zn^{2+} ions in carboxypeptidase G_2 (Rowsell et al. 1997). However, there is only 19% sequence identity between the two enzymes.

The N-terminal residues of the recombinant enzyme have been modified to enhance expression in *E. coli* using the vector pUCcer11. The recombinant enzyme has an insertion of four amino acids (Glu-Pro-Leu-Phe) between the N-terminal methionine and the second residue lysine of the wild type. The molecular mass of the enzyme was calculated to be 43,814.57 Da according to its sequence. SDS-PAGE analysis of the purified L-aminoacylase showed a single band at 43 kDa. However, gel filtration analysis

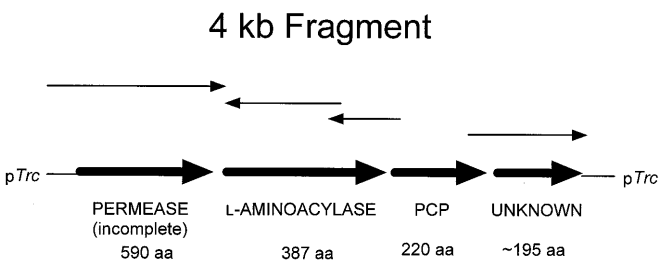

Fig. 1. Gene organization of the *Thermococcus litoralis* genomic fragment in p*Trc* 99 showing the relative direction of transcription. The *black arrows* indicate the sequencing strategy around the *pcp* gene

estimated the size of the enzyme to be 170 kDa, suggesting the enzyme exists as a homotetramer of 43 kDa. Sedimentation studies confirmed that the enzyme exists in vitro as a tetramer.

Optimal reaction conditions

Heat treatment of cell-free extracts was performed initially to clarify the solutions and partially purify the enzyme. However, heat treatment also caused a loss of at least 50% of the activity of the L-aminoacylase, which is thermostable under these conditions, possibly a result of coprecipitation with the large quantity of precipitated *E. coli* proteins. The average yield of activity is 2,470 U ml^{-1} extract (24,700 U g^{-1} cell paste) in the absence of heat treatment.

Figure 3A shows the pH profile of L-aminoacylase using the substrate ADLTrp in several buffers. This result shows that the optimal pH is 8.0 with Tris-HCl or phosphate buffers and pH 7.5 with Mes buffer (adjusted at room temperature). At least 70% of the activity is present between pH 6.5 and 9.5, suggesting the enzyme has a broad pH tolerance. Figure 3B shows the activity of L-aminoacylase using ADLTrp as the substrate in the temperature range 65°–95°C. This result shows that the optimal temperature for a 1 h reaction is 85°C. The reaction is linear throughout the 1-h time period at 85°C (results not shown).

Thermostability

Figure 4 shows the thermostability of recombinant L-aminoacylase at 60°–95°C. This result shows that the enzyme is thermostable, with a half-life of 25 h and 1.7 h at 70° and 85°C, respectively, in the absence of the substrate. This enzyme is less stable than the carboxypeptidase/aminoacylase from *P. horikoshii* OT3, which shows no loss of activity after 24–48 h at 90°C (Ishikawa et al. 2001).

Inhibitor studies

Table 1 shows the effect of inhibitors on the activity of L-aminoacylase. These results show that although a number of compounds will inhibit the aminoacylase, no compound that was tested completely inhibited the enzyme. Competitive

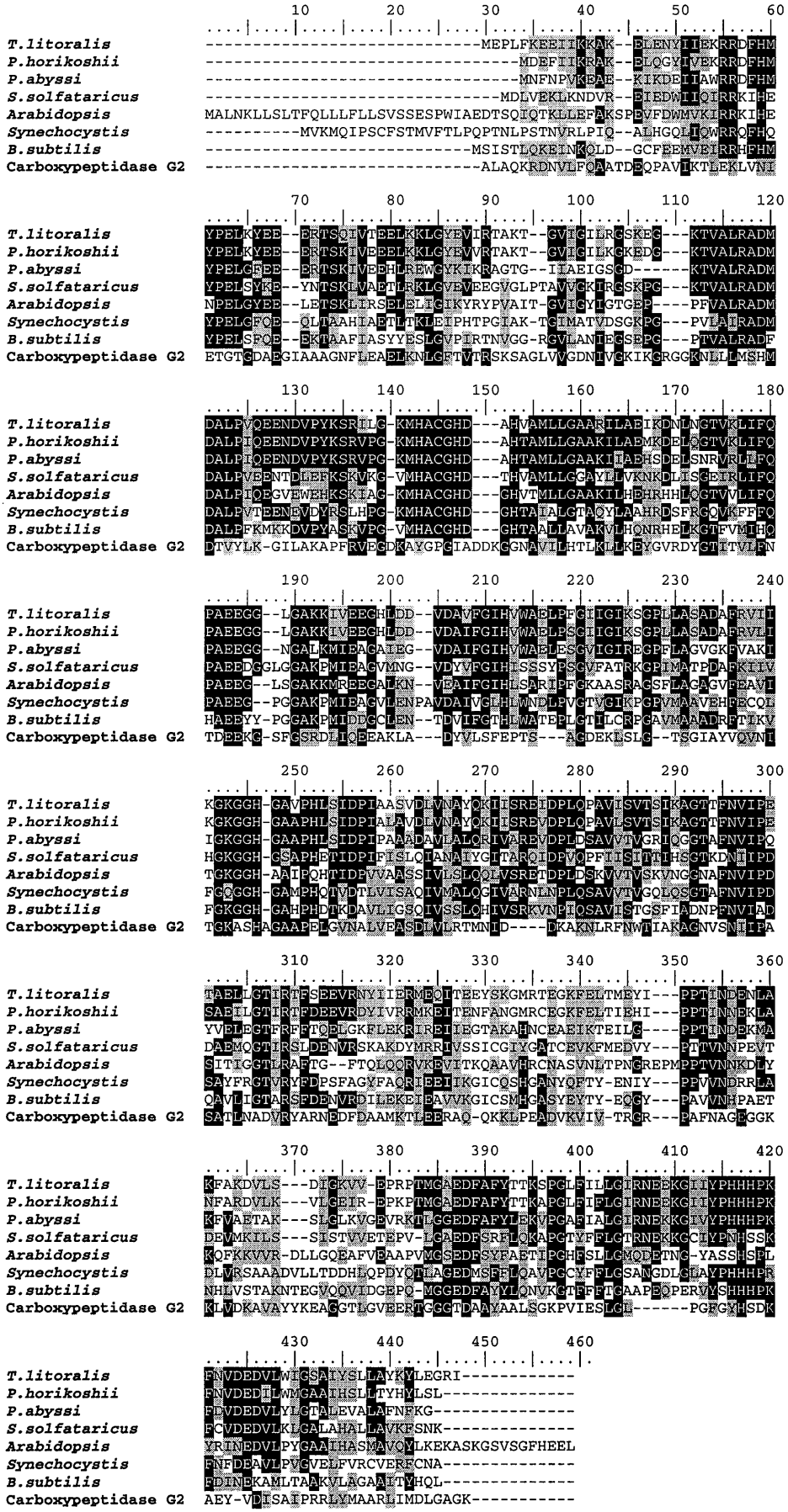

Fig. 2. Alignment of the *T. litoralis* L-aminoacylase with 6-aminoacylase and carboxypeptidase protein sequences. Residues *boxed in black* are entirely conserved. Sequence was created using ClustalW (Thompson et al. 1994)

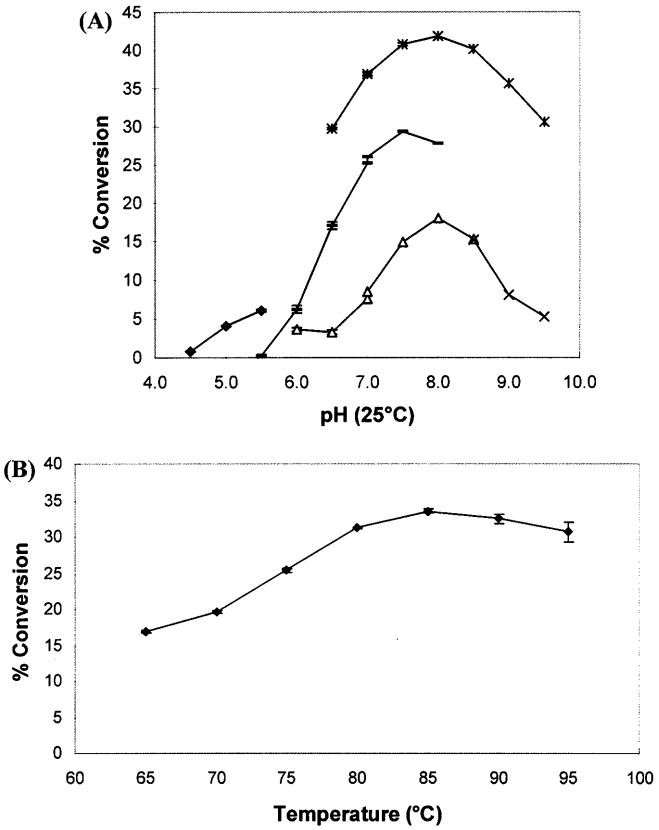

Fig. 3. Optimization of the reaction conditions of *T. litoralis* aminoacylase pH (**A**) profile and temperature profile (**B**). Diamonds, acetate buffer (pH 4.5–5.5); short bars, Mes buffer (pH 5.5–8.0); triangles, phosphate buffer (pH 6.5–9.5); crosses, borate buffer (pH 8.5–9.5); asterisks, Tris buffer (pH 7.0–8.5)

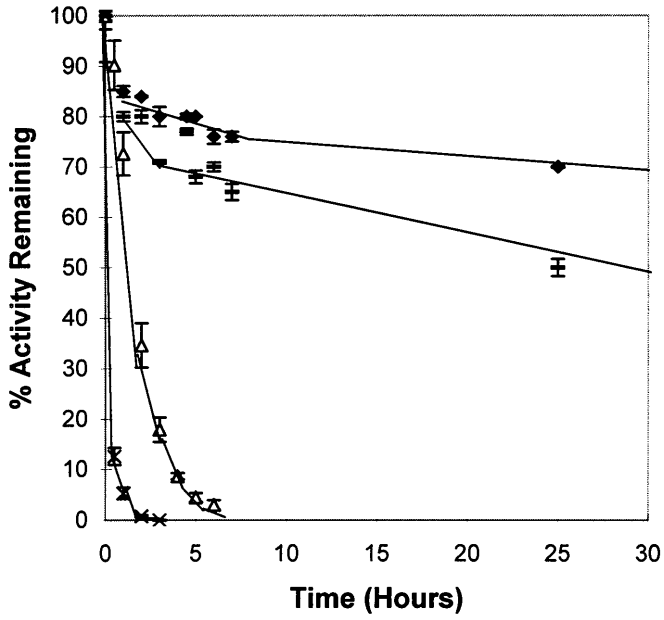

Fig. 4. Thermostability of the aminoacylase from *T. litoralis* at 60°–95°C. Diamonds, 60°C; short bars, 70°C; triangles, 85°C; crosses, 95°C

and/or slow-binding inhibitors of hog kidney acylase I, such as mono-*tert*-butyl malonate, mono-benzyl malonate, and *N*-(*p*-toluenesulfonyl)-DL-Met (Wu and Tsou 1993), had little or no effect on the enzyme. The inhibitor 1-chloro-2-tosylamido-7-amino-2-heptanone had only a slight effect on activity.

Thiol inhibitors such as *p*-hydroxymercuribenzoate and dithiothreitol caused a significant decrease in activity, which suggests that the conserved cysteine residue (Cys106) plays a role in the activity of the enzyme. The metal chelators ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline inhibited the enzyme by approximately 50%, suggesting the presence of at least one metal ion playing an indirect role in catalysis.

Effect of metal ions on activity

Table 2 shows the effect of metal ions on the activity of L-aminoacylase. The apo-enzyme was found to be free of Zn^{2+} ions (results not shown) but still retained about 80% of its activity. The addition of 10–100 μM Zn^{2+} and 100 μM Co^{2+} significantly increased its activity. The addition of the same amount or more of Zn^{2+} to the holo-aminoacylase caused an inhibition of activity whereas the addition of up to 100 μM Co^{2+} caused a 30% increase in activity (results not shown). This result was also seen with acylase I from hog kidney (Wu and Tsou 1993). Co^{2+} may have a lower affinity for the Zn^{2+}-binding site or may be binding to a different region of the enzyme. The addition of other metal ions, namely, Mg^{2+}, La^{3+}, and Ba^{2+}, to the apoenzyme slightly inhibited activity. The heavy metals Sn^{4+}, Fe^{3+}, and Cu^{2+} almost completely inhibited enzyme activity.

Substrate specificity

Table 3 shows a comparison of the substrate specificities of *T. litoralis* and Amano L-aminoacylases. The activities were calculated as activity per 1,000 standard units, as defined in the Materials and methods. These results show that neither aminoacylase was able to cleave substrates containing D-amino acids, confirming they are both L-aminoacylases.

For *T. litoralis* aminoacylase, the order of preferred N-blocking groups is benzoyl ≅ chloroacetyl >> acetyl > CBZ > BOC. The Amano aminoacylase has an order of preferred N-blocking groups of acetyl > chloroacetyl >> BOC > benzoyl. No activity was detected with substrates containing a CBZ- group for the latter enzyme. This enzyme appears to be very specific for acetyl and chloroacetyl substrates and has only weak activity toward other groups. This enzyme compares to *T. litoralis* aminoacylase, which is more specific for benzoyl and chloroacetyl substrates. Chloroacetyl substrates are the preferred N-protecting group of many aminoacylases (Anders and Dekant 1994, and references within). This protection may occur because the chlorine group has a strong electron-withdrawing potential that weakens the scissile carbon–nitrogen bond, making cleavage easier. Several BOC-substituted amino acids are known to inhibit aminoacylases (Anders and Dekant 1994), which

Table 1. Effect of inhibitors on the activity of *Thermococcus litoralis* aminoacylase

Inhibitor	Class of inhibitor	Activity remaining (%)
Control (no inhibitor)	–	100
1 mM *p*-Hydroxymercuribenzoate	Thiols	33
25 mM *mono-tert*-Butyl malonate	Aminoacylase	86
25 mM *mono*-Benzyl malonate	Aminoacylase	109
25 mM *N*-(*p*-Toluenesulfonyl)-D,L-Met	Aminoacylase	86
25 mM 1-Chloro-2-tosylamido-7-amino-2-heptanone	Aminoacylase	93
1 mM EDTA	Metallo-enzymes	55
1 mM *o*-Phenanthroline	Zinc enzymes	46
1 mM Phenylmethylsulfonyl fluoride	Serine-cysteine proteases	78
0.1 mM Leupeptin	Serine-cysteine proteases	105
1 mM Dithiothreitol	Cysteine proteases	58
1 mM Iodoacetic acid	Cysteine proteases	111
1 mM Benzamidine	Serine proteases	93
1 μM Aprotinin	Serine proteases	98
0.1 mg ml^{-1} Trypsin-chymotrypsin inhibitor	Serine proteases	117
0.1 mM Antipain	Papain and trypsin	105

EDTA, ethylenediaminetetraacetic acid

Table 2. Effect of metal ions on the activity of *T. litoralis* aminoacylase

Metal ion	Activity of Apoenzyme (%)	
	10 μM metal	100 μM metal
None	100	100
Zn^{2+}	128	126
Co^{2+}	107	131
K^{+}	76	–
Cs^{+}	53	–
Ca^{2+}	55	–
Mn^{2+}	46	–
Cu^{2+}	5	–
Na^{+}	81	–
Fe^{2+}	67	–
Fe^{3+}	1	–
Li^{+}	49	–
Ni^{2+}	73	–
Mg^{2+}	92	–
La^{3+}	83	–
Ba^{2+}	83	–
Sn^{4+}	1	–

10–100 μM metal refers to the final concentration of the metal ion in the assay

may be why these two aminoacylases had only weak activity toward BOC-L-Phe.

For *T. litoralis* aminoacylase, the order of preferred amino acids is Phe >> Met > Cys > Ala ≅ Val > Tyr > Propargylglycine > Trp > Pro > Arg. The Amano aminoacylase has an order of preferred amino acids of: Met > Cys > Phe > Trp >> Ala > Val > propargylglycine > Arg. No activity was detected with substrates containing proline with the latter enzyme. Although both enzymes have high activity toward substrates containing methionine and cysteine, *T. litoralis* aminoacylase is most specific for phenylalanine-containing substrates; it has a K_m of approximately 0.37 mM with BLPhe at 85°C (results not shown). Activity was determined up to 80 mM BLPhe with no substrate inhibition apparent. Cysteine is only partially charged at pH 8.0 whereas other amino acids such as arginine are fully charged at this pH. Thus, this enzyme appears to prefer hydrophobic, uncharged or partially charged amino acids. The Amano aminoacylase is less specific for the amino acid component of the substrate. However, both aminoacylases do not cleave *N*-acetyl-*tert*-Leu and have poor or no activity toward substrates containing proline and arginine. *T. litoralis* aminoacylase was able to cleave the dipeptide CBZ-Phe-Gly, but not the tripeptide benzoyl-Phe-Ala-Pro, although the latter substrate contains benzoyl-Phe, its most preferred combination.

The aminoacylase from *T. litoralis* contains esterase activity with *p*-nitrophenyl-palmitate and *p*-nitrophenyl-laurate, but not Phe-ethyl ester at 85°C. Both esterase and aminoacylase activity was detected with the substrate *N*-acetyl-L-Phe methyl ester (ALPheOMe) with purified aminoacylase (results not shown). *T. litoralis* aminoacylase showed no significant protease activity against azocasein, even after long incubations (16 h). This result shows that the enzyme does not appear to have native carboxypeptidase activity.

Activity and stability in organic solvents

Figure 5A and 5B shows the activity and stability, respectively, of *T. litoralis* aminoacylase in the presence of 10% organic solvents at 60°C over a 24-h period. These results show that the presence of 10% organic solvents reduces the activity of the enzyme. The most inhibitory solvent was isopropanol. Under these conditions, no significant activity was detected with ADTrp. However, an incubation of the enzyme in 10% organic solvents at 60°C before activity determination, under standard conditions, actually increased the specific activity of the enzyme as compared to the nonheated sample. Over the 24-h time period, the aqueous sample at 60°C lost 38% of the enzyme activity. However, the actual activities of all the samples in 10% organic

Table 3. Substrate specificity of *T. litoralis* and Amano L-aminoacylases

Enzyme	*T. litoralis* L-aminoacylase			Amano L-aminoacylase		
Substrate	μmol/min/1,000 U	μmol/min/g cell paste	% Ac-L-Phe activity	μmol/min/1,000 U	μmol/min/g cell paste	% Ac-L-Phe activity
N-Acetyl-DL-Phe	46.8	1,160	40.6	502.9	1.8	64.9
N-Acetyl-L-Phe	115.2	2,840	100.0	774.7	2.8	100.0
N-Acetyl-D-Phe	0.0	0	0.0	0.0	0.0	0.0
N-Benzoyl-DL-Phe	523.7	12,940	454.6	5.3	<0.1	0.7
N-Benzoyl-L-Phe	847.6	20,940	735.7	8.1	<0.1	1.0
N-CBZ-DL-Phe	19.1	470	16.6	0.0	0.0	0.0
N-CBZ-L-Phe	39.1	970	33.9	0.0	0.0	0.0
N-CBZ-D-Phe	0.0	0	0.0	0.0	0.0	0.0
N-BOC-L-Phe	1.8	45	1.6	36.5	0.1	4.7
N-BOC-D-Phe	0.0	0	0.0	0.0	0.0	0.0
N-Chloroacetyl-L-Phe	839.5	20,741	728.7	683.8	2.5	88.3
N-Acetyl-DL-Trp	2.6	63	2.2	693.2	2.5	89.5
N-Acetyl-L-Trp	3.6	89	3.1	644.8	2.3	83.2
N-Acetyl-D-Trp	0.0	0	0.0	0.0	0.0	0.0
N-Acetyl-DL-Val	11.2	278	9.8	63.6	0.2	8.2
N-CBZ-L-Val	19.4	4,790	16.8	0.0	0.0	0.0
N-Acetyl-DL-Pro	0.5	12	0.4	0.0	0.0	0.0
N-BOC-L-Pro	0.4	11	0.4	0.0	0.0	0.0
N-Acetyl-DL-Ala	11.6	286	10.1	187.2	0.7	24.2
N-Acetyl-L-Cys	21.4	530	18.6	945.1	3.4	122.0
N-Acetyl-DL-Met	18.8	465	16.3	1,072.8	3.9	138.5
N-Acetyl-L-Met	46.7	1,151	40.5	1,276.7	4.6	164.8
N-CBZ-L-Tyr	5.1	127	4.5	0.0	0.0	0.0
N-Acetyl-L-Arg	0.2	1.8	0.2	30.2	0.1	3.9
N-CBZ-Phe-Gly	56.8	1,404	49.3	–	–	–
N-Benzoyl-Phe-Ala-Pro[a]	0.0	0	0.0	–	–	–
N-Acetyl-DL-Propargyl glycine	5.1	120	4.4	51.7	0.2	6.7
N-Acetyl-DL-*tert*-Leu	0.0	0	0.0	0.0	0.0	0.0
N-Benzoyl-pipecolate	0.8	18	0.7	–	–	–

Specific activity was standardized to activity per 1,000 standard units (U) of each enzyme as described in the Materials and methods
CBZ, benzyloxycarbonyl; BOC, benzoyl- or chloroacetyl
[a] Cleavage between the benzoyl group and phenylalanine only

solvent and the water control were within 4% of each other after 24 h at 60°C (results not shown). This result occurred because the initial activity of the enzyme in 10% organic solvents was significantly lower than in 100% aqueous conditions. The presence of 50% organic solvents almost completely abolished activity toward ALTrp at 60°C (results not shown). Stability was also compromised as the average half-life was <1 h at 60°C (results not shown).

Scale-up biotransformations

Figure 6 shows the effect of the substrate concentration on the activity of *T. litoralis* aminoacylase with the substrate *N*-acetyl-DL-propargylglycine (ADLPropgly). The results show that increasing the substrate concentration (up to concentrations used in current industrial biotransformations) decreased the product yield, even when the ratio of substrate to enzyme was kept constant. This finding shows that the enzyme was susceptible to substrate inhibition. Other results showed that increasing the enzyme concentration (0.002–0.08 U ml^{-1}) with constant substrate concentration, or increasing the temperature (to 80°C) increased the reaction rate (results not shown). The scale-up biotransformation with 50 g of ADLPGly with 213 U L-aminoacylase showed 45% conversion of racemic substrate after 24 h at 75°C. Industrial-scale biotransformations have been carried out with this enzyme (Chirotech Technology, Cambridge, UK) and have proved to be successful (results not shown).

Discussion

This study describes the cloning and initial characterization of an L-aminoacylase from *T. litoralis*. The recombinant enzyme was evaluated for its effectiveness in catalyzing biotransformations for potential industrial use. For these latter studies, cell-free enzyme extracts were used as opposed to the purified enzyme because any industrial use of this enzyme would be with a partially purified preparation due to the prohibitive costs of large-scale enzyme purification procedures.

There have been many studies addressing the role of the conserved cysteine residue in aminoacylases (Sakanyan

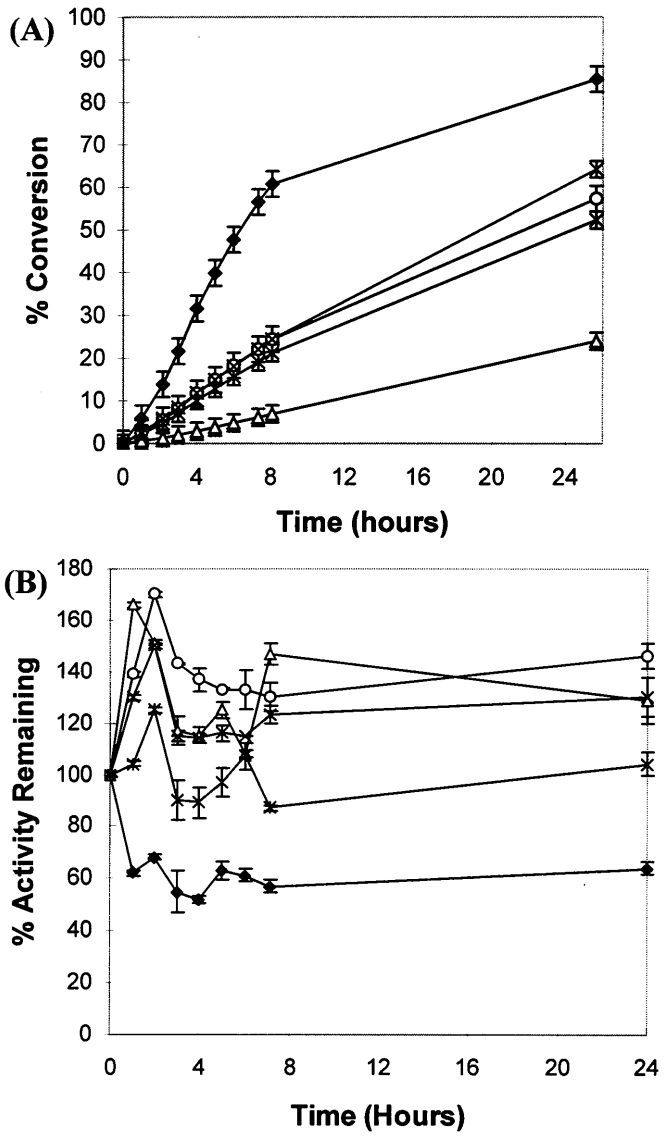

Fig. 5. Effect of 10% organic solvents on activity (**A**) and stability (**B**). Diamonds, 100% water; circles, 10% methanol; triangles, 10% isopropanol; crosses, 10% acetone; asterisks, 10% acetonitrile

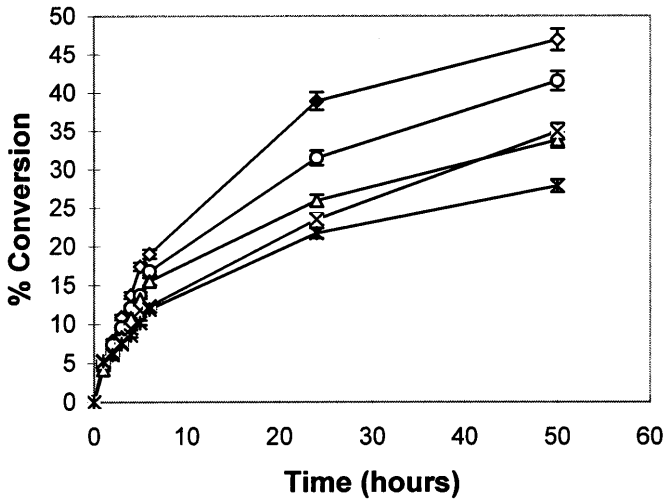

Fig. 6. Effect of substrate concentration on % conversion of *N*-acetyl-DL-propargylglycine. Diamonds, 0.5 M; circles, 0.75 M; triangles, 1.0 M; crosses, .2 M; asterisks, 1.4 M

et al. 1993; Wu and Tsou 1993; Wang et al. 1992; Tang et al. 1995; Heese and Rohm 1989; Heese et al. 1990). Some researchers have suggested the cysteine residue plays a direct role in catalysis of aminoacylases (Henseling and Rohm 1988; Anders and Dekant 1994), while others suggest that cysteine is important but not essential for activity (Yang et al. 1996). Chemical modification and site-directed mutagenesis of Cys102 in the carboxypeptidase/aminoacylase from *Pyrococcus horikoshii* OT3 showed that this cysteine residue was located at the active site and was involved in activity (Ishikawa et al. 2001). The aminoacylase from *T. litoralis* is partially inhibited by thiol reagents, suggesting that the cysteine residue has an indirect role in catalysis.

Several aminoacylases and carboxypeptidases are known to contain Zn^{2+} ions (Rowsell et al. 1997; Colombo et al. 1992). Bovine carboxypeptidase A contains one Zn^{2+} ion that binds to its one cysteine residue (Vallee 1963). The conclusions from these studies showed that the Zn^{2+} ion does not appear to directly participate in substrate binding or catalysis; rather, it plays a structural role by maintaining or stabilizing the catalytically active conformation of the active site. The *P. horikoshii* OT3 carboxypeptidase/aminoacylase is known to contain one bound zinc ion per enzyme molecule (Ishikawa et al. 2001) and retains only 1% of its activity in the presence of EDTA. In contrast, the *T. litoralis* L-aminoacylase is only inhibited by 44%–55% in the presence of EDTA or *o*-phenanthroline. This result suggests the Zn^{2+} ions may play a structural, rather than a catalytic, role in this enzyme.

The substrate specificity of several aminoacylases has been extensively characterized (Drauz and Waldmann 1995). For example, the aminoacylase from *Aspergillus oryzae* has an amino acid preference of Met > Phe > norleucine > Trp > Leu > Ala. Acylase I from hog kidney (Anders and Dekant 1994) is more specific for chloroacetyl-amino acids; it prefers amino acids that are neutral and aliphatic such as alanine, 2-aminobutanoic acid, valine, leucine, norleucine, and methionine (Anders and Dekant 1994). The thermostable aminoacylase from *Bacillus stearothermophilus* NCIB8224 (Sakanyan et al. 1993) is more specific for hydrophobic, aromatic amino acids, with a specificity of Tyr ≅ Ala > Phe > Val > Leu > His > Met. It has a ninefold higher activity with chloroacetyl- over acetyl-amino acids. It can also cleave some dipeptides (Sakanyan et al. 1993). None of these enzymes is most specific for benzoyl-amino acids. Thus, aminoacylases from different sources have a wide variety of specificities. Thermostable carboxypeptidases from *S. solfataricus* and *Thermus aquaticus* YT-1 (carboxypeptidase *Taq*) are capable of cleaving a wide variety of CBZ-amino acids. For example, *S. solfataricus* carboxypeptidase cleaves CBZ-amino acids with the following preference: Arg > Glu > Tyr ≅ Phe > Gly > Leu ≅ Ser > Ala (Colombo et al. 1992). The carboxypeptidase/aminoacylase from *P. horikoshii* OT3 exhibits native

carboxypeptidase activity by cleaving amino acids sequentially from the C-terminus of peptides (Ishikawa et al. 2001), in contrast to *T. litoralis* aminoacylase, which showed no detectable protease or peptidase activity. The high sequence homology between some aminoacylases and carboxypeptidases could be attributed, at least in part, to the similarity in their substrates (see Table 3).

The aminoacylase from *T. litoralis* was found to be rapidly inactivated by the presence of 50% organic solvents at 60°C, as was also found with the carboxypeptidase from *S. solfataricus* at suboptimal temperatures (Colombo et al. 1992). Boross et al. (1998) studied the effect of solvents such as dimethylformamide (DMF) and dioxane on the aminoacylase from hog kidney and found that 10% DMF caused a decrease in stability of more than 70% after 50 min at 25°C. It is generally considered that thermostable proteins tend to be more resistant to organic solvents than mesophilic proteins (Huber and Stetter 1998). In some cases, enzymes described as solvent-resistant were characterized at temperatures well below (at least 40°C below) normal assay temperatures (Colombo et al. 1992; Khan et al. 2000). Other studies have shown that some hyperthermophilic proteins are stable to organic solvents only at suboptimal temperatures (Guagliardi et al. 1989). It appears that some thermostable proteins may be just as sensitive as mesophilic proteins to organic solvents at their optimal temperature but may be quite resistant to these solvents at significantly lower temperatures.

The aminoacylase from *T. litoralis* was subject to substrate inhibition at high substrate concentrations of ADLPGly but not of BLPhe, possibly because the enzyme has a significantly lower specific activity with the former substrate (4.4 and 736 µmol/min/g cell paste, respectively). In spite of this, increasing the enzyme loading in the biotransformations enabled 45% conversion of the racemic mixture of ADLPGly in a 50-g scale-up trial.

The cloning, overexpression, and characterization of the aminoacylase from *T. litoralis* has enabled this enzyme to be successfully used in biotransformations at an industrial scale.

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