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Functional Expression and Characterization of Keratinase from *Pseudomonas aeruginosa* in *Pichia pastoris*

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Recombinant keratinase (rK) from *Pseudomonas aeruginosa* was secreted by *Pichia pastoris* SMD1168H with a final yield of 580 mg/L (1.03 kU/mL) after 72 h of induction. The rK was purified after nickel affinity chromatography and was stable at pH 6.0−9.0 and 10−60 °C. It was nonglycosylated protein with a molecular mass of 33 kDa and had an optimal pH and temperature at 8.0 and 60 °C, respectively. Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, dithiothreitol, glutathione, and β-mercaptoethanol activated, while Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, ethylene glycol tetraacetic acid, ethylene diamine tetraacetic acid, and *p*-chloromercuribenzoate inhibited its activity. rK could hydrolyze broad substrates and cleave hydrophobic and aromatic amino acids at P_1 position, behaving as those from the wild type strain and *E. coli* transformant.

KEYWORDS: ***Pseudomonas aeruginosa*; *Pichia pastoris*; functional expression; keratinase**

INTRODUCTION

One million tons of feathers are produced in the poultry industry and are currently processed into feather meals by high pressure and temperature. However, these kinds of processing conditions substantially lowered their nutritional value (*1*). Keratin, the main component of feathers, has a high degree of disulfide bonds and is poorly hydrolyzed by most proteolytic enzymes such as trypsin, pepsin, and papain (*2*). Bacterial keratinases were found to be able to hydrolyze insoluble keratin and a broad range of protein substrates (*3*). They have already stepped on to one of the most important groups of industrial enzymes because of their multipurpose applications in the feed, fertilizer, detergent, leather, and pharmaceutical industries (*4*).

Pseudomonas aeruginosa, a Gram-negative bacterium, is an opportunistic pathogen and easily causing fatal infections in vulnerable hosts because of the endotoxin it produces. *P. aeriginosa* is found to easily cause pneumonia among postoperative patients and patients suffering from burns, cancer, and cystic fibrosis (*5*). Although keratinase was obtained from a feather-degrading strain of *P. aeruginosa* and characterized (*6*), the utilization of this strain is still difficult because of its pathogenic risk.

Many mature and active proteases for industrial application have been successfully expressed in methylotrophic yeast, *Pichia pastoris* (*7*). This expression system is simple and inexpensive and is consequently employed in the overexpression of heterologous proteins (*8*). Furthermore, it is easy for further purification since *P. pastoris* itself only secretes a few extracellular proteins (*9*). Many studies have successfully expressed the keratinases from the Gram-positive bacterium *Bacillus licheniformis* (*8*), actinomycetes *Streptomyces fradiae* (*7*), fungi *Microsporum canis* (*10*), and *Aspergillus fumigatus* (*11*) in *P. pastoris*. This study aimed to clone and express the keratinase of *P. aeruginosa* in *P. pastoris* and further to characterize its recombinant keratinase.

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MATERIALS AND METHODS

Bacterial Strains and Vectors. *P. aeruginosa*, a keratinase-producing bacterium, was obtained from our laboratory (*6*). *Escherichia coli* Top10F′ and *P. pastoris* SMD1168H were purchased from Invitrogen Co. (Carlsbad, CA, USA). All media for bacteria cultivation were obtained from Difco Laboratories (BD Co., MD, USA). The synthesis of oligonucleotide primers and amino acid N-terminal sequencing were performed by Mission Biotech Inc. (Taipei, Taiwan). ExSel, a high fidelity DNA polymerase, was the product of JMR Holdings, Inc. (London, UK). T4 DNA ligase, pGEM-T Easy Vector, protein marker, and all restriction enzymes were obtained from Promega Co. (Madison, WI, USA), while the pPICZαC vector and Zeocin were purchased from Invitrogen Co. (Carlsbad, CA, USA). Methanol, sorbitol, ampicillin, kanamycin, and other chemicals were the products of Sigma-Aldrich Inc. (St. Louis, MO, USA). The resin for Ni 6 Sepharose Fast Flow was from Amersham Biosciences (GE Healthcare BioSciences Corp., MA, USA).

Molecular Cloning with the pGEM-T Vector. The sequence containing pro-peptide and mature protein was amplified by polymerase chain reaction (PCR) using pET-43b(+)-keratinase (*12*) as the template with 28 cycles (denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 70 °C for 90 s) and finally by a 10 min DNA thermal cycler at 70 °C (Perkin-Elmer, GeneAmp PCR system 2400). The forward primer was designed as 5′-(ATT *CTC GAG* AAA CGT GAG GCT GAA GCA GCC GAC CTG ATC GAC GTG TCC AAA CTC CCC)-3′ [with the *Xho*I (in italic) cutting site at the 5′ terminal and removal of the native signal peptide from the full sequence of the keratinase gene], while the reverse primer was 5′-(AAT *TCT AGA* AAC AAC GCG CTC GGG

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CAG GTC ACG)-3′ [with the *Xba* I (in italic) cutting site at the 3′ terminal and insertion of the His-tag at the 3′ terminal]. The other reverse primer with the *Xba*I recognition sequence (in italic) and without the His-tag gene at the 3′ terminal was used in PCR and for the construction of pPICZαC expression vectors. After being amplified, the PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), ligated with pGEM-T easy vector, transformed into *E. coli* Top 10F′, and screened according to Lin et al. (*12*).

Construction of the pPICZαC-Keratinase Expression Vector, Transformation, and Screening. The techniques for construction, transformation, and screening were according to the manufacturer's instructions (Invitrogen Co., Carlsbad, CA, USA). To construct the pPICZαC-keratinase expression vector, the DNA of keratinase was ligated into the pPICZαC vector at *Xho*I and *Xba*I cutting sites with an α-factor leader sequence by using T4 DNA ligase. After transformation and identification of positive colonies, the resulting plasmid, pPICZαC-keratinase expression vector, was prepared for yeast transformation. The plasmid of pPICZαC-keratinase was linearized by *Sac* I digestion and then transformed into *P. pastoris* SMD1168H by using a MicroPulser (Bio-Rad, USA) at 2 kV with a 0.2-cm cuvette. Negative control strains with/without an empty vector were also linearized by *Sac*I digestion and transformed into *P. pastoris* SMD1168H. After 2 h of incubation at 30 °C in 1 M sorbitol without shaking, cells were transferred to YPDS agar (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 1.5% agar) containing 100, 500, 1000, and 2000 μg/mL zeocin and incubated at 30 °C. After 2 to 3 days of incubation, it was confirmed by PCR using their genomic DNA.

Expression of Recombinant Keratinase (rK). After 2 days of incubation on YPD medium at 30 °C, the transformants were inoculated into 25 mL of BMGY medium and incubated at 30 °C and 200 rpm for 16 h. The cells were collected and resuspended in BMMY medium. Methanol was added to a final concentration of 0.5% every 24 h for induction during 5 days of incubation. The colonies were measured (CFU/mL), and keratinase activity and pH during incubation were monitored to examine optimal expression. The culture supernatants after 3 days of incubation were collected for further keratinase purification and characterization.

Purification of Recombinant Keratinase. After being filtered through a 0.45-μm membrane (Gelman Sciences, Ann Arbor, MI, USA) and concentrated with an Amicon ultrafiltration system (cutoff 5,000) (Amicon Div., W. R. Grace and Co., Beverly, MA, USA), the resulted samples were dialyzed against buffer A (25 mM potassium phosphate buffer, pH 7.5) containing 10 mM imidazole and 300 mM NaCl. rK was chromatographed on a nickel affinity column (2.6 × 4.7 cm), pre-equilibrated with buffer A containing 10 mM imidazole and 300 mM NaCl. After being washed with the same buffer, rK was eluted with 4 vol of buffer A containing 250 mM imidazole and 300 mM NaCl. Resulting samples were dialyzed against 20 mM phosphate buffer (pH 7.5) for further characterization.

Assay of Keratinase Activity. Keratinase activity was determined by measuring the azokeratin hydrolysis ability according to Sangali and Brandelli (*13*). Azokeratin was prepared according to Riffel et al. (*14*). One unit of activity was defined as the amount of keratinase that caused an increase in absorbance of 0.01 at 450 nm within 60 min of reaction at 50 °C.

Determination of Protein Concentration. Protein concentration was determined by the dye binding method (*15*). Bovine serum albumin was used as the standard protein.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed in a 12.5% (w/v) polyacrylamide gel according to Laemmli (*16*). Ten micrograms of samples was applied to each well of the gels. An SDS−PAGE standard protein kit (Bio-Rad, Hercules, CA, USA) was used as the marker. The procedure for estimation of molecular mass (*M*) was according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The gels were stained with Coomassie blue R-250 and glycoprotein staining following the method suggested by Zacharius et al. (*17*).

N-Terminal Amino Acid Sequence Analysis. Purified rK was subjected to SDS−PAGE analysis and then electro-transferred onto a polyvinylidene difluoride (PVDF) membrane. Protein bands were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences of proteins electro-transferred on to PVDF were analyzed by Edman degradation (*18*).

Effects of pH and Temperature. The optimal pH was determined by measuring the activity of purified rK at pH 3.0−11.0 (pH 3.0−6.0, 20 mM citrate buffer; pH 6.0−8.0, 20 mM sodium phosphate buffer; pH 7.0−9.0, 20 mM Tirs-HCl buffer; pH 9.0−11.0, 20 mM sodium carbonate buffer), while the optimal temperature of purified rK in 20 mM sodium phosphate buffer (pH 7.5) was measured at 10−90 °C using the azokeratin substrate according to Sangali and Brandelli (*13*).

pH and Thermal Stabilities. To determine pH stability, rK in various pH values of buffer (as shown above) was incubated at 37 °C for 30 min. Equal volume of 0.2 M sodium phosphate buffer (pH 7.5) was added to maintain the pH at 7.5. The residual activity was measured according to Sangali and Brandelli (*13*).

To determine the thermal stability, rK in 20 mM phosphate buffer (pH 7.5) was incubated at 10−90 °C for 30 min and then chilled in ice water immediately for 5 min. The residual activity was measured according to Sangali and Brandelli (*13*).

Effects of Metal Ions, Reducing Agents, and Inhibitors. Purified rK with 1, 5, and 10 mM of Ag^+, K^+, Li^+, Na^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{3+}, and with 1, 2, and 5 mM of dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), ethylene diamine tetraacetic acid (EDTA), glutathione (GSH), *N*-ethylmaleimide (NEM), β-mercaptoethanol (β-Me), *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonylfluoride (PMSF), and *N*-toyl-L-lysine chloromethyl ketone (TLCK) were incubated at 37 °C for 30 min. The residual activity was measured according to Sangali and Brandelli (*13*).

Substrate Specificity. Bovine serum albumin (BSA), casein, collagen, elastin, feather meal, and hemoglobin (Hb) were used as substrate. Substrates (final concentration, 0.5%) in 20 mM phosphate buffer (pH 7.5) were hydrolyzed at 50 °C by adding 10 μg of purified rK. After 60 min of reaction, equal vol of 15% trichloroacetic acid (TCA) was added to stop the reaction and then centrifuged at 10,000*g* at 4 °C for 10 min. A 0.5 mL reaction mixture was added to 1.0 mL of Cd-ninhydrin reagent and incubated at 84 °C for 5 min. Absorbance at 507 nm was measured after cooling (*19*). One unit of activity was defined as the amount of enzyme that could cause a release of 1 μg of tyrosine after 60 min of reaction at 50 °C (*20*).

To 0.1 mM of synthetic substrates in 20 mM phosphate buffer (pH 7.5), 10 μg of purified rK was added. After 4 min of incubation at 25 °C, absorbance at 405 nm was measured to determine the hydrolysis ability against synthetic substrates according to Lottenberg et al. (*21*).

RESULTS

Screening and Expression of Keratinase in *P. pastoris*. The pPICZαC-keratinase transformant, screened by zeocin hyper-resistant and PCR (data not shown), was able to secret rK. The yield of rK with/without the His-tag from *P. pastoris* was similar (data not shown). Keratinase activity in the culture supernatant of the pPICZαC-keratinase-His-tag transformant was monitored during methanol induction. During 72 h of induction, rK activity and protein concentration increased steadily up to 1.03 kU/mL and 0.58 mg/mL, respectively (**Figure 1**), while no rK activity was observed on the transformant only with mature keratinase (without its pro-peptide) (data not shown). The pH of broths decreased during the initial 30 h of incubation and then rapidly increased between 30 and 120 h of incubation at 30 °C. The death of the transformant was observed after 72 h of incubation, corresponding to the rapid decrease of keratinase activity.

Purification of the Recombinant Keratinase. A specific activity of 1.8 kU/mg was obtained after passing through a 0.45-μm membrane. rK was purified to electrophoretical homogeneity with a specific activity of 22.8 kU/mg, 73.8% recovery, and 12.8-fold purification after nickel affinity chromatography (**Figure 2** and **Table 1**). The *M* of purified rK was 33 kDa, estimated by SDS−PAGE (**Figure 3A**), which was almost similar to that predicted. No glycosylation was observed in rK (**Figure 3B**).

The N-terminal sequence (AEAGGPGG) of rK was similar to that of the wild type. This study was the first report on the expression of functional keratinase from *P. aeruginosa* in a *P. pastoris* system.

Characterization of the Recombinant Keratinase. The purified rK had an optimal pH and temperature at 8.0 and 60 °C, respectively, and was stable at pH 6.0−9.0 and 10−50 °C (**Figures 4** and **5**). It was moderately inhibited by Cd^{2+}, Co^{2+}, Ni^{2+}, NEM, and 10 mM of Zn^{2+} but highly inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, EGTA, EDTA, and *p*CMB. It was activated by Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, DTT, GSH, and β-Me (**Table 2**). Purified rK could hydrolyze a broad range of protein substrates including soluble proteins and a few insoluble proteins such as elastin and feather meal (**Table 3**). The specific activity of this rK on *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and Suc-Ala-Ala-Ala-*p*NA was 117.0 and 29.3 nmol//mg/min, respectively (**Table 3**).

DISCUSSION

It was clear that *P. pastoris* could be used for the expression of keratinase from *P. aeruginosa*. The pPICZαC expression vector with an inducible AOX1 promoter and the α-factor leader sequence could produce a higher yield than pGAPZαC with constituted GAP promoter (*8*). In comparison with the over-expression of rK from *P. pastoris* (22.8 kU/mg), less expression and secretion of rK in the cytoplasm of *E. coli* (0.148 kU/mg) (*12*) and *P. aeruginosa*, wild type (0.13 kU/mg) (*6*), were observed because of the formation of an insoluble inclusion body. The yield of rK obtained from the *P. pastoris* SMD1168H transformant of pPICZαC-keratinase was higher than those of previous studies by Evans et al. (*22*), Porres et al. (*8*), and Lin et al. (*12*) using *B. subtilis* FDB-22, *P. pastoris* X-33, and *E. coli* AD494(DE3) pLysS as expression hosts, respectively. This study also confirmed that the pro-peptide of the enzyme was definitely necessary for the functional expression of rK in the *P. pastoris* transformant and was considered as a helper for correct folding of the mature protease (*7*). In accordance with previous reports (*7*, *23*), the

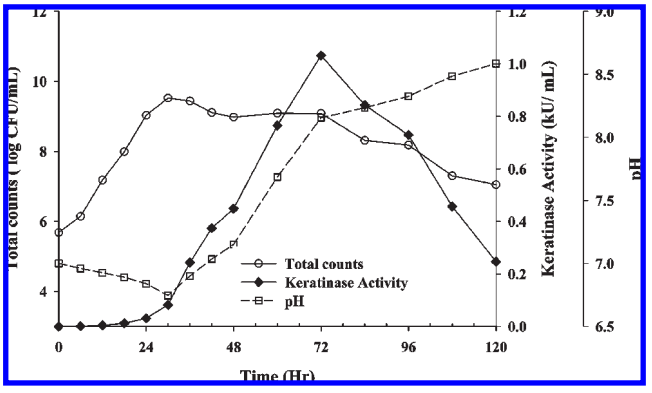

Figure 1. Time course of change in total count, keratinase activity, and pH during the incubation of the *P. pastoris* SMD1168H transformant at 30 °C.

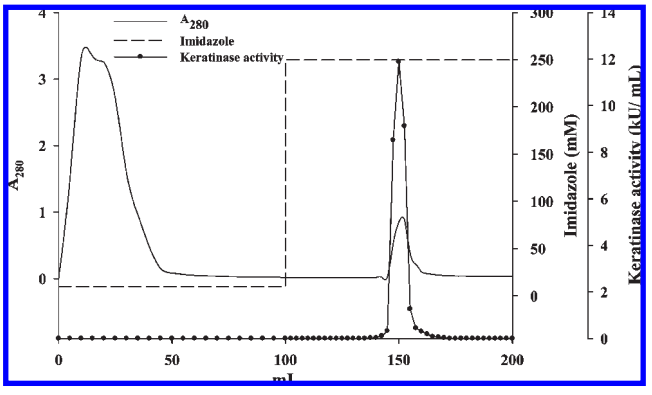

Figure 2. Chromatogram of recombinant keratinase on a nickel affinity column.

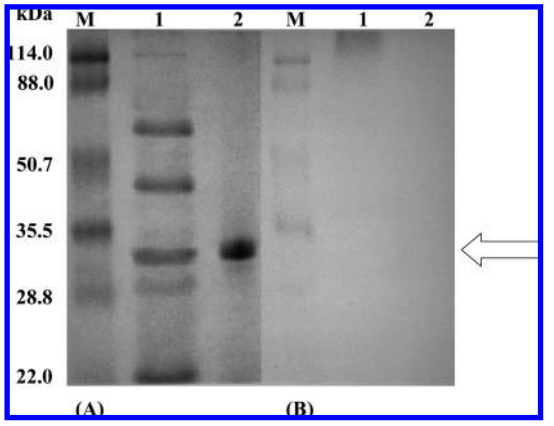

Figure 3. Profile of SDS−PAGE of recombinant keratinase. (**A**) Coomassie blue staining. (**B**) Glycoprotein staining. M, prestained SDS−PAGE marker; lane 1, crude enzyme secreted from *P. pastoris* SMD1168H transformant; lane 2, purified enzyme obtained after the nickel affinity column.

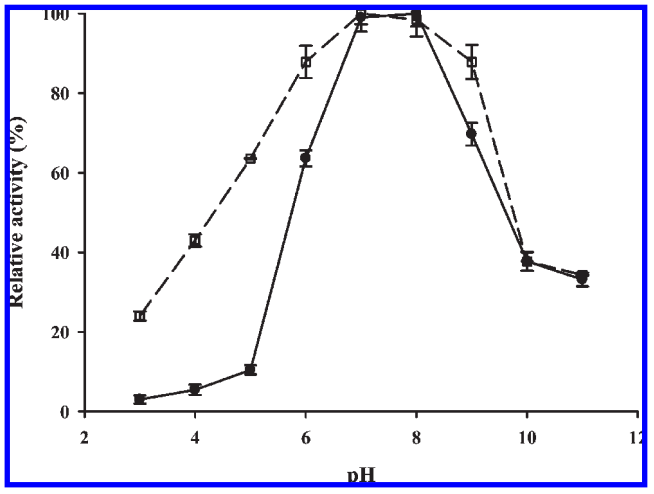

Figure 4. Effect of pH on recombinant keratinases. (−●−), optimal; (−□−), stability; the vertical bars show the standard deviations from 3 measurements.

Table 1. Summary of the Purification of Keratinase from *P. pastoris* SMD1168H Transformant

procedures	total protein (mg)	total activity (kU)	specific activity (kU/mg)	recovery (%)	purification (fold)
crude enzyme	278	493.9	1.8	100.0	1.0
ultrafiltration	161	403.2	2.5	81.6	1.4
nickel affinity column	16	364.7	22.8	73.8	12.8

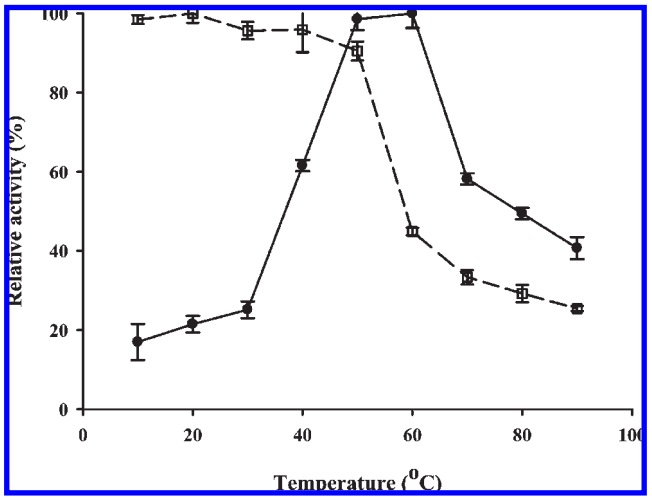

Figure 5. Effect of temperature on recombinant keratinases. (−●−), optimal; (−□−), stability; the vertical bars show the standard deviations from 3 measurements.

Table 2. Effect of Metal Ions and Chemicals on Recombinant Keratinases

	relative activity (%)		
metals	1 mM	5 mM	10 mM
none[a]	100	100	100
Ag^{+}	102.7 ± 1.5[b]	98.6 ± 2.6	99.0 ± 2.2
K^{+}	100.9 ± 2.2	98.6 ± 1.9	103.1 ± 1.9
Li^{+}	102.2 ± 1.9	101.4 ± 2.6	102.3 ± 1.9
Na^{+}	100.1 ± 0.7	99.1 ± 0.7	96.3 ± 0.7
Ba^{2+}	98.6 ± 1.1	105.9 ± 3.3	110.5 ± 7.1
Ca^{2+}	111.8 ± 2.2	115.4 ± 0.4	118.6 ± 1.1
Cd^{2+}	87.3 ± 1.1	81.8 ± 0.4	71.4 ± 2.6
Co^{2+}	77.7 ± 0.4	72.7 ± 1.5	60.9 ± 0.7
Cu^{2+}	56.8 ± 1.1	6.4 ± 0.7	1.4 ± 1.1
Fe^{2+}	81.4 ± 2.6	9.6 ± 1.1	0.9 ± 0.4
Hg^{2+}	60.5 ± 5.6	25.0 ± 0.4	18.2 ± 2.2
Mg^{2+}	100.1 ± 2.2	110.4 ± 0.7	117.4 ± 0.4
Mn^{2+}	96.4 ± 0.4	100.5 ± 0.4	109.6 ± 1.5
Ni^{2+}	101.8 ± 1.5	65.9 ± 0.4	57.7 ± 1.1
Zn^{2+}	114.1 ± 1.9	94.9 ± 2.6	75.5 ± 0.4
Fe^{3+}	98.2 ± 3.7	87.7 ± 1.9	23.6 ± 0.7
chemicals[c]	1 mM	2 mM	5 mM
DTT	106.0 ± 0.3	115.3 ± 0.7	125.4 ± 1.0
EGTA	87.2 ± 1.3	74.4 ± 0.7	48.8 ± 0.7
EDTA	90.8 ± 0.3	69.6 ± 1.3	44.8 ± 1.3
GSH	107.5 ± 1.6	113.3 ± 2.6	118.4 ± 1.0
NEM	93.2 ± 1.3	88.4 ± 0.4	70.1 ± 0.3
β-ME	146.4 ± 1.3	159.2 ± 2.6	171.6 ± 2.9
*p*CMB	92.0 ± 2.0	66.8 ± 0.3	37.6 ± 0.7
PMSF	95.6 ± 2.9	101.6 ± 0.7	98.4 ± 1.3
TLCK	107.2 ± 2.0	106.8 ± 5.6	109.6 ± 3.3

[a] The activity of that without metal ion or chemical added was defined as 100%. [b] Values in this table are mean values from 3 measurements ± SD. [c] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; GSH, glutathione; NEM, *N*-ethylmaleimide; β-ME, β-mercaptoethanol; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonylfluoride; TLCK, *N*-toyl-L-lysine chloromethyl ketone.

strategy design for expressing the pro-mature sequence could increase the production of keratinase and protect from proteolysis in expression hosts. Furthermore, the use of the protease deficient strain (*P. pastoris* SMD1168H) as the expression host and the insertion of His-tag at the C-terminal of rK were helpful for reducing the degradation during expression (*24*) and simplifying the purification by nickel affinity chromatography, respectively.

Table 3. Effect of Various Substrates on Recombinant Keratinase: (a) Different Protein Substrates and (b) Synthetic Substrates

(a) protein substrate	specific activity (kU/mg)[a]
casein	17.8 ± 0.2 (100%)
hemoglobin	12.6 ± 0.4 (70.8%)
bovine serum albumin	10.1 ± 0.1 (56.5%)
elastin	7.7 ± 0.4 (43.5%)
feather	6.8 ± 0.3 (38.0%)
collagen	3.5 ± 0.1 (19.5%)
(b) synthetic substrates	specific activity (nmol/mg/min)[a]
N-succinyl-Ala-Ala-Pro-Phe-*p*NA	117.0 ± 7.3
Suc-Ala-Ala-Ala-*p*NA	29.3 ± 2.8

[a] Values in this table are mean values from 3 measurements ± SD.

The gradual death of transformed yeast cells might be due to the damage and toxicity of recombinant keratinase to cell membranes (*23*), and the rapid decrease of keratinase activity might be due to self-keratinolysis (autolysis) and poor stability in the alkaline state (*23*). Although the yield of rK from the *P. pastoris* transformant (580 mg/L) was less than that of other heterologus proteins from *P. pastoris* (*23*), it was almost 6-fold higher than *ker*A of *B. licheniformis* from *P. pastoris* (124 mg/L) (*8*). According to SDS−PAGE and glycoprotein staining as well as predicted *M*, although there were 4 potential N-glycosylation sites in the amino acid sequence of keratinase from *P. aeruginosa*, no glycosylation occurred in rK. Hence, modification of amino acids at the protease cutting site, optimization of codon usage frequently used in *P. pastoris*, and engineering additional glycosylation of recombinant protein in the *P. pastoris* expression system were considered to be the strategies for efficient recombinant protease secretion, improvement in its stability, decrease of proteolysis, and enhancement of expression (*8*, *23*, *25*, *26*).

The optimal pH and temperature of rK from the *P. pastoris* transformant were similar to those of the wild type, *P. aeruginosa* (pH 9.0; 60 °C) (*6*) or *B. licheniformis* (pH 8.5, 60 °C) (*20*). In addition, the pH and temperature stability of rK (6.0−9.0 and 10−60 °C) were close to those of *P. aeruginosa* (pH 5.5−9.0, 10−50 °C) (*6*), or *Streptomyces albidoflavus* (pH 6.0−9.5, 40−70 °C) (*27*). Keratinases from *Vibrio* kr2 (*13*) and *Bacillus* sp. SCB-3 (*28*) had similar keratinolytic characteristics including activation by Ca^{2+} and inhibition by a high concentration of Zn^{2+}. Additionally, a previous study indicated that some metallopeptidases were inhibited by excess metals, particularly at pH values from neutral to alkaline (*14*). Heavy metals such as Cu^{2+}, Hg^{2+} (*14*), and Co^{2+} (*28*) could inhibit keratinase activity. This might be because Hg^{2+} could bind the thiol groups and interact with tryptophan residues or carboxyl group of amino acids (*29*). In contrast, activation of keratinase by reducing agents might be due to the reduction of disulfide bonds by sulfitolysis, which consequently increased the contact with substrate for keratinolysis (*14*, *28*). Since rK was inhibited by EDTA and EGTA and activated in the presence of divalent metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, it was considered to be a metalloprotease (*14*, *30*).

The rK from the *P. pastoris* transformant easily hydrolyzed a broad range of protein substrates including soluble proteins and a few insoluble proteins such as elastin and feather meal, which was in accordance with those from other studies (*4*, *20*). The rK from this study showed much higher hydrolytic ability against elastin

than those from *Bacillus licheniformis* (*20*) and *Bacillus* sp. SCB-3 (*28*). Therefore, increasing metal salts or pretreatments of substrates with physical methods and the addition of reducing agents or detergents might be able to improve the degradation of insoluble substrates and activation of keratinase (*20*). According to the synthetic substrate specificity, purified rK had high specificity against aromatic and hydrophobic amino acids at the P1 substrate position, which corresponded to that of another study (*4*). According to the data obtained in this study, the purified rK behaved as that from wild type, *P. aeruginosa* (*6*).

The keratinase from *P. aeruginosa* was successfully expressed and secreted by *P. pastoris* in an inducible expression system. rK was nonglycocylated, which was similar to that from the wild type. Production of rK by this expression system is feasible and has high potential to hydrolyze feathers and subsequently promote the nutritional values of feather meal.

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