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ORIGINAL ARTICLE

Purification and characterization of *Stenotrophomonas maltophilia*-derived L-amino acid ester hydrolase for synthesizing dipeptide, isoleucyl-tryptophan

Md Saddam Hossain[1] · Takahiro Tanaka[1] · Kazuyoshi Takagi[2] · Junji Hayashi[1] · Mamoru Wakayama[1]

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Abstract
In the present study, we purified α-amino acid ester hydrolase (AEH) from cell-free extracts of the *Stenotrophomonas maltophilia* strain HS1. The approximately 70-kDa AEH from *S. maltophilia* HS1 (SmAEH) was homogeneous in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses, and was present as a tetramer in gel-filtration experiments. The activity of the SmAEH enzyme was then determined by monitoring the synthesis of the antihypertensive agent dipeptide isoleucyl-tryptophan (Ile-Trp) from isoleucyl methyl ester (Ile-OMe) and tryptophan (Trp). In these experiments, SmAEH had wide substrate specificity for acyl donors, such as Gly-OMe, β-Ala-OMe, Pro-OMe and Trp-OMe and Ile-OMe, and maximal activity were observed under conditions of pH 9.0 and 30 °C. SmAEH also showed the greatest stability at pH 9.0, whereas its activity was reduced by 40% after 10-min incubation at approximately 50 °C. In subsequent activity assays in the presence of various metal ions, Ag^+ strongly inhibited enzyme activity. Finally, SmAEH activity was completely inhibited by phenylmethanesulfonyl fluoride (PMSF), suggesting that the protein is a serine protease.

Keywords α-Amino acid ester hydrolase · *Stenotrophomonas maltophilia* · Antihypertensive dipeptide · Ile-Trp

Introduction

Peptides are important sources of nutrition, and are increasingly considered for biomedical applications (Santos et al. 2012). Peptides are used as pharmaceuticals, flavour-active ingredients in natural health products, nutraceuticals, and functional foods (Mine et al. 2011; Santos et al. 2012), and dipeptides and tripeptides attract considerable attention because of their cost effectiveness and potential oral activity, and because they are easily characterised in structural, molecular and quantitative functional studies (Kurochkina et al. 2013).

Multiple methods are available for synthesising peptides, and peptide bonds between amino acids are widely produced using chemical and chemo-enzymatic techniques (Guzmán et al. 2007; Kurochkina et al. 2013; Yagasaki and Hashimoto 2008). However, these methods require the introduction and elimination of protective groups, or the synthesis of intermediates, and therefore, have limited industrial utility (Yokozeki and Suzuki 2010). As alternatives, methods using complete enzymatic reactions exploit molecular recognition between enzymes and their substrates (Gill et al. 1996; Guzmán et al. 2007), and peptide-hydrolysing enzymes, such as proteases and esterases, are used to trigger peptide-bond formation between amino acids (Yagasaki and Hashimoto 2008). Peptide synthesis using proteases has been extensively performed by protecting or freeing either one or both N- and C-terminal ends of acyl donors and acceptors, respectively, and peptide bonds have been formed using Nα-free amino acid esters with Cα-protected amino acids (Gill and Vulfson 1993; Sheldon et al. 2001), and using α-protected amino acid esters and Nα with Cα-free amino acids (Hansler and Jakubke 1996). However, proteases fail to meet industrial requirements of productivity, reaction rates, yields, proportions of acyl acceptors to acyl donors, and numbers of

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✉ Mamoru Wakayama
wakayama@sk.ritsumei.ac.jp

[1] Department of Biotechnology, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan

[2] Department of Applied Chemistry, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan

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peptides that can be synthesised using this approach. In addition, these methods are hampered by temporary accumulation of dipeptides and degradation of dipeptides by proteases (Yagasaki and Hashimoto 2008). In contrast, α-amino acid ester hydrolase (AEH) can be used to synthesise dipeptides efficiently and irreversibly using Nα-free amino acid ester as an acyl donor and Nα- and Cα-free amino acids as acyl acceptors (Barends et al. 2003; Yokozeki and Hara 2005).

Several studies report the use of AEH to synthesise dipeptides (Abe et al. 2011; Hirao et al. 2013; Sugihara et al. 2001; Yokozeki and Hara 2005). Herein, we identified microorganisms that produced AEH efficiently for use in the synthesis of the dipeptide isoleucyl-tryptophan (Ile-Trp), which is an important antihypertensive agent. Recently, the antihypertensive effects of dipeptides have attracted considerable attention (Sato et al. 2002), and hydrolysates of fish meat, seaweed and mushrooms have been shown to lower blood pressure. Among active components of protein hydrolysates, the dipeptides Ile-Trp, Ile-Tyr, Lys-Trp and Val-Tyr have been identified (Yagasaki and Hashimoto 2008), and their antihypertensive effects follow inhibition of angiotensin-I-converting enzyme (ACE), which regulates blood pressure (Kitts and Weiler 2003; Sato et al. 2002). Extracts and hydrolysates containing these dipeptides have been approved as foods for specified health uses in Japan (Yagasaki and Hashimoto 2008), and represent healthier natural alternatives to ACE inhibitor drugs. In particular, the potent ACE-inhibitory activities of Ile-Trp have been studied comprehensively (Ono et al. 2006; Enari et al. 2008), and when administered to spontaneously hypertensive rats at its IC_{50} value of 4.7 μM, Ile-Trp exerted antihypertensive activities within 2 h (Iroyukifujita et al. 2000). However, methods for the synthesis of Ile-Trp have not been reported previously. Moreover, few enzymatically produced dipeptides are currently available due to the lack of cost-effective manufacturing processes, and no standard protocols have been established for scaling up and optimising dipeptide production (Yagasaki and Hashimoto 2008). Because efficient dipeptide production will facilitate the identification of novel applications, we optimised the production, purification and characterisation of AEH from the bacterial strain *Stenotrophomonas maltophilia* HS1.

Materials and methods

Reagents

L-Isoleucine methyl ester (L-Ile-OMe) and L-tryptophan (L-Trp) were purchased from Wako Pure Chemicals (Tokyo, Japan). Isoleucyl-tryptophan (Ile-Trp) was obtained from Watanabe Chemical Industry (Hiroshima, Japan) and acetic acid, acetonitrile, tetrahydrofuran and Coomassie Brilliant Blue (CBB) were purchased from Nacalai Tesque (Kyoto, Japan). DEAE-Cellufine and Butyl-Cellufine columns were purchased from JNC (Tokyo, Japan), and Superdex 200 HR 10/30 columns were obtained from GE Healthcare (Tokyo, Japan). All other reagents were of analytical grade.

Screening of AEH-producing bacterial strains

Samples of soil were collected from a hill-tract forest area in Shiga prefecture, Japan. Briefly, one loop of soil was suspended in 1 mL of sterile water. Subsequently, one loop of the resulting soil suspension was then transferred to 1 mL of liquid medium 1 containing 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.2% L-Ile-OMe (pH 7.0) and was incubated at 30 °C for 48–72 h with shaking at 165 rpm. The mixture was then suspended in 1 mL of fresh liquid medium 1 and was incubated as above, and pure colonies were isolated after loop streaking the resulting suspension onto agar plates containing 0.5% glucose, 0.5% polypeptone, 0.5% yeast extract, 0.1% $MgSO_4{\cdot}7H_2O$ and 1.5% agar (pH 7.0) and incubating at 30 °C for 16–20 h. One loop of pure colony was then transferred to 5 mL of liquid medium 2 containing 0.5% glucose, 0.5% yeast extract, 0.5% polypeptone, 0.2% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.15% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7.0) and was incubated at 30 °C at 250 rpm for 20–22 h. The resulting culture was then centrifuged at 10,000*g* for 2 min, and intact cells were mixed with reaction mixture containing 50-mM L-Ile-OMe (pH 7.0), 100-mM L-Trp (pH 9.0) and 100-mM Tris–HCl buffer (pH 9.0). After 1 h at 30 °C, synthesised Ile-Trp was quantitatively analysed as described below.

Identification of isolates and production of SmAEH

Isolates were identified using 16S ribosomal RNA sequencing and phylogenetic analyses were performed using multiple sequence alignments in the MUSCLE server (Edgar 2004). A phylogenetic tree was created and visualised using the Wagner parsimony method in the PHYlip package (Felsenstein 1989), and all tools were used in their default settings. To produce SmAEH, seed cultures were prepared by incubating freshly isolated colonies in 5-mL aliquots of liquid medium 2 at 30 °C with shaking at 250 rpm for 20 h. Seed cultures were then inoculated into 500 mL of the same media in a 2-L flask, and were incubated at 30 °C with shaking at 100 rpm for 20 h. Cells were then harvested by centrifugation at 10,000*g* for 20 min, were washed two times and dissolved in 50-mM Tris–HCl buffer (pH 8.0), and were then treated with lysozyme (0.5 mg/mL) at 4 °C for 30 min. Sonication was performed with optimised parameters, and cell-free extracts with AEH activity were obtained by centrifugation at 12,000*g* for 30 min followed by removal of cell debris.

Purification of SmAEH

All SmAEH purification steps were performed at 4 °C unless otherwise stated. Cell-free extracts were loaded onto a DEAE-Cellufine column that was pre-equilibrated with 20-mM Tris–HCl buffer (pH 8.0) after dialysing against 20-mM Tris–HCl buffer (pH 8.0). Prior to elution, nonbinding proteins were washed from the column using equilibration buffer, and bound proteins were eluted stepwise using 0–0.3-M NaCl in the same buffer at a flow rate of 1 mL/min. Active fractions were then collected and dialysed against 20-mM Tris–HCl buffer (pH 8.0) and were assayed for AEH activity. Prior to Butyl-Cellufine chromatography, enzymes and columns were equilibrated with 20-mM Tris–HCl buffer (pH 8.0) containing 2-M ammonium sulphate, and the enzyme was then eluted with a linear gradient of 2–0 M ammonium sulphate. Active fractions were then collected and dialysed against 20-mM Tris–HCl buffer (pH 8.0) and the enzyme was then applied to Superdex 200 HR 10/30 gel-filtration columns in 20-mM Tris–HCl buffer containing 150-mM NaCl. Active fractions were then collected and dialysed against the same buffer, and enzyme samples were concentrated by ultrafiltration and were stored at 4 °C.

Analysis

Protein concentrations were determined using the Lowry method with egg albumin as a standard (Lowry et al. 1951). The molecular mass of denatured enzyme was estimated using SDS-PAGE and gels were stained with Coomassie Brilliant Blue. Synthesised Ile-Trp in reaction mixtures was quantitatively measured and monitored using high-performance liquid chromatography (HPLC) with a COSMOSIL 5C18-MS-II column (4.6 × 250 mm; Nacalai Tesque, Inc., Kyoto, Japan) equilibrated with 0.1-M acetate buffer (pH 6.0) containing 7% (v/v) acetonitrile and 3% (v/v) tetrahydrofuran. The derivative Ile-Trp was eluted using a linear gradient of acetonitrile (7–47%) at a flow rate of 1 mL/min. Before analysis, samples (5 µL) were quenched and diluted 100-fold by the addition of derivatized fluorescence compound using 10-mM Boc-Cys-OH, 20-mM phthalaldehyde in 400-mM borate buffer (pH 9.0) and 5-µL aliquots of sample mixtures were then applied to HPLC columns. Standard reaction mixtures for Ile-Trp synthesis contained 100-mM Tris–HCl buffer (pH 9.0), 50-mM L-Ile-OMe, 100-mM Trp (pH 9.0) and enzyme in a final volume of 0.1 mL. After 1 h at 30 °C, reactions were terminated by boiling for 10 min and one unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of Ile-Trp per min under standard assay conditions. Data were presented as means ± standard errors of the mean of at least three independent experiments, or as noted in the figure legends. Differences were identified using Student's *t* test and were considered significant when $P < 0.05$.

Characterisation of SmAEH

The thermal stability of SmAEH was determined after exposure to 20–55 °C for 10 min followed by cooling for 2 min on ice. Enzyme reactions were then allowed to proceed for 1 h. The effects of varying pH on AEH activity were determined after adjusting pH to between pH 7.0–10.5 using buffer and allowing reactions to proceed at 30 °C for 1 h. In further experiments, pH stability of AEH was determined by incubating 100 µL aliquots of enzyme for 12 h with 100 µL aliquots of various buffers with a pH range of 4.0–10.0 at 4 °C. Reactions were then performed at 30 °C for 1 h, and residual enzyme activity was determined as described above.

To investigate the acyl donor specificity of SmAEH, hydrolysis reactions were performed with various amino acid methyl ester substrates and hydrolytic activities of SmAEH were assayed according to methanol release from acyl donors at 30 °C for 1 h. In separate experiments, AEH activity was assayed spectrophotometrically using assay mixtures comprising 0.25 U of alcohol oxidase and 1 U of horseradish peroxidase in 100-mM potassium phosphate buffer (pH 7.0). Subsequently, SmAEH reaction solution (10 µL) was added to the assay mixture, was supplemented with 1-mM *o*-phenylenediamine and was incubated at 30 °C for 30 min. These analyses showed that alcohol oxidase catalyses the oxidation of methanol to the corresponding carbonyl compound formaldehyde and hydrogen peroxide. Finally, *o*-phenylenediamine signals were amplified by hydrogen peroxide and horseradish peroxidase, and concentrations of the resulting intermediate were determined at 420 nm.

Results

Screening and identification of AEH-expressing bacterial isolates

A total of 200 soil samples were screened according to rates of Ile-Trp synthesis from Ile-OMe and Trp. From these analyses, ten microbial strains were selected as Ile-Trp producers and four strains with higher Ile-Trp-synthetic activity were identified using 16S ribosomal RNA sequencing. *S. maltophilia* HS1 was a remarkably efficient producer of Ile-Trp (2 mM) under the present screening conditions (Supplementary data Table 1), and was selected for further experiments. Phylogenetic analyses placed *S. maltophilia* HS1 in distinct position on the tree, although sequence identity with relevant strains was more than 96% (Supplementary data Fig. 1).

Optimisation of culture conditions for SmAEH production

The effects of various carbon and nitrogen sources on SmAEH production were examined (Table 1), and the highest specific activity (approximately 0.12 U/mg) was observed in medium-containing glucose, casamino acid and yeast extract. Conversely, media with casamino acid led to the lowest specific activity (0.007 U/mg). No activity or growth was observed in media supplemented with glucose–$(NH_4)_2SO_4$, glucose–NH_4Cl and glucose–urea.

The cultivation period for optimal production of AEH by *S. maltophilia* HS1 was 20 h (Supplementary data Fig. 2) and production was highest during the exponential cell growth phase. Moreover, at 22 h, SmAEH productivity was decreased and after 26 h, enzyme activity was greatly decreased, although cell numbers continued to increase.

Purification of SmAEH

Enzymes were purified using DEAE-Cellufine and Butyl-Cellufine chromatography columns followed by gel filtration. During these purification steps, specific activity increased and finally reached approximately 5.34 U/mg at a purity of 116 fold (Table 2). Although the enzyme yield was 1.90%, the purified enzyme was homogeneous in SDS-PAGE analyses (Fig. 1), as indicated by a single band of 70 kDa under denaturing conditions. In addition, the molecular mass of the purified enzyme was approximately 300 kDa in gel-filtration analyses, suggesting that the enzyme is active as a tetramer.

Characterisation of SmAEH

We determined optimal physicochemical conditions for purified enzyme activity, and showed that SmAEH is most active at 30 °C. Our data also show that the enzyme is stable for 10 min between 20 and 35 °C (Fig. 2a), and 60% of enzyme activity remained after 10-min incubation at 50 °C.

Table 1 Effects of carbon and nitrogen sources on AEH productivity

C source	N source	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
D-Glucose	Yeast extract	0.281	3.31	0.084
D-Glucose	Polypeptone	0.121	3	0.040
D-Glucose	$(NH_4)_2SO_4$		No growth	
D-Glucose	NH_4Cl		No growth	
D-Glucose	Urea		No growth	
D-Glucose	Casamino acid	0.234	3.2	0.07
D-Glucose	Casamino acid + 0.05% yeast extract	0.395	3.21	0.12
Glycerol	Casamino acid	0.347	3	0.11
Polypeptone	Yeast extract	0.059	3.3	0.017
Polypeptone	$(NH_4)_2SO_4$	0.166	2.9	0.057
Polypeptone	Casamino acid	0.229	3.15	0.072
–	Casamino acid	0.008	1.1	0.007
–	Casamino acid + 0.05% yeast extract	0.025	2	0.012

Cells were grown in a test tube containing 5-mL culture medium on a reciprocal shaker at 30 °C for 24 h. Concentration of carbon and nitrogen sources was 0.5%. However, the composition and amount of salt in each medium remained constant, and only the effects of carbon and nitrogen in AEH production were tested. The resulting culture was harvested and activity data were determined. Experiments were performed in duplicate

Table 2 Steps of purification

Steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell-free extract	1804	84.1	0.046	100	–
DEAE-cellufine (pH 8.0)	145	51.75	0.35	61	7.60
Butyl-cellufine (pH 8.0)	2.8	7	2.5	8.32	54.34
Gel filtration	0.3	1.6	5.34	1.90	116.08

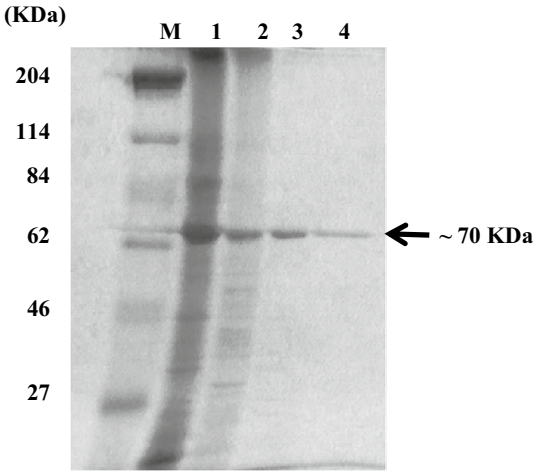

Fig. 1 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of SmAEH. Cell-free extracts from *S. maltophilia* HS1 (lane 1), DEAE-Cellufine (lane 2), Butyl-Cellufine (lane 3) and gel filtration (lane 4) are shown. The SmAEH band is indicated. Molecular mass markers were loaded in lanes labelled M, and their masses (KDa) are shown on the left

However, enzyme activity was decreased to approximately 75% after 10-min treatment at 55 °C.

SmAEH was highly active in the pH range of 7.0–10.0, and was most active at pH 9.0 (Fig. 2b). The enzyme was also stable at pH between 5.0 and 10.0, although approximately 25 and 10% decreases in enzyme activity were observed following treated at pH 4.0 and 10.0, respectively.

Substrate specificity for acyl donors was investigated using a colorimetric method with 10 differing L-amino acid esters. In these experiments, all tested acyl donors were rapidly hydrolysed by SmAEH and the enzyme accepted a wide range of amino acid methyl ester substrates, including β-Ala-OMe (Fig. 3).

To investigate the effects of metal ions on enzyme activities, reactions were performed in the presence of various metal ions. These activity assays showed little preference of SmAEH for specific metals (Fig. 4), although Ag^+ abolished SmAEH activity completely and Na^+ slightly enhanced SmAEH activity. Finally, SmAEH activity was completely abolished after 1-h treatment with 1-mM PMSF (Fig. 4).

Analysis of the reaction products

In HPLC analyses of SmAEH reaction mixtures (Fig. 5), products were formed in a time-dependent manner. In particular, the Ile-Trp (peak no. 3) was detected in 30-min reactions and increased further after 1-h reactions. Moreover, in comparisons with standards and zero-min HPLC peaks, time-dependent reaction peak profiles showed a new peak with a retention time of 34 min, presumably reflecting the presence of Il-Trp at this time point. After 1 h reactions,

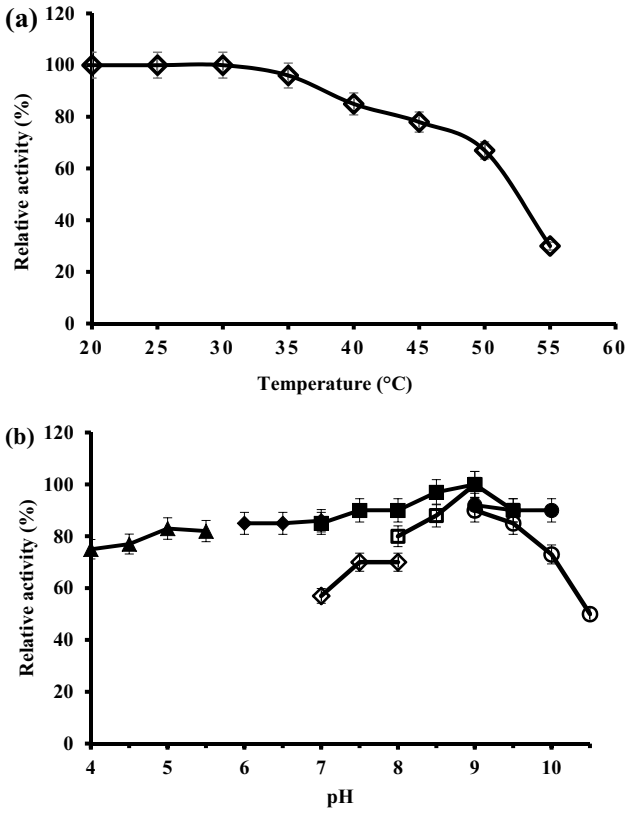

Fig. 2 Temperature stability and effects of pH on SmAEH enzyme activity and stability. **a** Temperature stability of SmAEH; **b** effects of pH on SmAEH activity and stability; reactions were adjusted to various pH values using potassium phosphate buffer (pH 7.0–8.0; open diamond), Tris–HCl buffer (pH 8.0–9.5; open square) and borate buffer (pH 9.0–10.5; open circle). SmAEH stability was also examined in the presence of citrate buffer (pH 4.0–5.5; closed triangle), potassium phosphate buffer (pH 6.0–7.0; closed diamond), Tris–HCl buffer (pH 7.0–9.0; closed square) and borate buffer (pH 9.0–10.0; closed circle)

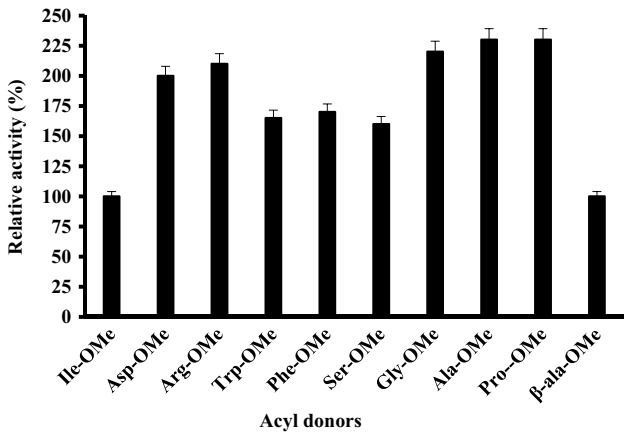

Fig. 3 Substrate specificity of SmAEH in hydrolysis reactions. Acyl donor specificity; hydrolysis on SmAEH was performed in 100-μL aliquots of reaction buffer containing 50-mM acyl donor and 100-mM Tris–HCl buffer (pH 9.0) at 30 °C for 1 h. Reaction mixtures without enzyme were used as controls

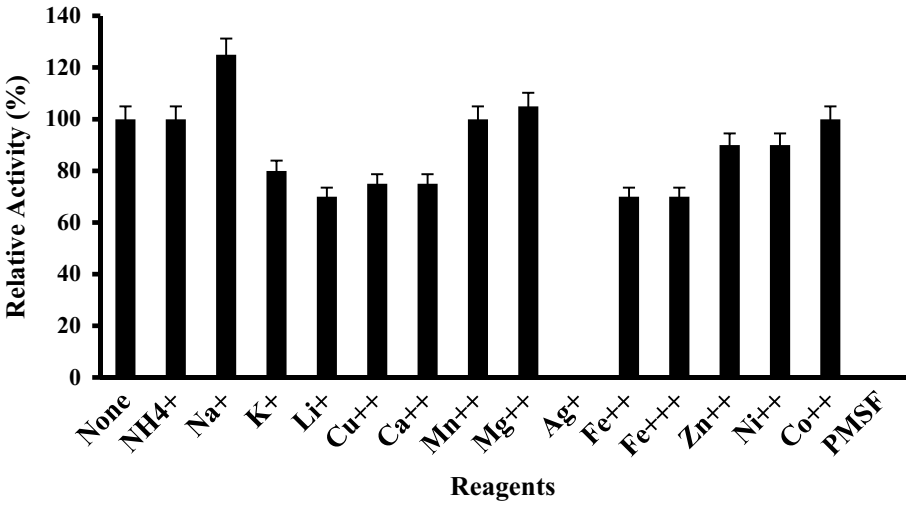

Fig. 4 Effects of metal ions and phenylmethane sulfonyl fluoride (PMSF) on SmAEH activity. Reactions were performed separately with 50-mM Ile-OMe, 100-mM Trp, individual metal ions at 5 mM and PMSF at 1 mM in Tris–HCl buffer (pH 9.0) at 30 °C for 1 h. Relative activity of SmAEH was then determined and data were presented as means ± standard errors of the mean from at least two independent experiments

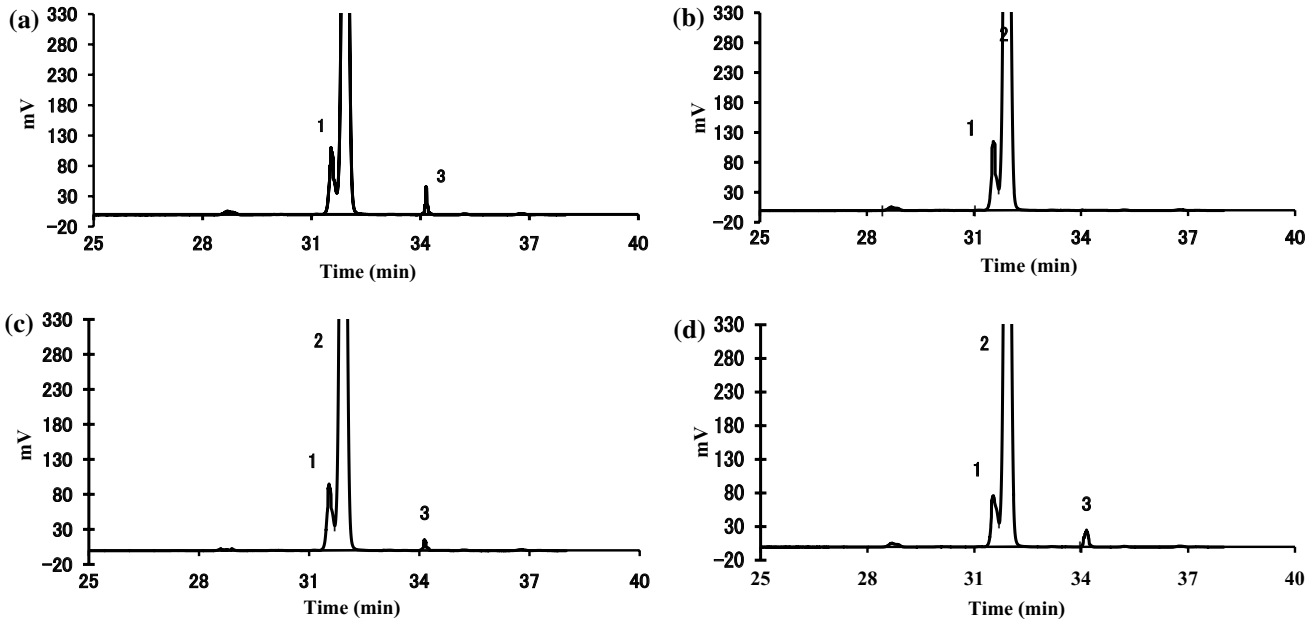

Fig. 5 High-performance liquid chromatography (HPLC) profiles of SmAEH reactions over time. HPLC analyses of the reaction products were classified in the following four sections: **a** standard, **b** 0-min reactions, **c** 30-min reactions, and **d** 1-h reactions. Peaks 1, 2 and 3 correspond with Ile-OMe, Trp and Ile-Trp, respectively. Standard concentrations for Ile-OMe, Trp and Ile-Trp were 50, 100 and 10 mM, respectively

product concentrations reached approximately 2 mM, indicating that SmAEH produces Ile-Trp.

Discussion

AEH was first discovered by Takahashi et al. in *Acetobacter turbidans* (Takahashi et al. 1974) and was shown to catalyse amino-acyl transfer from α-amino acid esters to 7-amino-3-deacetoxy-cephalosporanic acid, along with the hydrolysis of corresponding α-amino acid esters. Later, Kato et al. isolated and characterised AEH from *Xanthomonas citri* and demonstrated hydrolysis reactions of L-amino acid ester, but could show no caseinolytic activity (Kato et al. 1980).

In the present study, we isolated *S. maltophilia* HS1 by screening AEH activities in more than 200 soil samples. We also found AEH activity in *Pseudomonas* spp. (Supplementary data Table 1), and previous studies show AEH activity in the bacterial strains *Empedobacter brevis* (Yokozeki and Hara 2005), *A. turbidans* (Polderman-Tijmes et al. 2002), *Bacillus mycoides* (Sugihara et al. 2001) and *Sphingobacterium siyangensis* (Abe et al. 2011), and in yeast

Saccharomyces spp. (Yokozeki and Hara 2005). Biochemical, physicochemical and catalytic properties of AEHs have been studied previously and by 2013, dipeptide-synthetic activities were documented in ten wild-type and mutant strains, which are known to express AEHs comprising multiple subunits (Kurochkina et al. 2013). The present data show that AEH from *S. maltophilia* is a tetramer, whereas other studies show that AEH from *Pseudomonas melanogenum* comprises two identical subunits of 72 kDa (Kim and Byun 1990). Enzymes from the *Xanthomonas* family comprise four identical subunits of 70–72 kDa, with total molecular weights of 260–280 kDa (Kato et al. 1980). However, in *A. turbidans*, AEH reportedly comprises a tetramer of two 70-kDa subunits and two 72-kDa subunits, with a total molecular weight of 280–290 kDa (Ryu and Ryu 1988). In contrast, more recent data suggest that purified native AEH from *A. turbidans* is a multimer of 70-kDa subunits, and that both dimers and tetramers have enzymatic activity (Polderman-Tijmes et al. 2002). These AEHs have been considered for industrial enzyme production of semi-synthetic β-lactam antibiotics (Barends et al. 2003; Polderman-Tijmes et al. 2002), but were not used for peptide synthesis until 2001 (Sugihara et al. 2001), reflecting the lack of data confirming that the enzyme synthesises and hydrolyses peptides. Accordingly, Sugihara et al. was the first to demonstrate that AEH from *Bacillus mycoides* catalyses the formation of dipeptides (Sugihara et al. 2001). Among other enzymatic methods for synthesising dipeptides, L-amino acid ligase (Lal)-catalysed synthesis of dipeptides leads to the formation of unprotected L-amino acids in an ATP-dependent manner, and several Lals have been used for the synthesis of dipeptides such as Ala-Gln, Phe-Ala, Met-Gly and Leu-Gly (Kino et al. 2008a, b; Tabata et al. 2005). However, Lals suffer from low accumulation and productivity of the target dipeptide (Yagasaki and Hashimoto 2008), reflecting the technological nascence of dipeptide formulations. However, Ile-Trp potently inhibits ACE activity, reduces matrix metalloproteinase-2 activity and improves coronary flow reserves (Sato et al. 2002; Li et al. 2004; Murray and Fitz Gerald 2007), and could be administered as a food additive.

Because Ile-Trp is an important ACE inhibitor with applications as a drug pressure lowering drug, we investigated the synthesis of Ile-Trp by AEH from *S. maltophilia*. Subsequently, we characterised the enzyme for industrial application. AEHs from different microbial sources show similar biochemical, physicochemical and catalytic properties (Kurochkina et al. 2013), and the present data show that SmAEH has high activity under conditions of pH 9.0 at 30 °C. Although AEH from *S. siyangensis* showed maximal activity at pH 8.5 and 25 °C (Hirao et al. 2013), optimal pH and temperature values may differ between substrates (Malathi and Chakraborty 1991). We also examined pH and temperature stability of the enzyme and found that SmAEH activity decreases dramatically after exposure to temperatures above 50 °C, whereas the enzyme remained stable between pH 5.0 and 10.0. In agreement, Kato et al. isolated AEH from *X. citri* and showed complete inactivation after incubation at 50 °C for 15 min. These authors also report a broad range of pH stability from 4.0 to 9.0 (Kato 1980). Finally, because serine residues play active roles in the catalytic activities of α-β hydrolases (Ollis et al. 1992), we performed experiments with the serine protease inhibitor PMSF and confirmed that serine residues are critical to the activity of SmAEH. Similarly, Sugihara et al. showed that PMSF strongly inhibits AEH from *B. mycoides* (Sugihara et al. 2001).

Colorimetric assays of acyl donor hydrolysis by SmAEH showed relatively high substrate preference for non-bulky amino acid methyl esters, and Ala-OMe was preferred over the other substrates. However, further studies with standard dipeptide stocks are required to confirm the preference of SmAEH for acceptor substrates. Such studies of acceptor preferences are of high priority, particularly because these will inform the synthesis of other biologically important dipeptide derivatives.

The present findings indicate noteworthy utility of SmAEH for the synthesis of Ile-Trp and suggest that other dipeptide derivatives may be biologically significant. However, enzyme production and dipeptide yields were inadequate under the present conditions. Hence, future studies are required to clone the *SmAEH* gene for the production of dipeptides in large scale with minimum production costs and high yields. These aims will be facilitated by the current development of an expression system for recombinant SmAEH in *Escherichia coli*.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests.

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