Prolyl Aminopeptidase from *Streptomyces thermoluteus* subsp. *fuscus* Strain NBRC14270 and Synthesis of Proline-Containing Peptides by Its S144C Variant[†]

Yukihiro Yamamoto, Hirokazu Usuki, Masaki Iwabuchi, and Tadashi Hatanaka*

Okayama Prefectural Technology Center for Agriculture, Forestry and Fisheries, Research Institute for Biological Sciences (RIBS), 7549-1 Kibichuo-cho, Kaga-gun, Okayama 716-1241, Japan

Received 26 May 2010/Accepted 19 July 2010

We specifically examined an exopeptidase, prolyl aminopeptidase (PAP), as a target for synthesis of proline-containing peptides. A PAP from *Streptomyces thermoluteus* subsp. *fuscus* NBRC14270 (PAP14270) was obtained using sequence-based screening. From PAP14270, 144Ser was replaced by Cys (scPAP14270) to give aminolysis activity. In contrast to wild-type PAP14270, scPAP14270 produced a polymer of proline benzyl ester and cyclo[Pro-Pro]. The product mass was confirmed using liquid chromatography-mass spectrometry (LC/MS). Several factors affecting the reaction, such as the pH, concentration of the substrate, and reaction time, were measured to determine their effects. Furthermore, a correlation was found between substrate specificity in proline peptide synthesis and the log *D* value of acyl acceptors in aminolysis catalyzed by scPAP14270. Results showed that dipeptide synthesis proceeded in a weakly acidic environment and that cyclization and polymerization occurred under alkaline conditions. Furthermore, results suggest that almost all amino acid esters whose log *D* value is greater than 0, except hydroxyproline benzyl ester (Hyp-OBzl), can be recognized as acyl acceptors. These findings support the use of PAPs as a tool for production of physiologically active proline peptides.

Prolyl aminopeptidase (PAP) (EC 3.4.11.5), belonging to the S33 family, is an exopeptidase that catalyzes the hydrolysis of the N terminus prolyl residue of peptides or proteins. This family has catalytic Ser. To date, few applications of this enzyme for peptide synthesis have been reported. However, from the perspective of biotechnology, PAP might be a good tool for synthesizing proline-containing peptides by catalyzing aminolysis.

Recently nutraceutical properties of peptides containing proline have received increasing attention. For example, prolyl hydroxyproline (Pro-Hyp) stimulates the growth of fibroblasts from mouse skin (11). Pro-Arg can protect against oxidative stress/damage and H₂O₂-induced human diploid fibroblast cell death (13). Furthermore, the lactotripeptides Ile-Pro-Pro and Val-Pro-Pro exhibit angiotensin I-converting enzyme-inhibiting activity (9). In addition to these dipeptides and tripeptides, a cyclic dipeptide (namely, diketopiperazine) containing proline shows several physiological functions. Cyclo[Pro-Pro] (cPP) exerts antibacterial activity against *Micrococcus luteus* and *Pseudomonas aeruginosa* (8). Caspase-3 activation by cyclo[Pro-Phe] in HT-29 cells has been described (3). However, its synthesis method has not been established. Enzymatic peptide synthesis presents a useful and desirable strategy be-

cause it can conduct specific reactions under milder conditions than those of chemical synthesis.

Engineered endoserine proteases that have Cys substituted for catalytic Ser have also been applied for peptide synthesis since subtiligase was constructed by Abrahmsén et al. (1). Because of the weakened hydrolytic activity of the parent enzyme, it is considered that Ser/Cys-substituted protease can trap the substrate (acyl donor). Then, a nucleophilic reaction occurs between another substrate (acyl acceptor) and the trapped acyl donor (2). This is a so-called “aminolysis” reaction. Although aminolysis can conduct peptide synthesis in an aqueous solution, the problem of the necessity of using an N-protected amino acid as an acyl donor remains when using endoproteases.

These problems would be solved using exoprotease as a catalyst, because N-terminal free amino groups of acyl donors are recognized by enzymes. It is rarely reported that exoprotease was applied for peptide synthesis, except in the report of Oshiro et al., in which Pro-Phe, Pro-Tyr, and Pro-Trp were synthesized (10). Recently our group reported that the Ser/Cys variant of exoprotease, aminolysin-S, has been constructed and has produced L-Phe-L-Phe ethyl ester and their derivatives from non-N-protected phenylalanine and phenylalanine ethyl ester as acyl donors in aqueous solution (12). However, aminolysin-S cannot produce proline-containing dipeptides.

In this study, we describe a PAP from *Streptomyces thermoluteus* subsp. *fuscus* strain NBRC14270 (PAP14270). Furthermore, synthesis of various proline peptides was attempted through catalysis by its Ser/Cys variant (scPAP14270) from proline ester and several amino acids and their esters in aqueous solution. A basic characterization to determine the effect of pH and the amount of substrate was conducted. Moreover,

* Corresponding author. Mailing address: Okayama Prefectural Technology Center for Agriculture, Forestry and Fisheries, Research Institute for Biological Sciences (RIBS), 7549-1 Kibichuo-cho, Kaga-gun, Okayama 716-1241, Japan. Phone: 81 866 56 9452. Fax: 81 866 56 9454. E-mail: hatanaka@bio-ribs.com.

[†] Supplemental material for this article may be found at <http://aem.asm.org/>.

[‡] Published ahead of print on 30 July 2010.

correlation was found between proline peptide synthesis and the log *D* value, which is the distribution coefficient between octanol and water, of acyl acceptors in aminolysis mediated by scPAP14270.

MATERIALS AND METHODS

Materials. Amino acids and amino acid esters used for this study were purchased as shown in Table S1 in the supplemental material. A PCR cloning kit was purchased from Invitrogen Corp. (Zero Blunt II TOPO; Life Technologies Inc., Carlsbad, CA). The plasmid pET28a(+) and *Escherichia coli* Rosetta-Gami-2 (DE3) were obtained from Novagen Inc. (Darmstadt, Germany). All solvents and other chemicals used for this study were of analytical grade.

Cloning of the PAP14270 gene using inverse PCR. Genomic DNA of *Streptomyces thermoluteus* subsp. *fuscus* NBRC14270 was prepared using a method described in an earlier study (7). The internal sequence of genomic DNA of PAP14270 was amplified using PCR with two primers, including the degenerated primers 5'-CAGWSSTTCGGGGTTCTGC-3' and 5'-ACGTACATGTCGTC GTGGTA-3', which were designed from alignment of primary sequences of annotated PAPs from *Streptomyces coelicolor* (gene ID SCO5122), *Streptomyces avermitilis* (gene ID SAV3144), and *Streptomyces griseus* (gene ID SGR2407). In fact, PCR was performed using 1 μ M primers and LA *Taq* DNA polymerase with GC buffer 1 (TaKaRa Holdings Inc., Kyoto, Japan). The PCR program was as follows: 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 of 5 min at 72°C. The partial PAP14270 gene was sequenced using the set of PCR primers. Next, the genomic DNA was digested by CpoI overnight at 37°C, followed by phenol-chloroform-isoamyl alcohol extraction. The digested DNA was self ligated. In the same PCR program, the ligated DNAs were amplified with a set consisting of a sense primer (5'-AACG AGGTCCCGTCCGCCG-3') and an antisense primer (5'-GGCGAGGGAG AGGTACGACA-3') designed from internal sequence information of the partial PAP14270 gene. The PCR product (ca. 2 kbp) was obtained and sequenced using the set of primers described above. The entire sequence of the PAP14270 gene has been assigned accession number AB519645 in the DDBJ database (see Fig. S1 in the supplemental material).

Expression using *E. coli* and purification of protein. The PAP14270 gene was amplified using PCR (Prime STAR GXL DNA polymerase; Takara Bio Inc., Shiga, Japan) and a set consisting of a sense primer containing the NdeI site (CATATG; the start codon is underlined) and an antisense primer containing the HindIII site downstream of the stop codon. The resultant DNA fragment was cloned and sequenced using a PCR cloning kit (Zero Blunt II TOPO; Invitrogen Corp.). The DNA fragment encoding the PAP was digested by NdeI and HindIII and ligated into the NdeI-HindIII gap of the expression vector pET-28a(+).

The expression vector was transformed into *E. coli* Rosetta-Gami-2 (DE3) using a Gene Pulse Xcell electroporator (Bio-Rad Laboratories Inc., CA). Transformants were inoculated into 50 ml of Overnight Express instant TB medium using Overnight Express system 1 (Novagen Inc.). Cultivation and induction were conducted at 25°C for 48 h at 180 rpm. Cells were collected by centrifugation and disrupted using sonication (Elestin NP035SP; Nepa Gene Co. Ltd., Chiba, Japan) at 1-min intervals for 5 min to a total time of 45 min at maximum output. The obtained cell extract was purified using cobalt affinity resin (Talon; Clontech Japan) according to the manufacturer's instructions. Using this method, PAP14270 was obtained as 20 mM phosphate buffer (pH 8.0) solution. The purified proteins were analyzed using SDS-PAGE under reducing conditions with Coomassie blue staining (see Fig. S2 in the supplemental material). The protein concentration was measured using a Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc.) according to the manufacturer's protocol.

Mutagenesis on catalytic Ser of PAP 14270. Catalytic Ser144 on PAP14270 in pET28a(+) was replaced by Cys using site-directed mutagenesis (Prime Star GXL DNA polymerase kit; Takara Bio Inc.) with a set consisting of a sense primer (5'-GGCCAGTGCTTCGGCGGCTTC-3') and an antisense primer (5'-GAAGCCGCCGAAGCACTGGCC-3'). The PCR program was as follows: 30 cycles of 10 s at 98°C, 15 s at 60°C, and 7 min at 68°C. The PCR product was treated with DpnI at 37°C overnight and extracted using phenol-chloroform-isoamyl alcohol. After precipitation by ethanol, the precipitate was transfected into competent cell JM109 (Competent high; Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's protocol. The extracted plasmid (NucleoSpin extract II; Macherey-Nagel GmbH and Co. KG, Dürren, Germany) was transformed into *E. coli* Rosetta-Gami-2 (DE3) using a Gene Pulse Xcell electroporator (Bio-Rad Laboratories Inc.). Expression and purification of mutated PAP14270 (scPAP14270) was conducted identically to the process used for wild-type materials.

Proline peptide synthesis mediated by scPAP14270. Typical reaction conditions were the following: 5 μ l of 100 mM proline benzyl ester (Pro-OBzl) as the acyl donor, 5 μ l of dimethyl sulfoxide (DMSO) or 100 mM amino acids (or their esters) as the acyl acceptor, 50 μ l of 200 mM Tris-HCl buffer (pH 8.5), and 40 μ l of enzyme solution containing 8.7 μ g of scPAP14270, at 900 rpm for 3 h at 37°C. The final volume was adjusted up to 100 μ l with distilled water. The reaction was terminated by addition of 200 μ l of methanol. After centrifugation at 10,000 rpm, 20 μ l of supernatant was applied to liquid chromatography-mass spectrometry (LC/MS) analysis. Amino acids and their esters were dissolved using water or DMSO.

LC/MS analysis. A separation module (2690; Waters), an absorbance detector (2487 Dual λ ; Waters), and an API 2000 LC/MS/MS system (AB Sciex, Foster City, CA) were used. The column used for this study was a TSKgel ODS-120T column (5 μ m, 2.0 by 150 mm; Tosoh Corp., Tokyo, Japan). The mobile phase was water containing 0.1% (vol/vol) HCOOH (solvent A) and methanol containing 0.1% (vol/vol) HCOOH (solvent B) at a flow rate of 0.2 ml/min. The LC conditions were as follows: 5% (B) for 0 to 5 min, a linear increase to 50% (B) for 5 to 30 min, a hold at 50% (B) for 30 to 35 min, and a linear increase to 80% (B) for 35 to 40 min.

LC/MS monitoring of reaction product. To monitor reaction products, corresponding peak areas of the extracted ion chromatogram (XIC) were calculated according to an API 2000 LC/MS/MS device system. For XIC, the $m/z \pm 0.25$ value of $[M+H]^+$ was adopted. For example, an $[M+H]^+$ value of 205.85 to 206.35 was adopted for XIC of Pro-OBzl; the theoretical mass of Pro-OBzl is 205.1.

RESULTS

Proline peptide synthesis catalyzed by scPAP14270. The total ion chromatogram of LC/MS analysis showed six peaks with retention times (RTs) of 17.2, 24.2, 30.9, 33.2, 35.0, and 36.2 (min), respectively (Fig. 1A, a to f), although they did not appear when the reaction was carried out without scPAP14270. The main molecular ions of these six peaks were, respectively, 195.2, 206.2, 303.2, 400.1, 497.3, and 594.3 (Fig. 1B, a to f). These values, respectively, coincided with the predicted molecular formulas of cPP, Pro-OBzl, prolyl-prolyl benzyl ester (P2-OBzl), P3-OBzl, P4-OBzl, and P5-OBzl. Additionally, the MS spectra at RT 17.2, 30.9, 33.2, 35.0, and 36.2 corresponding to $[M+Na]^+$ and/or $[M+K]^+$ were detected.

On the other hand, these spectra were not detected when proline was used as a substrate, which means that the products were synthesized using aminolysis, not a reverse reaction of hydrolysis (data not shown). Furthermore, under similar conditions, wild-type PAP14270 did not produce these compounds (data not shown).

Effects of pH on proline peptide synthesis catalyzed by scPAP14270. The effect of pH on the proline peptide synthesis catalyzed by scPAP14270 was examined using citrate-phosphate-borate buffer (pH 3.5 to 11.5). Under these reaction conditions, the optimum pH was different with each resultant peptide (Fig. 2). The P2-OBzl production was highest at pH 6.5 and was not detected at pHs of >9.5, although cPP, P3-OBzl, and P4-OBzl production peaked at around pH 8.5 to 9.5. The amount of P5-OBzl was highest at pH 9.5. At the range of pHs of <3.5 and >11, no products were observed. All products were detected at pH 8.5 to 9.5. Therefore, 200 mM Tris-HCl buffer (pH 8.5) was adopted for the following study.

Effect of the substrate concentration on the proline peptide synthesis catalyzed by scPAP14270. Effects of substrate concentration on the proline peptide production catalyzed by scPAP14270 were examined using final concentrations ranging from 5 mM to 100 mM Pro-OBzl.

At final concentrations of 5 to 25 mM, the amounts of

FIG. 1. LC-MS profile of reaction mixture of aminolysis catalyzed by scPAP14270. (A) Total ion chromatogram. (B) Mass spectrum of each compound. Reaction conditions: 5 μ l of 100 mM Pro-OBzl, 5 μ l of DMSO, 50 μ l of 200 mM citrate-phosphate-borate buffer (pH 7.5), and 40 μ l of enzyme preparation containing 8.7 μ g of scPAP14270 at 900 rpm for 3 h at 37°C.

proline peptides, including cPP, P3-OBzl, and P4-OBzl, were increased dose dependently (Fig. 3). At those of 50 to 100 mM, the amount of P2-OBzl was increased dose dependently, although those of cPP, P4-OBzl, and P5-OBzl decreased concomitantly with the amount of substrate.

Time course of proline peptide synthesis catalyzed by scPAP14270. The time course of the proline peptide production catalyzed by scPAP14270 was examined for reaction times of 0.5 to 24 h.

The amount of cPP increased with the reaction time. It reached a plateau at a reaction time of 6 h (Fig. 4). Amounts of P3-OBzl and P4-OBzl decreased with reaction time after peaking at 1 to 3 h. The amounts of P2-OBzl and the substrate Pro-OBzl decreased rapidly at the first stage of the reaction. They were not detected at reaction times greater than 6 h.

Correlation between substrate specificities and log *D* values of acyl acceptors upon aminolysis catalyzed by scPAP14270. To investigate the preference for an acyl acceptor in proline

FIG. 2. Effect of pH on the proline peptide synthesis catalyzed by scPAP14270. Reaction conditions: 5 μ l of 100 mM Pro-OBzl, 5 μ l of DMSO, 50 μ l of 200 mM citrate-phosphate-borate buffer (pH 3.5 to 11.5), and 40 μ l of enzyme preparation containing 8.7 μ g of scPAP14270 at 900 rpm for 3 h at 37°C.

peptide synthesis catalyzed by scPAP14270, the reaction was conducted using various amino acids or their esters as acyl acceptors. In Fig. 5, the reaction conditions under which heteroproline peptide was observed are shown as closed symbols; those in which no heteroproline peptide was observed are shown as open symbols. As an example of the resultant heteropeptide, Pro-Phe-OMe is shown in Fig. S3 in the supplemental material. Under reaction conditions using an acyl acceptor with a log *D* value of more than 0 (a compound with a high log *D* value is more hydrophobic; values were calculated using software supplied at <http://sparc.chem.uga.edu/sparc/>), heteroproline peptides were detected, with the exception of hydroxyproline benzyl ester (Hyp-OBzl) and Pro-OMe. At final concentrations of 5 mM Met-OMe, Ala-OBzl, and Gly-OBzl, used as acceptors, proline peptides were not observed.

FIG. 3. Effect of substrate concentration on the proline peptide synthesis catalyzed by scPAP14270. Reaction conditions: 10 μ l of 50–1,000 mM Pro-OBzl, 50 μ l of 200 mM Tris-HCl buffer (pH 8.5), and 40 μ l of enzyme preparation containing 8.7 μ g of scPAP14270 at 900 rpm for 3 h at 37°C.

FIG. 4. Time course of the proline peptide synthesis catalyzed by scPAP14270. Reaction conditions: 5 μ l of 100 mM Pro-OBzl, 5 μ l of DMSO, 50 μ l of 200 mM Tris-HCl buffer (pH 8.5), and 40 μ l of enzyme preparation containing 8.7 μ g of protein at 900 rpm for 0.5 to 24 h at 37°C.

There also were no proline peptides using final concentrations of 50 mM Cys-OMe, Hyp-OBzl, Arg-OMe, Glu-OMe, and Asp-OMe. Furthermore, heterocycloprolyl (such as cPF) was detected when Trp-OBzl, Trp-OMe, Phe-OEt, Phe-OMe, Tyr-OBzl, Leu-OBzl, Leu-OMe, Pro-OBzl, Ala-OBzl, and Gly-OBzl were used as acyl acceptors.

In addition, the influence of pH on production of heteroproline dipeptide was examined using serine, hydroxyproline, valine, and tryptophan and their esters. When Trp-OBzl was used as an acceptor, Pro-Trp-OBzl increased with pH and log *D* value and decreased at pHs of >8.5 (Fig. 6). At pHs of >8.5, a molecular ion corresponding to Pro-Trp (*m/z* 302.2) was also observed. The amount increased with the pH (data not shown). The same results were obtained using Ser-OBzl, Val-OBzl, and Trp-OMe as acceptors (data not shown). When hydroxyproline and its esters were used, no heteroproline dipeptide was observed.

DISCUSSION

To give the enzyme peptide synthesis capability, Cys was substituted into PAP14270 using site-directed mutagenesis, thereby replacing 144Ser. The LC/MS analysis results suggest that scPAP14270 has the synthetic capability of a variety of proline peptide esters (Fig. 1 and 5). When only Pro-OBzl was used as the substrate, scPAP14270 exhibited intermolecular cyclization and polymerization activity for Pro-OBzl, which means that scPAP14270 recognized Pro-OBzl as both an acyl donor and acceptor (Fig. 1). It is an especially notable finding that scPAP14270 produced cPP and other cyclic dipeptides. Of the few known enzymes that catalyze cyclization of dipeptide, nonribosomal peptide synthase (NRPS) is known to produce cyclic dipeptides (4, 12). Recently cyclodipeptide synthase (CDPS) in the albonoursin pathway has also been found (6). However, industrial applications of these enzymes might be difficult because they require ATP as a substrate for the NRPS-mediated reaction or aminoacyl-tRNA for that of CDPS. Therefore, this Ser/Cys substitution aminopeptidase presents

FIG. 5. Correlation between substrate specificity and log D value of acyl acceptors on aminolysis catalyzed by scPAP14270. Reaction conditions: 5 μ l of 100 mM Pro-OBzl, 5 μ l of 100 mM amino acid or amino acid ester, 50 μ l of 200 mM Tris-HCl buffer (pH 8.5), and 40 μ l of enzyme preparation containing 8.7 μ g of scPAP14270 at 900 rpm for 3 h at 37°C. Circles, amino acid benzyl esters; triangles, amino acid methyl esters; diamonds, free amino acids; closed symbols, reaction condition under which predicted m/z of proline peptide was observed; open symbols, reaction condition under which predicted pseudomolecular ions of proline peptide were not observed; "C," reaction condition under which cyclo[Pro-Xaa] was observed; *, reaction condition for which a 50 mM (final concentration) of the acyl acceptor was adopted. Log D values were obtained from the web site at <http://sparc.chem.uga.edu/sparc/>.

the possibility of altered enzymes for cyclic dipeptide production. Our research group has demonstrated the enzymatic synthesis of cyclo[Phe-Phe] and cyclo[Phe-Tyr] with a Ser/Cys substitution aminopeptidase, aminolysin-S (12). However, the mechanism of this cyclization reaction remains unclear. We investigated mutations of two other residues consisting of a catalytic triad (see Fig. S2 in the supplemental material). In PAP14270, when catalytic 364Asp or 411His was replaced, respectively, by Ala, Asn, and Glu or Ala, Lys, and Arg, its hydrolysis activity toward proline *p*-nitroanilide (Pro-*p*NA) was

FIG. 6. Influence of pH and log D value for acceptor. Reaction conditions are as follows: 5 μ l of 100 mM Pro-OBzl, 5 μ l of 100 mM Trp-OBzl, 50 μ l of 200 mM citrate-phosphate-borate buffer (pH 5.5 to 10.5), and 40 μ l of enzyme preparation containing 8.7 μ g of scPAP14270 at 900 rpm for 3 h at 37°C. The solid line shows the detected peak area. The broken line shows the log D value at respective pH.

diminished. None of these mutated PAP14270s showed aminolysis activity for Pro-OBzl (data not shown), which suggests that the combination of 114Cys, 364Asp, and 411His is necessary for aminolysis activity.

A detailed investigation of the effect of pH on the aminolysis reaction suggests that "dipeptide" synthesis was done in a weakly acidic environment whereas cyclization and polymerization took place under alkaline conditions (Fig. 2). For observation of aminolysis reactions catalyzed by variants of *Streptomyces griseus* protease B, ligation requires a deprotonated amine nucleophile for the acceptor (5). In this study, under an alkaline condition where deprotonated amine nucleophiles exist, P2-4-OBzl was preferably serially recognized as an acceptor. In addition, dipeptide production was dominant at high concentrations of the substrate, whereas other peptides were suppressed (Fig. 3). It is important to produce the desired peptide alone if the peptides are to be applied in several industries. These results indicate that peptide production can be controlled by optimizing the pH and concentration of the substrate in a reaction medium.

Reaction time is another important factor for controlling peptide production. At an early stage of the reaction, P2-OBzl decreased rapidly whereas P3-OBzl and P4-OBzl increased with time (Fig. 4). This observation suggests that P2-OBzl was spent for P3-OBzl production and P3-OBzl was spent for P4-OBzl. Subsequently, hydrolysis of P3-OBzl and P4-OBzl might be predominant because Pro-OBzl also decreased, perhaps because of both alkaline and enzymatic hydrolysis. The rapid decrease in P2-OBzl and the lack of an increase in P5-OBzl

implied that scPAP14270 prefers P2-OBzl and P3-OBzl to P4-OBzl as an acyl acceptor. After 6 h of the reaction, all production was decreased except for that of cPP. Di-ketopiperazines, such as cPP, are known to be thermodynamically stable and cannot be an acceptor. Consequently, the amount of cPP would not be decreased once production achieves equilibrium.

The substrate specificity of scPAP14270 for proline peptide production is revealed to be broad, suggesting that almost all amino acid esters with a log *D* value greater than 0, irrespective of the kind of ester, are recognized as acyl acceptors. Therefore, scPAP14270 prefers hydrophobic amino acids as acyl acceptors (Fig. 5). The exception was Hyp-OBzl. A possible reason is steric hindrance to the substrate pocket by the C-4 hydroxyl group on Hyp-OBzl, the only difference in structure in comparison to Pro-OBzl. The other reason might be the remaining hydrolytic activity of scPAP14270, which suppresses Pro-Hyp-OBzl formation. Wild-type PAP14270 showed weaker hydrolytic activity toward Hyp-MCA than toward Pro-pNA (data not shown). Furthermore, based on the finding that serine or valine, whose log *D* value at pHs of >8.5 is less than -1, was not recognized as an acceptor even at a pH of >8.5, it is suggested that hydrophobicity of the acceptor compound is an important factor in addition to deprotonation of the acceptor in aminolysis using scPAP14270.

Although aminolysis, which is targeted to peptide production using Ser/Cys substitution endoprotease, has been well characterized, this article describes how using an exopeptidase resolves several problems that remain as obstacles to conventional synthesis methods. These findings will support the use of PAPs as a leading tool in proline peptide production.

ACKNOWLEDGMENTS

This research was supported financially by a Grant-in-Aid for Scientific Research (no. 20550158). H.U. is a research fellow of the Japan Society for the Promotion of Science (JSPS).

REFERENCES

1. Abrahmsén, L., J. Tom, J. Burnier, K. A. Butcher, A. Kossiakoff, and J. A. Wells. 1991. Engineering subtilisin and its substrates for efficient ligation of peptides bounds in aqueous solution. *Biochemistry* **30**:4151–4159.
2. Alfonso, I., and V. Gotor. 2004. Biocatalytic and biomimetic aminolysis reactions: useful tools for selective transformations on polyfunctional substrates. *Chem. Soc. Rev.* **33**:201–209.
3. Brauns, S. C., G. Dealtry, P. Milne, R. Naudé, and V. M. de Venter. 2005. Caspase-3 activation and induction of PARP cleavage by cyclic dipeptide cyclo (Phe-Pro) in HT-29 cells. *Anticancer Res.* **25**:4197–4202.
4. Duerfahrt, T., K. Eppelmann, R. Müller, and M. A. Marahiel. 2004. Rational design of a bimodular model system for the investigation of heterocyclization in nonribosomal peptide biosynthesis. *Chem. Biol.* **11**:261–271.
5. Elliott, R. J., A. J. Bennet, C. A. Braun, A. M. MacLeod, and T. J. Borgford. 2000. Active-site variants of *Streptomyces griseus* protease B with peptide-ligation activity. *Chem. Biol.* **7**:163–171.
6. Gondry, M., L. Sauguet, P. Belin, R. Thai, R. Amouroux, C. Tellier, K. Tophile, M. Jacquet, S. Braud, M. Courçon, C. Masson, S. Dubois, S. Lautru, A. Lecoq, S. Hashimoto, R. Genet, and J. L. Pernodet. 2009. Cyclo-dipeptide synthases are a family of tRNA-dependent peptide bond-forming enzymes. *Nat. Chem. Biol.* **5**:414–420.
7. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kiser, C. J. Bruton, H. M. Kiser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schempf. 1985. Genetic manipulation of *Streptomyces*: a laboratory manual, p. 70–84. The John Innes Foundation, Norwich, United Kingdom.
8. Huberman, L., N. Gollop, K. Y. Mumcuoglu, E. Breuer, S. R. Bhusare, Y. Shai, and R. Galun. 2007. Antibacterial substances of low molecular weight isolated from the blowfly, *Lucilia sericata*. *Med. Vet. Entomol.* **21**:127–131.
9. Meyer, J., U. Bütikofer, B. Walther, D. Wechsler, and R. Sieber. 2009. Hot topic: changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese variations. *J. Dairy Sci.* **92**:826–836.
10. Oshiro, T., K. Mochida, and T. Uwajima. 1992. Enzymatic peptide synthesis by proline iminopeptidase. *Biotechnol. Lett.* **14**:175–178.
11. Shigemura, Y., K. Iwai, F. Morimatsu, T. Iwamoto, T. Mori, C. Oda, T. Taira, E. Y. Park, Y. Nakamura, and K. Sato. 2009. Effect of prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin. *J. Agric. Food Chem.* **57**:444–449.
12. Usuki, H., Y. Uesugi, J. Arima, Y. Yamamoto, M. Iwabuchi, and T. Hatanaka. 2010. Aminolysin-S, an engineered transaminopeptidase catalyzes the synthesis of diverse dipeptides and cyclic dipeptides by one-pot reaction manner. *Chem. Commun. (Camb.)* **46**:580–582.
13. Wang, Y., F. Zhu, J. Chen, F. Han, and H. Wang. 2009. Effects of Pro-Arg, a novel dipeptide derived from protamine hydrolysate on H₂O₂-induced oxidative stress in human diploid fibroblasts. *Biol. Pharm. Bull.* **32**:389–393.