Efficient Extracellular Expression of Metalloprotease for Z-Aspartame Synthesis

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Supporting Information

ABSTRACT: Metalloprotease PT121 and its mutant Y114S (Tyr114 was substituted to Ser) are effective catalysts for the synthesis of Z-aspartame (Z-APM). This study presents the selection of a suitable signal peptide for improving expression and extracellular secretion of proteases PT121 and Y114S by Escherichia coli. Co-inducers containing IPTG and arabinose were used to promote protease production and cell growth. Under optimal conditions, the expression levels of PT121 and Y114S reached >500 mg/L, and the extracellular activity of PT121/Y114S accounted for 87/82% of the total activity of proteases. Surprisingly, purer protein was obtained in the supernatant, because arabinose reduced cell membrane permeability, avoiding cell lysis. Comparison of Z-APM synthesis and caseinolysis between proteases PT121 and Y114S showed that mutant Y114S presented remarkably higher activity of Z-APM synthesis and considerably lower activity of caseinolysis. The significant difference in substrate specificity renders these enzymes promising biocatalysts.

KEYWORDS: extracellular expression, metalloprotease, Escherichia coli, arabinose, Z-aspartame

INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester, APM) is a protected dipeptide sweetener, which is 200 times sweeter than sucrose.¹ Since its approval, APM has been widely used as a low-calorie sweetener in soft drinks and food products.² More than 19,000 t of APM are produced every year, making it the most highly synthesized peptide in the world.³ APM can be easily obtained by deprotection of carboxybenzyl from Z-aspartame (N-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, Z-APM).⁴ Members of metalloprotease family M4 (also known as the thermolysin family) such as thermolysin and pseudolysin, as efficient biocatalysts, were utilized to catalyze the synthesis of Z-APM from N-carbobenzoxy-L-aspartic acid (Cbz-Asp) and L-phenylalanine methyl ester (Phe-OMe).⁵ A previous study developed mutant Y114S, which can efficiently catalyze the synthesis of Z-APM, by site-directed mutagenesis of protease PT121 from Pseudomonas aeruginosa.⁶ However, due to the limited expression level, these metalloproteases are one of the limiting factors in Z-APM enzymatic preparation. Various alkaline proteases have been produced at industrial scale.⁷ However, to the best of our knowledge, the expression levels of many thermolysin family metalloproteases, such as thermolysin-like protease and pseudolysin, are still limited. In Bacillus subtilis and Pichia pastoris hosts, extracellular expression of thermolysin family proteases can reach 10−50 mg/L.⁸−¹⁰ As the most commonly used host, Escherichia coli is also utilized to express thermolysin family metalloproteases, but most of these enzymes are expressed either in intracellular form, ranging from 6 to 20 mg/L,¹¹−¹³ or in inclusion body form.¹⁴ Because of its high proteolysis, mature proteases located inside cells could cause recombinant cell growth inhibition, or even cell lysis. Targeting the protein to the culture medium may overcome this obstacle.¹⁵ Additionally, extracellular expression may facilitate downstream processing and achieve higher produc-

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of inclusion bodies and significantly improved the activity of penicillin acylase. To our best knowledge, no study has reported the arabinose-induced expression of protease.

In the present study, replacement of signal peptide was applied to promote the extracellular expression of metalloprotease PT121 and its mutant Y114S. Arabinose supplementation significantly increased the expression level of proteases and improved the purity of secreted proteases. Possible mechanisms are also discussed. Additionally, the substrate specificity of PT121 and Y114S in the catalyzed synthesis of Z-APM and caseinolytic activity was investigated and rationalized by assessing kinetic parameters and conducting molecular dynamic simulation.

**MATERIALS AND METHODS**

**Chemicals, Reagents, and Materials.** N-Carboxybenzoyloxys-aspartic acid (Cbz-Asp) and L-phenylalanine methyl ester (Phe-OMe) were obtained from GL Biochem Co., Ltd. (GLS, Shanghai, China). o-Nitrophenyl-β-D-galactopyranoside (ONPG) and N-phenyl-α-naphthylamine (NPN) were purchased from Aladdin Co., Ltd. (Aladdin, Shanghai, China). All primers were synthesized at Invitrogen (Invitrogen, Shanghai, China). DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Takara (Takara, Dalian, China).

**Molecular Techniques.** All primers used in this study are shown in Table S1. Genomic DNA of _P. aeruginosa_ PT121 was used as template for PCR. The 1497 bp fragment of gene _lasB_ was amplified with cds-F and cds-R as the nucleotide primers. The amplified DNA product was inserted into the pMD-18T vector (Takara). The recombinant plasmid was used as a template for PCR. The 1425 bp fragment containing the gene encoding the pro-peptide and mature peptide was amplified using the primers _lasB-F1_ and _lasB-R1_. Thus, the original _pcdB_ signal sequence of PET-22b (Takara) was retained. The product of PCR was digested with restriction enzymes _NcoI_ and _BamHI_ and then ligated into PET-22b. Primers _lasB-F2_ and _lasB-R2_ were used to obtain the DNA product with its native signal peptide by PCR. The product was digested with the restriction enzymes _NdeI_ and _BamHI_ and then ligated into PET-22b. As a result, the native signal peptide of metalloprotease PT121 was retained. The nucleotide sequence of signal peptide ompA was fused to the _lasB_ gene without its native signal peptide by overlap PCR using primer-rF1, primer-rR1, primer-rF2, and primer-rR2. Then the constructed fragment was cloned into vector pET-22b. The recombinant _E. coli_ BL21 containing the constructed plasmid was transformed into _E. coli_ BL21. The correct clones were selected for DNA sequencing (GenScript, Nanjing, China).

**Medium and Culture Conditions.** The transformed BL21 was inoculated in 5 mL of LB medium and grown with shaking at 37 °C overnight to serve as the seed. A 2% (v/v) concentration of the inoculated was inoculated in 50 mL of LB medium containing 100 μg/ mL ampicillin and then incubated at 37 °C until an optical density at 600 nm (OD_{600}) reached 1.0. Then, 10 μM isopropyl thioc-β-D-galactopyranoside (IPTG) was added to induce protein expression at 30 °C for 24 h. The _E. coli_ transformed with PET-22b was taken as the control group for analysis.

The pooled supernatant was dialyzed against 50 mM Tris-HCl (pH 8.0) buffer to remove salt and other small molecules, and then the sample was submitted for assessment of its casein hydrolysis activity. The purity and molecular weight of the target protein were measured using SDS-PAGE with 12.5% separating gel as described by Laemmli.

**Purification of Protease.** The protease purification was performed as described by Tang et al. with slight modification. The culture supernatant was harvested by centrifugation at 12000 g and 4 °C for 10 min and loaded onto a Phenyl Sepharose column (GE Healthcare, Uppsala, Sweden), which was equilibrated with 50 mM Tris-HCl (pH 8.0) containing 1.0 M NaCl. After binding, the column was eluted with 50 mM Tris-HCl (pH 8.0) at a flow rate of 1.0 mL/min. The absorbance of eluent at 280 nm was measured, and the active fractions were collected and dialyzed for further research. Bradford’s method was used for assaying the protein concentration.

**Assay of Caseinolytic Activity.** The activity of the supernatant toward casein was measured as previously described. Two milliliters of the diluted protease was added to 2 mL of Tris-HCl buffer (50 mM, pH 8.0) containing 2% (w/v) casein. The reaction mixture was incubated at 40 °C for 10 min and terminated by adding 4.0 mL of TCA mixture containing trichloroacetic acid (0.11 M), sodium acetate (0.22 M), and acetic acid (0.33 M). The mixture was then centrifuged at 12000 g for 10 min. The absorbance at 280 nm of the reaction supernatant was measured against a blank control. The amount of enzyme that hydrolyzes casein and produces 1 μg of tyrosine per minute is defined as 1 unit (U) of protease activity. All above assays were performed in triplicates for calculating the mean and standard deviation.

**Effect of IPTG and Arabinose on Expression of Proteases.** To examine the effect of IPTG and arabinose on the expression of proteases PT121 and Y114S, IPTG (0–100 μM) and arabinose (0–5.0 mg/mL) were applied to induce the expression of proteases, respectively. The flask cultures were further shaken at 30 °C for another 24 h. Then, the cultures were centrifuged at 4 °C and 12000 g for 10 min. The supernatant of culture was assayed for extracellular caseinolytic activity. The cell pellet was resuspended in Tris-HCl buffer (50 mM, pH 8.0). The cell suspension was sonicated for 5 min by using an ultrasonic processor and then centrifuged at 12000 g for 4 °C for 5 min. The supernatant of cell extracts was assayed for intracellular protease activity. All above assays were performed in triplicates for calculating the mean and standard deviation.

**Determination of Cell Lysis.** Cell lysis was determined as the percentage of extracellular activity versus the total activity containing extracellular and intracellular portion by using β-galactosidase as the reporter protein. Activity of β-galactosidase was assayed as previously reported.

**Assay of Membrane Permeability.** Permeability of the inner membrane was determined by measuring the influx of ONPG, a substrate of cytosolic β-galactosidase. β-Galactosidase localized within the cytoplasm can hydrolyze ONPG that passes the inner membrane, resulting in absorbance at 420 nm. Thus, an increase in the rate of absorbance indicates the permeability of the inner membrane. In this study, the permeability of the inner membrane was assayed by measuring the access of ONPG to the cytoplasm in accordance with a previously reported method with minor modifications. The recombinant _E. coli_ BL21 containing the constructed plasmid was obtained by centrifugation (12000 g), rinsed once, and then suspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{590} of 0.15. ONPG with a final concentration of 100 μg/mL was added to an ELISA plate containing 200 μL of the cell suspension. Substrate cleavage by β-galactosidase was measured by light absorption every 3 min at 420 nm in a microplate reader (BioTek, Winooski, VT, USA).

The hydrophobic fluorescent probe NPN was used as an indicator of outer membrane integrity. NPN has a low fluorescence quantum yield in aqueous solution, but its fluorescence is strong in the hydrophobic environment of cell membrane. Normally, NPN is excluded from the lipopolysaccharide layer of the outer membrane but can enter at points where membrane integrity is compromised. Thus, the permeability of the outer membrane can be indicated by fluorescence value and its increased rate. Outer membrane permeability was measured according to a previous method with minor modifications. NPN with a final concentration of 10 μM was added to a quartz cuvette containing 3 mL of cell suspension. Fluorescence absorption was assayed using a spectrophotometer Shimadzu RF-1501 (Shimadzu, Kyoto, Japan) with 5 nm slit widths. The emission and excitation wavelengths were set to 420 and 350 nm, respectively.

**Kinetic Constants of Recombinant PT121 and Y114S.** The initial rate of tyrosine formation, presented as the initial rate of caseinolysis, was determined at a fixed concentration of purified protease.
enzyme (0.1 mg/mL) and at different concentrations of casein (0.5–10.0 mg/mL) for 5 min. All above assays were performed in triplicates for calculating the mean and standard deviation. The determined value was plotted by nonlinear fitting using Origin 9.0.

To measure the kinetic parameters of the synthetic reaction for Z-APM, various Cbz-Asp and Phe-OMe concentrations of 10–100 mM at fixed concentrations of 500 mM Phe-OMe and 100 mM Cbz-Asp were used as substrates, respectively. Purified enzyme (0.9 μM) was added into the reaction mixture, and the reaction system was shaken at 37 °C and 180 rpm for 1 h. All samples were analyzed by high-performance liquid chromatography (HPLC) as previously reported.6 The kinetic constants $K_M$ were calculated from Lineweaver–Burk plots.

We calculated the $K_{M1}$ of Cbz-Asp (ZD) and the $K_{M2}$ of Phe-OMe (FM), respectively. Random rapid equilibration mechanism was applied to calculate the kinetic constants of Z-APM synthesis according to a previous method.32 The initial reaction rate $v_0$ of Z-APM synthesis was obtained at 100 mM Cbz-Asp and 500 mM Phe-OMe. The $k_{cat}$ would be obtained by the following equation:

$$v_0 = \frac{k_{cat}[E][ZD][FM]}{K_{M1}[E][ZD] + K_{M2}[E][FM] + [ZD][FM]}$$

RNA Secondary Structure Prediction. The secondary structure and Gibbs free energy ($\Delta G$) of the mRNA TIR were predicted and calculated using an online server (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form).

Molecular Simulation. The crystal structure (PDB code: 1EZM) determined by Thayer et al.33 was used for the molecular modeling of PT121 and Y114S. The 3D structure of Phe-OMe was stripped from the crystal structure of thermolysin (PDB code: 3QGO). Molecular docking of the substrate was performed using LeDock (http://lephar.com). The software in optimizations of the ligand pose (position and orientation) and its rotatable bonds is based on simulated annealing as well as genetic algorithm.34 All crystal waters were removed from the crystal structure, and polar hydrogen atoms were added to the structure of protein by LePro module in the docking suite LeDock. All catalytic residues were included in the active pocket. Molecular dynamic simulation was performed using Amber 12 with the Amber 99SB force field.35 Ligand topology was generated using the Leap Program. Sodium ions were added to maintain system neutrality. The neutral system was subjected to energy minimization with the steepest descent of 1000 steps and conjugate gradient of 2000 steps. Then, the 50,000 steps position-restrained dynamic simulation was applied to the system at 300 K. Finally, MD was performed for 40 ns at 300 K. Postprocessing and analysis were performed using standard Amber tools and customized scripts.

RESULTS AND DISCUSSION

Effect of Signal Peptides on Expression of PT121 and Y114S. The effect of signal peptides (pelB, ompA, and its native signal peptide) on the expression of PT121 and Y114S was studied. The recombinant E. coli cells harboring Sp-PT121 (BL21/Sp-PT121), ompA-PT121 (BL21/ompA-PT121), pelB-PT121 (BL21/pelB-PT121), and pelB-Y114S (BL21/pelB-Y114S) were induced for 24 h. In Figure 1, SDS-PAGE shows that most of the proteases PT121 and Y114S were extracellularly expressed by using pelB or ompA as signal
Supernatant reached 5200 activities of pelB-PT121 and pelB-Y114S in the culture peptide was observed as shown in Figure 1. The protease the highest expression of protease PT121 using pelB as signal promoted the extracellular secretion of protease PT121, and by comparison, the signal peptides pelB/ompA e.

Mutant Y114S exerts little e. on the expression of protease PT121 using pelB as signal peptide was observed as shown in Figure 1. The protease activities of pelB-PT121 and pelB-Y114S in the culture supernatant reached 5200 ± 230 and 2700 ± 170 U/mL, respectively, which constituted 91.5 and 88.4% of the total activity. Even though the concentrations of PT121 and Y114S increased, and a maximum value was obtained at 10 μM (Table 1). Further increase of IPTG concentration beyond 10 μM significantly decreased the expression level of protease. On the other hand, the cell density (OD600) significantly decreased even when 5 μM IPTG was added. High concentrations of IPTG negatively influenced the expression of the target protein. This result may be attributed to the fact that overexpression of target protease inhibits cell growth or even causes cell lysis. The effects of IPTG and arabinose on the expression of protease PT121 were investigated.

As the concentration of IPTG increased, the expression level increased, and a maximum value was obtained at 10 μM (Table 1). Further increase of IPTG concentration beyond 10 μM significantly decreased the expression level of protease. On the other hand, the cell density (OD600) significantly decreased even when 5 μM IPTG was added. High concentrations of IPTG negatively influenced the expression of the target protein. This result may be attributed to the fact that overexpression of target protease inhibits cell growth or even causes cell lysis. The effects of IPTG and arabinose on the expression of protease PT121 were investigated.

Effect of Inducers (IPTG and Arabinose) on Expression of Protease PT121. Mutant Y114S exerts little effect on protein expression. Thus, protease PT121 was selected as a model to investigate. IPTG is commonly used as an inducer of protein expression regulated by T7 promoter, and arabinose was also able to induce penicillin acylase expression regulated by T7 promoter in E. coli. The effects of IPTG and arabinose on the expression of protease PT121 were investigated.

As the concentration of IPTG increased, the expression level increased, and a maximum value was obtained at 10 μM (Table 1). Further increase of IPTG concentration beyond 10 μM significantly decreased the expression level of protease. On the other hand, the cell density (OD600) significantly decreased even when 5 μM IPTG was added. High concentrations of IPTG negatively influenced the expression of the target protein. This result may be attributed to the fact that overexpression of target protease inhibits cell growth or even causes cell lysis. The effects of IPTG and arabinose on the expression of protease PT121 were investigated.

Signal peptide not only promoted extracellular secretion but also affected the S’mRNA secondary structure. In the present study, the secondary structures of the mRNA TIR (from nucleotide points −35 to +36) of three constructs named TIR-pelB (pelB signal peptide), TIR-ompA (ompA signal peptide), and TIR-Sp (native signal peptide of protease PT121) were predicted theoretically. The essential architecture of the TIR is shown in Figure 2. The ΔG values of mRNA TIR in TIR-pelB, TIR-ompA, and TIR-Sp were −9.5, −9.7, and −10.5 kcal/mol, respectively. Previous studies demonstrated that increasing the ΔG of TIR increases protein expression. In addition, the start AUG codon was demonstrated as a key limiting factor for protein expression in E. coli. The AUG start codons in TIR-pelB and TIR-ompA were fully exposed, compared with that in TIR-Sp. The exposure of initiator AUG was speculated to reduce the blockade element for gene translation and to improve protein expression. Therefore, the pelB signal peptides were more suitable for secretory expression of proteases PT121 and Y114S. To our knowledge, this study is the first report on the extracellular expression of thermolysin-like protease from P. aeruginosa in an E. coli system. The strategies described herein may be feasible for rational selection of a suitable signal peptide for efficient protein expression.

**Table 1. Expression of Protease PT121 under Various Inducer Conditions**

<table>
<thead>
<tr>
<th>Inducer</th>
<th>IPTG (μM)</th>
<th>arabinose (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>protease activity</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>210 ± 6</td>
<td>4900 ± 150</td>
</tr>
<tr>
<td>protease activity/OD₆₀₀</td>
<td>5.4 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

*Experiments were performed in triplicate and are presented as the mean ± standard deviation.*
expression is unclear. In this study, the effects of arabinose concentration on cell growth and protease production were investigated (Table 1). The extracellular protease activity and cell density initially increased with increasing arabinose concentration and then decreased when this concentration exceeded 3.0 g/L. The maximum protease activity achieved was 2200 U/mL. Although the protease activity in the arabinose-induced culture was lower than that in the IPTG-induced culture, the cell density was >2-fold that when IPTG was used as an inducer. The result indicates that arabinose not only enhances cell growth but also induces protease PT121 expression.

Conventionally, cell growth is severely inhibited by protease expression because of its high proteolytic activity. To overcome these deficiencies, arabinose and low-concentration IPTG (10 μM) were used as co-inducers for protease expression. As shown in Figure 3, the supernatant activity and total activity increased with increasing arabinose concentration. The optimum arabinose concentration was 3.0 mg/mL, and the total activity reached 11900 ± 500 U/mL, which was 2.1-fold that of the arabinose-free culture. Significantly, the extracellular protease reached 10300 ± 460 U/mL (468 mg/L). Similarly, the total activity/OD<sub>600</sub> increased when the arabinose concentration was increased from 0.0 to 3.0 mg/mL, but remarkably decreased when the concentration was further increased to 4 mg/mL. The maximum total activity/OD<sub>600</sub> was achieved at 3.0 mg/mL arabinose and was 1.7-fold that without arabinose addition. The OD<sub>600</sub> was 1.2-fold that without arabinose addition. The production of mutant Y114S was also determined under the same conditions. The total and extracellular activities of Y114S reached 6600 ± 240 and 5400 ± 200 U/mL, respectively, both of which were almost 2.0-fold that of the arabinose-free culture (Figure 4). In particular, the specific activity of protease PT121 in culture supernatant with 3.0 g/L arabinose was 22,000 U/mg, which was almost equal to that of purified native protease PT121. These results suggest that arabinose may prevent cell lysis by decreasing the leakage of intracellular proteins, thus improving the purity of target protease in the culture supernatant. Arabinose enhances the production of penicillin acylase by mediating the processing and correct folding of this enzyme. However, the decrease of the leakage of intracellular proteins in E. coli by arabinose has not been reported. In early papers, the full length of the lasB gene with the native signal sequence integrated into vector pUC19 was intracellularly expressed in E. coli JM109 with 23.7 U/mL. Odunuga et al. cloned the mature pseudolysin coding sequence of lasB gene into pET28a and achieved the yield of 40 mg/L in E. coli BL21 as inclusion bodies followed by refolding. In P. pastoris, P. aeruginosa elastase was successfully expressed and secreted into cultures with 330 U/mL, and the expressed protease was glycosylated. In this study, addition of arabinose significantly promoted expression of protease PT121 and kept cells from leakage. High-purity extracellular protein would considerably simplify the process of purification. This study may provide a useful strategy in expressing proteases.

**Figure 3.** Synergistic effect of IPTG and arabinose on protease PT121 production: extracellular activity (cross-hatched bars); total activity (black bars); total activity/OD<sub>600</sub> (light gray bars); pH of supernatant (slashed bars). Results are expressed as the mean ± standard deviation from three independent experiments.

**Figure 4.** SDS-PAGE analysis of proteins in the culture supernatant. Lanes: M, protein marker; 1 and 3, culture supernatant of BL21/pelB-PT121 and BL21/pelB-Y114S induced by IPTG; 2 and 4, culture supernatant of BL21/pelB-PT121 and BL21/pelB-Y114S induced by IPTG and arabinose.

**Determination of Cell Lysis and Membrane Permeability.** Extracellular expression of protease may cause hydrolysis of membrane proteins, causing cell lysis. To explore this possibility, β-galactosidase was taken as the reporter protein. β-Galactosidase is a cytoplasmic protein, and its level in the culture medium is very low under normal conditions; thus, the presence of extracellular β-galactosidase activity would be an indication of cell lysis. In this study, 91.5% of the total expressed protease PT121 and 13.6% of the total expressed β-galactosidase were found in the arabinose-free culture medium. However, 86.9% of the total expressed protease PT121 and <1% of the total β-galactosidase were found in the arabinose-supplemented culture medium, and this amount of β-galactosidase was similar to that of the control E. coli cells harboring pET-22b (BL21/pET22b) without the target gene inserted. The addition of arabinose reduced the percentage of β-galactosidase in the culture supernatant, but the percentage of target protease in the culture supernatant remained almost the same. These results suggest that the presence of extracellular protease is not due to cell lysis. Additionally, supplementation of arabinose could prevent cell lysis.

We analyzed cell membrane permeability to further explore the mechanism by which arabinose prevents cell lysis and
increases the purity of the recombinant enzyme. As shown in Figure 5, under the arabinose-free supplemented culture, the inner and outer membrane permeabilities of BL21/pelB-PT121 were significantly higher than those of the control BL21/pET22b, which may be the result of hydrolysis by expressed protease. However, when BL21/pelB-PT121 was cultured in arabinose-supplemented LB medium at the inductive phase, inner membrane permeability significantly decreased to a level even lower than that of BL21/pET22b in the culture without addition of arabinose. The outer membrane permeability also decreased when arabinose was added. However, the outer membrane maintained its higher permeability even when arabinose was added. Proteases of the thermolysin family are folded and matured in periplasmic space, and then a correctly folded mature protease is secreted.44 The higher permeability of the outer membrane promoted the secretion of the recombinant protease located in periplasmic space into culture. The inner membrane maintained its lower permeability with the addition of arabinose, and this phenomenon might have two benefits. One is prevention of cytoplasmic protein from leaking out of the cytoplasm to express purer secreted proteases; the other is maintenance of bacterial growth.

Mutant Y114S with Efficient Synthesis of Z-APM and Lower Caseinolysis. The activities of Z-APM synthesis and caseinolysis were compared between protease PT121 and its mutant Y114S with the same concentrations of proteins. As shown in Figure 6, protease PT121 presented a higher initial reaction rate of caseinolysis than that of Y114S. The specific activity of PT121 on caseinolysis was 22,000 U/mg, which was almost 2-fold that of Y114S (12,000 U/mg). The results may demonstrate that PT121 has higher hydrolysis activity compared with Y114S.

Interestingly, mutant Y114S facilitates efficient synthesis of Z-APM. As shown in Table 2, $v_0$ and $k_{cat}$ values of Y114S in Z-APM synthesis were 8.2- and 8.5-fold those of PT121, respectively, whereas both $K_M1$ and $K_M2$ of mutant Y114S were slightly declined compared with those of PT121. Mutant Y114S showed remarkably higher activity of Z-APM synthesis and considerably decreased the activity of caseinolysis. This difference may be the result of alteration in substrate specificity.

In the present study, the metalloprotease presented efficient peptide synthesis through site-directed mutagenesis, which may illustrate that the mutation site Tyr114 is a key residue for mediating the substrate specificity of protease PT121.

To rationalize this difference between PT121 and Y114S, molecular docking was applied to obtain the enzyme–substrate complex as shown in Figure S1. Molecular dynamic simulation was used to calculate the binding free energies of two enzymes toward Phe-OMe, and the binding free energies were $-20.23$ and $-23.34$ kcal/mol for PT121 and Y114S, respectively, by sampling the last 5 ns of trajectories. This result illustrates that mutant Y114S has stronger binding ability to Phe-OMe than...
that of PT121. Previous studies also reported only one mutation that considerably affects substrate specificity.\textsuperscript{45} Protease PT121 and its mutant Y114S might be useful in the degradation and engineering of food proteins\textsuperscript{46} and in the synthesis of bioactive peptides, such as aspartame, respectively. Further work will be conducted to engineer proteases PT121 and Y114S for enhancing yield in the synthesis of other active peptides.

In summary, we demonstrated that replacement of the signal peptide promotes the extracellular expression of metalloprotease PT121 and its mutant Y114S. The addition of arabinose significantly promoted the expression of proteases and improved the purity of the proteases in \textit{E. coli}. In addition, the residue 114 has been well characterized as a key residue to alter substrate specificity. Molecular simulation suggested that residue 114 plays a positive role in substrate binding.

## ASSOCIATED CONTENT

\textbf{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04164.

Table S1: Primers used in this study. Figure S1: (A) 3D structure of Y114S-Phe-OMe complex interaction; (B) structure of Phe-OMe in the active center; (C) RMSDs of the Ca atoms for PT121 and Y114S over all trajectories at 300 K (40 ns) (PDF)

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**Notes**
The authors declare no competing financial interest.

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