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Extracellular Production and Characterization of *Streptomyces* X-prolyl Dipeptidyl Aminopeptidase

Tadashi Hatanaka · Akihiro Yamasato · Jiro Arima · Hirokazu Usuki · Yukihiro Yamamoto · Yuya Kumagai

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Abstract X-prolyl dipeptidyl aminopeptidases (X-PDAPs) are useful in various food industries. In this study, we performed sequence-based screening to obtain a stable X-PDAP enzyme from thermophilic *Streptomyces* strains. We found three genes that encoded X-PDAP from *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 (14270 X-PDAP), *Streptomyces thermocyaneoviolaceus* NBRC 14271 (14271 X-PDAP), and *Streptomyces thermocoerulescens* NBRC 14273, which were subsequently cloned and sequenced. The deduced amino acid sequences of these genes showed high similarity, with ~80% identity with each other. The isolated X-PDAPs and an X-PDAP from *Streptomyces coelicolor* were expressed in *Streptomyces lividans* using a hyperexpression vector: pTONA5a. Among these genes, only 14270 and 14271 X-PDAPs caused overexpression and extracellular production without artificial signal peptides. We also characterized the biochemical properties of purified 14271 X-PDAP. In addition, we found that, in peptide synthesis via an aminolysis reaction, this enzyme recognized D-amino acid derivatives as acyl acceptors, similar to L-amino acid derivatives.

Keywords Peptidase · *Streptomyces* · Extracellular production · Dipeptidyl aminopeptidase · Aminolysis

T. Hatanaka (✉) · A. Yamasato · H. Usuki · Y. Yamamoto · Y. Kumagai
Okayama Prefectural Technology Center for Agriculture, Forestry, and Fisheries,
Research Institute for Biological Sciences (RIBS),
Okayama, 7549-1 Kibichuo-cho, Kaga-gun, Okayama 716-1241, Japan
e-mail: hatanaka@bio-ribs.com

H. Usuki
Japan Society for the Promotion of Science (JSPS), Tokyo, Japan

J. Arima
Department of Agricultural, Biological, and Environmental Sciences, Faculty of Agriculture,
Tottori University, 4-101 Koyama-minami, Tottori 680-8553, Japan

Introduction

Among the 20 naturally occurring amino acids, proline is unique because of its unusual cyclic structure. Because of this structure, peptide bonds involving proline residues are notoriously difficult to cleave using available enzymes. Casein, gluten, collagen, and gelatin are proline-rich proteins. Their hydrolysates, which are produced by peptidases, are useful in various food industries, although their application is limited because of their proline-rich properties. The X-prolyl dipeptidyl aminopeptidases (X-PDAPs; EC 3.4.14.5) are serine enzymes that remove Xaa-Pro dipeptides from the N-terminus of peptides. Consequently, these enzymes make an important contribution to the utilization of proline-rich proteins, including casein, soy, and gluten, by hydrolyzing peptides containing proline [1]. Furthermore, X-PDAPs catalyze acyl transfer from specific substrates to various nucleophiles via a covalent acyl-enzyme intermediate, a process also referred to as "aminolysis" [2]. Ota et al. previously demonstrated the synthesis of a bioactive tetrapeptide of morphiceptin via X-PDAP from *Aspergillus oryzae* [3]. Accordingly, X-PDAPs are also useful from an industrial perspective.

Recently, we developed a hyperexpression vector for *Streptomycetes* named pTONA5a. Using pTONA5a and *Streptomyces lividans*, we achieved overexpression and extracellular production of two *Streptomyces* aminopeptidases that possessed no signal peptide for secretion [4]. Herein, we describe our further attempt at overexpression and extracellular production of various useful *Streptomyces* enzymes in *S. lividans* using pTONA5a.

In this study, we cloned and expressed X-PDAP genes from *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 (14270 X-PDAP), *Streptomyces thermocyaneoviolaceus* NBRC 14271 (14271 X-PDAP), and *Streptomyces thermocoerulescens* NBRC 14273 (14273 X-PDAP). We subsequently attempted extracellular production of these X-PDAPs using *S. lividans*. The 14270 and 14271 X-PDAPs, but not 14273 X-PDAP or X-PDAP from *Streptomyces coelicolor* (SCO X-PDAP), are secreted extracellularly. In addition, we purified 14271 X-PDAP for further biochemical characterization and characterized an aminolysis reaction using this X-PDAP.

Materials and Methods

Sequence-Based Screening

We prepared genomic DNA of several thermophilic *Streptomyces* NBRC-type culture strains (*S. thermoluteus* NBRC 14269, *S. thermoluteus* subsp. *fuscus* NBRC 14270, *S. thermocyaneoviolaceus* NBRC 14271, *Streptomyces thermocyaneomaculatus* NBRC 14272, *S. thermocoerulescens* NBRC 14273, *Streptomyces thermolilacinus* NBRC 14274, and *Streptomyces thermogriseus* NBRC 100772) using a method described in an earlier study [5]. All strains were cultured at 45–50 °C, and their genomic DNAs were used as templates for screening. Two degenerated primers, 5′-GAGTTCRTCGCSGCSGAGGA GATG-3′ and 5′-CTCCTGSGGSGTCATGTGSGT-3′ (a sense and an anti-sense primer, respectively), were designed from alignment of primary sequences of predicted X-PDAPs from *S. coelicolor* (gene ID: SCO5122, SCO X-PDAP), *Streptomyces avermitilis* (gene ID: SAV3144), and *Streptomyces griseus* (gene ID: SGR2407). X-PDAPs with such specificity are classified into the following two distinct families in clan SC: S9B and S15 [6]. The *Streptomyces* X-PDAPs described above are members of the S9B family of dipeptidyl aminopeptidases IV (DPP-IV). We subsequently performed PCR with 1 μM primers and

LA Taq DNA polymerase with GC Buffer I (TaKaRa Holdings, Inc.) using the following protocol: 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C, and final extension for 5 min at 72 °C. Among the thermophilic *Streptomyces* strains that we examined, we obtained successful PCR products (ca. 1.5 kbp) from *S. thermoluteus* subsp. *fuscus* NBRC 14270, *S. thermocyaneoviolaceus* NBRC 14271, and *S. thermocoerulescens* NBRC 14273. We subsequently cloned the PCR products into pGEM-T Easy Vector (Promega Corp.) and sequenced them.

Cloning of X-PDAP Genes

The genomic DNA of *S. thermoluteus* subsp. *fuscus* NBRC 14270 was digested with *Bal*I and self-ligated. We amplified this ligation product using primer sets designed based on the sequence obtained through PCR-based screening. We also sequenced the inverse PCR product. Using sequence-based screening, we identified a 2,133-bp open reading frame from *S. thermoluteus* subsp. *fuscus* NBRC 14270 that encoded a predicted X-PDAP. Using inverse PCR, we obtained the genes encoded by 14271 and 14273 X-PDAP, which were similar to that of 14270 X-PDAP. The entire sequences of 14270, 14271, and 14273 X-PDAPs were assigned accession numbers AB510493, AB510494, and AB510495 in the DDBJ database, respectively. The deduced amino acid sequence of 14270 X-PDAP showed 80.0%, 84.2%, and 86.7% identities with the encoded 14271, 14273, and SCO X-PDAPs, respectively.

Construction of Expression Plasmids for *Streptomyces* X-PDAPs

For construction of expression plasmids, we amplified 14270, 14271, 14273, and SCO X-PDAPs using PCR with primer sets including a sense primer containing the *Nde*I site (CATATG; the start codon is underlined) and an anti-sense primer containing the *Hin*dIII site downstream of the stop codon. We subsequently cloned the resultant fragment and confirmed correct cloning by sequencing. The DNA fragments that encode the X-PDAPs were digested using *Nde*I and *Hin*dIII and were ligated into the *Nde*I–*Hin*dIII gap of pTONA5a [4].

Expression Using *S. lividans*

The expression vectors were transformed in *Escherichia coli* S17-1. The transformants were subsequently used to conjugate *S. lividans* 1326, using a procedure similar to a method described in a previous report [4]. The resultant *S. lividans* transformants were inoculated and grown in culture medium containing 0.8% K_2HPO_4, a 1.0–4.0% carbon source that was sterilized separately, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% polypeptone, and 0.5% yeast extract at 30 °C for 5 days at 180 strokes or 180 rpm. We used glycerol, fructose, and glucose as carbon sources. In this study, the above-mentioned medium containing 2.0% glucose was termed "PG medium."

Purification of Recombinant 14271 X-PDAP

We obtained a culture filtrate with PG medium using an ultrafiltration apparatus (0.45 μm pore size; Sartorius Corp.). The filtrate was dialyzed against 25 mM Tris–HCl buffer (pH 8.0). After centrifugation, the sample was loaded onto a Vivapure Q spin column (Millipore Corp.) that had been equilibrated using the same buffer used for dialysis. The

bound protein was eluted with 0.5 M NaCl in 25 mM Tris–HCl buffer (pH 8.0). The eluate was subsequently loaded onto a gel filtration column (HiLoad 16/10 superdex 200 prep grade; GE Healthcare Bio-Science). The active fraction was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) and loaded onto a Mono Q HR5/5 column that had been pre-equilibrated with dialysis buffer. The 14271 X-PDAP was eluted using a linear gradient of NaCl from 0 to 0.5 M. The active fraction was dialyzed against 10 mM Tris–HCl buffer (pH 7.5). The enzyme was purified to homogeneity on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), resulting in a 1.44-fold purification of X-PDAP with a yield of 6.2% (data not shown). We used the resultant enzyme solution for characterization.

Determination of N-terminal Amino Acid Sequences

After performing 10% SDS-PAGE under denaturing conditions, the sample was electro-blotted onto a polyvinylidene difluoride membrane. It was subsequently loaded onto a protein sequencer (model 491; Applied Biosystems) to identify its N-terminal amino acid sequence. The N-terminal sequence of secreted 14271 X-PDAP was determined to be TEPLS. The deduced N-terminal amino acid sequence of 14271 X-PDAP was MTTEPLSFPR (AB510494 in the DDBJ database). From the N-terminal amino acid sequence analysis, we found that the purified enzyme had two amino acid residues (Met-Thr) truncated from the starting Met residue.

Gel Filtration Analysis

Gel filtration was performed using a column (Superdex 200 10/300; GE Healthcare Bio-Science) that had been pre-equilibrated with 80 mM Tris–HCl (pH 7.0) containing 0.2 M NaCl. We purchased molecular-weight marker proteins (glutamate dehydrogenase [290 kDa], lactate dehydrogenase [142 kDa], enolase [67 kDa], myokinase [32 kDa], and cytochrome C [12.4 kDa]) from Oriental Yeast Co., Ltd.

Analysis of X-PDAP Activity

We determined the enzyme activities toward Xaa-Pro-*p*NA derivatives (Bachem AG) under the following conditions. We added an enzyme solution (80 μl) and a substrate solution in dimethyl sulfoxide (DMSO) (80 μl, 20 mM) to 640 μl of 100 mM Tris–HCl buffer (pH 7.5) and continuously monitored the increase in absorption at 405 nm caused by the release of *p*-nitroaniline per minute using a spectrophotometer. The initial activity rate was determined from the linear part of the optical density profile (ε_{405}nm=10,600 M^{-1} cm^{-1}), as previously described [7].

Assessment of X-PDAP Stability

We investigated the thermal stability of X-PDAP using the following method. Briefly, the purified enzyme solution (0.1 mg/ml in 10 mM Tris–HCl buffer [pH 7.5]) was incubated at different temperatures for 30 min. Subsequently, the samples were assayed for residual activity using the enzyme assay method described in the above section, and the residual activity of samples was assessed relative to that when it was stored on ice. We calculated the T_m value from a plot of relative inactivation (percent) versus temperature (°C), that is, the T_m value of each sample was determined as the temperature at which 50% of the total activity was lost when the enzyme was stored on ice after heat treatment.

Furthermore, we also investigated the pH stability of the enzyme. Briefly, the purified enzyme solution (0.1 mg/ml in 100 mM acetate buffer or Tris–HCl buffer) was incubated at 37 °C for 24 h, and the samples were subsequently assayed for residual activity using the enzyme assay method that is described above. We assessed the residual activity relative to that when the enzyme was stored at 4 °C and pH 7.5 for 24 h.

Inhibition Assay

We also performed an inhibition assay using the purified enzyme. Briefly, the purified enzyme solution (0.1 mg/ml in 10 mM Tris–HCl buffer [pH 7.5]) was incubated in the presence of 2 mM phenylmethylsulfonyl fluoride or 2 mM diprotin A at 37 °C for 30 min, and the samples were subsequently assayed for residual activity using the enzyme assay method described above. The residual activity of the enzyme was assessed relative to that in the absence of the inhibitor.

Assessment of Aminolysis Reaction

For assessment of the aminolysis reaction, we set up the following typical reaction conditions: 1 μl of 50 mM Val-Pro-O-*tert*-butyl ester (Val-Pro-O-tBu, Bachem AG) as an acyl donor, 25 μl of 200 mM amino acid amides as an acyl acceptor, 10 μl of 1 M Tris–HCl buffer (pH 7.5), and 10 μl of enzyme solution containing 10 μg of 14271 X-PDAP at 900 rpm for 17 h at 40 °C. We adjusted the final volume with distilled water up to 100 μl. Furthermore, the reaction was terminated by the addition of 100 μl of 1.0% (*v*/*v*) HCOOH. After centrifugation at 15,000 rpm for 5 min, we used 10 μl of the supernatant to liquid chromatography-mass spectrometry (LC/MS) analysis. Amino acids and their esters were dissolved using water or DMSO. We used the dialysate of the culture supernatant as an enzyme source.

LC/MS Analysis

For this analysis, we used a Waters ACQUITY UPLC system, a Waters TUV detector, and an API 2000 LC/MS/MS system (Life Technologies Inc., Carlsbad, CA, USA). Furthermore, the column used for this study was a BEH C_{18} ACQUITY UPLC 2.1× 50 mm column (Waters Corp., Tokyo, Japan). The mobile phase included both water-containing and methanol-containing 0.1% (*v*/*v*) HCOOH at a flow rate of 0.3 ml/min. Specifically, the LC condition involved only 5% of the latter component during 0–5 min, a linear increase to 95% of this component during 5–12 min, and finally 95% of this component during 12–13.5 min. To monitor the products, we calculated the corresponding peak areas of the extracted ion chromatogram (XIC) according to the device system of the API 2000 LC/MS/MS. For the XIC, the m/z ±0.5 value of $[M+H]^+$ was adopted.

Results and Discussion

Extracellular Production of X-PDAPs

Among the four X-PDAPs we tested, the 14270 and 14271 X-PDAPs were secreted using *S. lividans*. Despite the fact that these enzymes had highly similar primary structures, their quantities of secretion differed greatly. *Streptomyces* X-PDAP is a

cytoplasmic enzyme without a signal peptide. However, the 14270 and 14271 X-PDAPs were highly secreted. We determined that the activities of the supernatants were 10-fold higher than that of the cell-free extracts (data not shown). We currently have no understanding of their secretion mechanism. We found that the specific activity of 14271 X-PDAP in the supernatant toward AlaPro-*p*NA was 4.31±0.68 U/mg, whereas the activity of 14270 X-PDAP was 3.36±0.35 U/mg. Therefore, in terms of the quantity and quality of the activity in the extracellular products we obtained, 14271 X-PDAP showed superior properties.

We also examined the effect of carbon sources on extracellular production. As shown in Fig. 1a, the media containing 2.0–4.0% glucose provided suitable conditions for extracellular production. Furthermore, the recombinant 14271 X-PDAP was found to be the main constituent in culture supernatants (Fig. 1b).

Researchers have previously investigated other bacterial X-PDAPs. Some studies have been focused on X-PDAPs from lactic acid bacteria [8–11], which revealed that these X-PDAPs were cytosolic enzymes. Therefore, preparation of these X-PDAPs requires cell lysis accompanied by a complicated procedure. Furthermore, previous research shows that one particular X-PDAP is secreted extracellularly [12]; however, this X-PDAP is not suitable for food processing as it is produced by a pathogenic bacterium. In this study, we demonstrated convenient extracellular production of *Streptomyces* X-PDAP, which may likely be convenient for industrial production.

We succeeded in inducing extracellular expression of some *Streptomyces* enzymes without introducing artificial signal peptides, including proline aminopeptidase, aminopeptidase P (APP), and two asparaginases, by using pTONA5a and *S. lividans* [4, 13]. Except for the case of APP, the three enzymes were secreted with cleavage at Xaa-Ser. 14271 X-PDAP was secreted with cleavage at Thr-Thr. Although APP was only secreted in the form with a starting Met, it may be possible that extracellular expression without artificial signal peptides is accompanied with cleavage between Xaa-Ser or Xaa-Thr. What remains to be resolved is a mechanism of this abnormal secretion.

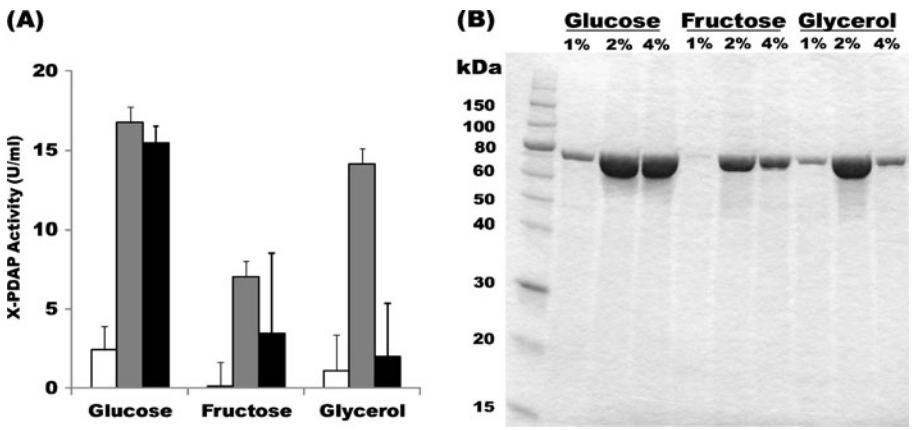

Fig. 1 Effect of carbon sources on the extracellular production of 14271 X-PDAP. **a** Comparison of X-PDAP activities in culture supernatants. The percent of carbon sources (1.0%, 2.0%, and 4.0%) were indicated as *white*, *gray*, and *black bars*, respectively. **b** SDS-PAGE of culture supernatants. All samples containing 10 μl of the supernatants were loaded onto a 12% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. The types and contents of the carbon sources are indicated in the *upper side* of the gel

Biochemical Characterization of 14271 X-PDAP

The basic properties of the examined enzymes, such as their optimum temperatures, optimum pHs, compositions, T_m values, inhibitors, and specific activities toward Xaa-Pro-*p*NAs, are presented in both Table 2 and Fig. 2. The kinetic parameters related to Ala-Pro-*p*NA (K_m, k_{cat}, and k_{cat}/K_m) were 0.25±0.10 (mM), 12.55±0.55 (1/s), and 50.59 ((1/s)×(1/mM)), respectively. These results indicate that 14271 X-PDAP has a broad specificity and moderate thermal and pH stabilities. Furthermore, this enzyme was inhibited by phenylmethylsulfonyl fluoride, which is an inhibitor of serine proteases and diprotin A, a typical substrate analogue for DPP-IV (Table 1). These results confirm that 14271 X-PDAP belongs to the S9B family of DPP-IV. When we attempted to express the SCO X-PDAP gene using *E. coli*, overexpression was achieved. The recombinant enzyme, which possessed a His-tag at the N-terminal and was purified using a metal affinity column, showed a complete loss of activity

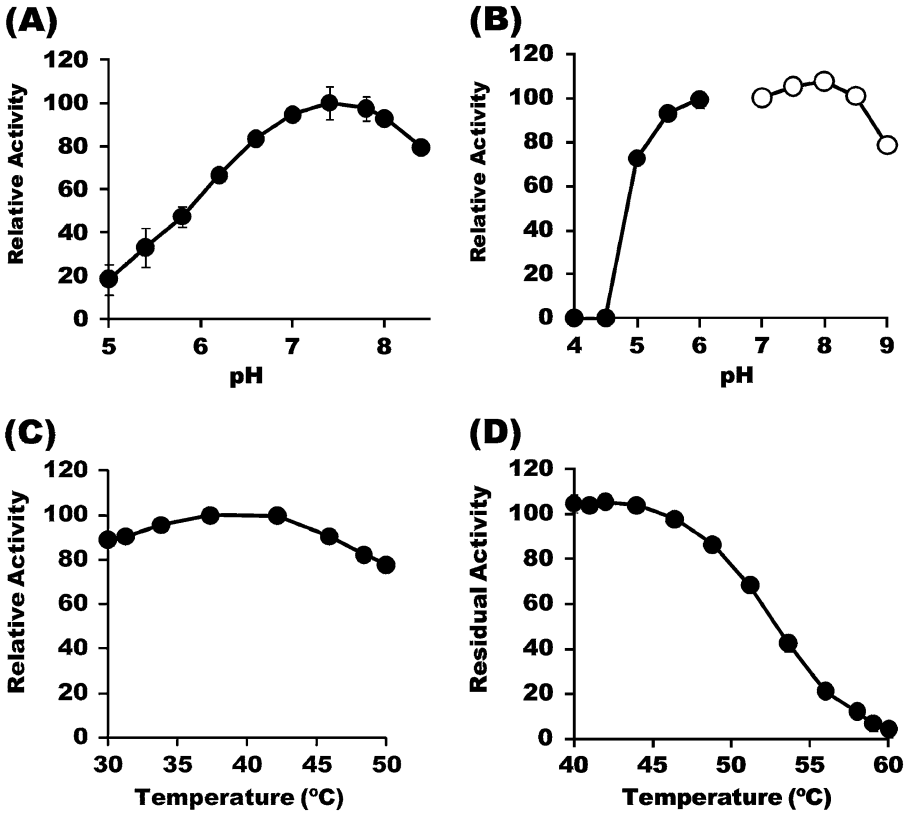

Fig. 2 Effects of pH and temperature on 14271 X-PDAP activity. **a** The optimum pH of the 14271 X-PDAP. The activity is presented as relative activity. The activity was determined using 2 mM Ala-Pro-*p*NA in 100 mM Tris–maleate buffer at 37 °C. **b** The pH stability of the 14271 X-PDAP. The full method was described in "Materials and Methods". Briefly, the residual activity was determined using 2 mM Ala-Pro-*p*NA in 100 mM Tris–HCl buffer (pH 7.5) at 37 °C. *Symbols*: *filled circle*, acetate buffer; *empty circle*, Tris–HCl buffer. **c** The optimum temperature of the 14271 X-PDAP. The activity is presented as relative activity to that at the highest temperature. The activity was determined using 2 mM Ala-Pro-*p*NA in 100 mM Tris–HCl buffer (pH 7.5). **d** Thermal stability of the 14271 X-PDAP. The residual activity was determined using 2 mM Ala-Pro-*p*NA in 100 mM Tris–HCl buffer (pH 7.5) at 37 °C

Table 1 Biochemical properties of 14271 X-PDAP

Properties	Purified 14271 X-PDAP
Molecular mass of protomer	77 kDa
Composition	Dimer
T_m (°C)	52.3
Inhibitors	
2 mM phenylmethylsulfonyl fluoride	10.5[a]
2 mM diprotin A	21.4[a]
Substrate specificity[b]	
AlaPro-*p*NA	9.28±0.12[c]
GlyPro-*p*NA	5.40±0.35[c]
ArgPro-*p*NA	7.82±0.57[c]
AspPro-*p*NA	6.09±0.31[c]
GluPro-*p*NA	8.98±0.63[c]

[a] Residual activity (percent)

[b] The 14271 X-PDAP did not hydrolyze Pro-*p*NA, AlaAlaPro-*p*NA, or AlaAla-*p*NA. All substrates were used at a concentration of 2 mM in 100 mM Tris–HCl buffer (pH 7.5) at 37 °C

[c] Specific activity (unit per milligram)

after 30 min of incubation at 40 °C (data not shown). Moreover, its specific activity toward AlaPro-*p*NA was found to be particularly low (0.10±0.01 U/mg). Furthermore, His-tagged 14271 X-PDAP was expressed by *E. coli* and purified in a manner similar to that of His-tagged SCO X-PDAP. The kinetic parameters related to Ala-Pro-*p*NA (K_m, k_{cat}, and k_{cat}/K_m) were 0.32±0.02 (mM), 11.15±0.2 (1/s), and 35.44 ((1/s)×(1/mM)), respectively. These parameters were similar to those of purified 14271 X-PDAP expressed using *S. lividans*. His-tagged 14271 X-PDAP was also similar to purified 14271 X-PDAP expressed using *S. lividans* with respect to optimum pH, optimum temperature, and thermal stability (T_m, 52.9 °C). This result suggests that the addition of His-tag to X-PDAPs has no effect on their enzymatic properties. Based on these results, it is likely that one important factor for secretion using pTONA5a and *S. lividans* was stability of the protein.

Because nonspecific exo-peptidases, such as leucine aminopeptidases, cannot hydrolyze Xaa-Pro peptides [14], protein degradation by these enzymes is interrupted at the Xaa-Pro sequence. Therefore, it is effective to combine nonspecific exo-peptidases with X-PDAP to produce protein hydrolysates [1]. Moreover, it is expected that the utilization of hydrolysates from proline-rich proteins can be improved by combining them with an X-PDAP and a prolidase or an APP that hydrolyzes the peptide bond between Xaa-Pro dipeptides. We previously reported the extracellular and over-production of both an APP from *Streptomyces costaricanus* TH-4 [14] and a leucine aminopeptidase from *Streptomyces cinnamoneus* TH-2 (formally called *Streptomyces septatus* TH-2), which was determined to be a nonspecific exo-peptidase [15]. However, further work is necessary to clarify the synergistic action of 14271 X-PDAP and the above-mentioned peptidases in the hydrolysis of proline-rich proteins, such as collagen.

Aminolysis Reaction

We also examined the substrate specificity of 14271 X-PDAP in aminolysis. The amino acid derivatives and synthesized peptides by 14271 X-PDAP that we tested are listed in

Table 2. Using a set of substrates (Val-Pro-O-tBu and L-Phe-NH_2) and Tris–HCl buffers (pH 7.0–8.5), we determined that the optimum pH in the aminolysis reaction was 7.0, based on the amount of Val-Pro-L-Phe-NH_2 (data not shown). Among the D-acyl acceptors we tested, D-Leu-NH_2, D-Leu-OH, D-Leu-OBzl, D-Met-NH_2, and D-Phe-NH_2 were recognized and generated the expected products (Table 2). We also separated a set of diastereomeric products by LC/MS analysis. For example, Val-Pro-L-Phe-NH_2 and Val-Pro-D-Phe-NH_2 showed different retention times (Fig. 3, Table 2). These data illustrate the synthesis of peptides containing D-amino acid derivatives using X-PDAP that had hydrolysis activity toward L-amino acid residues. To the best of our knowledge, D,D-carboxypeptidase from *Streptomyces* sp. R61, D-aminopeptidase from *Ochrobactrum anthropi*, and D-peptidase from *Bacillus cereus* have the function of aminolysis reaction that is specific toward D-amino acid residues [16–18]. These peptidases showed the activity only toward D-amino acid residues. Very recently, we have demonstrated that S9 aminopeptidase from

Table 2 Tested amino acid derivatives and synthesized peptides by 14271 X-PDAP

Substrate as an acyl acceptor			Product	Observed *m/z*, $[M+H]^+$	Intensity (c. p. s.)	Retention time (min)
Amino acid moiety	Types of derivatization of carboxyl group	Supplier				
Gly	$-NH_2$	N	n.d.	–	–	–
L-Ala	$-NH_2$	N	Val-Pro-L-Ala-NH_2	285.2	1.42×10^6	5.77
D-Ala	$-NH_2$	A	Val-Pro-D-Ala-NH_2	285.3	1.16×10^6	5.64
L-Leu	$-NH_2$	N	Val-Pro-L-Leu-NH_2	327.2	1.33×10^7	2.99
	–OH	W	Val-Pro-L-Leu-OH	328.4	5.68×10^5	4.37
	–OBzl	P	Val-Pro-L-Leu-OBzl	418.1	6.09×10^7	7.70
D-Leu	$-NH_2$	N	Val-Pro-D-Leu-NH_2	327.3	1.75×10^7	4.62
	–OH	W	Val-Pro-D-Leu-OH	328.4	1.00×10^5	5.34
	–OBzl	B	Val-Pro-D-Leu-OBzl	418.3	6.68×10^7	7.85
L-Gln	$-NH_2$	N	n.d.	–	–	–
D-Gln	$-NH_2$	N	n.d.	–	–	–
L-Met	$-NH_2$	W	Val-Pro-L-Met-NH_2	345.2	1.92×10^7	1.61
D-Met	$-NH_2$	Wa	Val-Pro-D-Met-NH_2	345.2	3.55×10^7	3.69
L-Phe	$-NH_2$	B	Val-Pro-L-Phe-NH_2	361.3	1.03×10^7	4.37
D-Phe	$-NH_2$	D	Val-Pro-D-Phe-NH_2	361.3	2.01×10^7	5.07
L-Pro	$-NH_2$	B	n.d.	–	–	–
	–OH	W	n.d.	–	–	–
	–OMe	T	n.d.	–	–	–
	–OBzl	B	n.d.	–	–	–
D-Pro	$-NH_2$	Wa	n.d.	–	–	–
	–OH	W	n.d.	–	–	–
	–OMe	B	n.d.	–	–	–
	–OBzl	B	n.d.	–	–	–
L-Tyr	$-NH_2$	B	Val-Pro-L-Tyr-NH_2	377.1	5.99×10^6	2.58
L-Phe-L-Phe	$-NH_2$	B	Val-Pro-L-Phe-L-Phe-NH_2	508.2	2.88×10^7	6.40

Suppliers: *A*, Sigma-Aldrich (St. Louis, MO, USA); *B*, Bachem AG (Bubendorf, Switzerland); *N*, Novabiochem (Darmstadt, Germany); *P*, Peptide Institute, Inc. (Osaka, Japan); *S*, Sigma Chemical Co. (St. Louis, MO, USA); *T*, Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); *W*, Wako Pure Chemical Industries, Ltd. (Osaka, Japan); *Wa*, Watanabe Chemical Industries Ltd. (Hiroshima, Japan); *n.d.*, not detectable by LC/MS analysis

S. thermocyaneoviolaceus NBRC 14271 was also catalyzed, and the aminolysis reaction was similar to that by 14271 X-PDAP [19]. The reaction was consisted of L-Pro-OBzl ester as an acyl donor and L- or D-amino acid esters as acyl acceptors, and produced L-Pro-D-amino acid esters. However, further work is necessary to troubleshoot the presented process to achieve maximal selectivity and yield.

When Gly-NH_2 was used as the acceptor, the expected product of Val-Pro-Gly-NH_2 was not detected, similar to the result when L-Pro-NH_2 was used. A previous study that used X-PDAP from *Lactococcus lactis* showed that Gly-NH_2 was the best acceptor [2]. Because *L. lactis* X-PDAP (accession number: AF401518), which also belongs to the S9B family of enzymes, shows very low homology (25.3%) with 14271 X-PDAP (based on their primary sequences), the two enzymes were different in substrate specificities.

In this study, we demonstrated the synthesis of Val-Pro-Xaa derivatives via the aminolysis reaction using 14271 X-PDAP. Diprotin A (Ile-Pro-Ile-OH) and diprotin B (Val-Pro-Ile-OH) have been reported as inhibitors of DPP-IV; however, it was reported that an apparent competitive inhibition by these compounds is a kinetic artifact [20]. Human DPP-IV (EC 3.4.14.5) is a membrane-associated peptidase and also exists in serum as a soluble form with a truncated N-terminal. This DPP-IV cleaves dipeptides from the N-terminus of peptides containing Pro or Ala at the penultimate position [21]. Of particular interest is the established role of DPP-IV in degrading the incretin hormone, glucagon-like peptide-1 (GLP-1). GLP-1 stimulates glucose-induced insulin biosynthesis and secretion, which leads to lower blood glucose levels [22]. However, using GLP-1 alone as a therapeutic agent is not practical, especially because it is rapidly inactivated by DPP-IV in vivo [23]. Therefore, inhibitors of DPP-IV are of considerable interest for use as supplemental anti-diabetic agents. Future studies are necessary to investigate the inhibitory action of Val-Pro-Xaa derivatives containing D-amino acids against human DPP-IV.

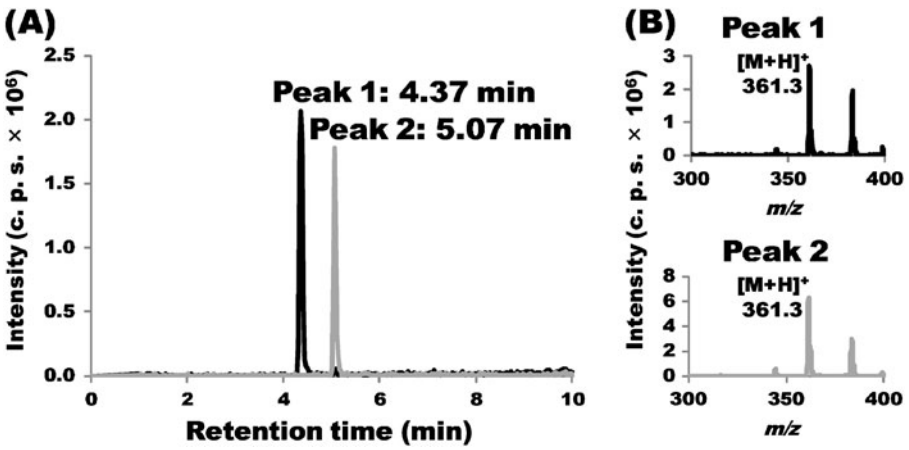

Fig. 3 **a** XICs of the reaction mixtures are shown. The 361±0.5 value of $[M+H]^+$, which was equivalent to that of Val-Pro-Phe-NH_2, was adopted. The *black line* represents a reaction mixture of Val-Pro-O-tBu and L-Phe-NH_2. The extracted peak (peak 1) was detected at 4.37 min. The *gray line* represents a reaction mixture of Val-Pro-O-tBu and D-Phe-NH_2. The retention time of the extracted peak (peak 2) was 5.07 min. **b** MS spectra of peak 1 (*upper*) and peak 2 (*lower*) are shown

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