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ARTICLE

Cloning, Expression, and Purification of *Pseudomonas aeruginosa* Keratinase in *Escherichia coli* AD494(DE3)pLysS Expression System

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The DNA encoding keratinase from *Pseudomonas aeruginosa* was ligated into pET-43b(+) expression vector and transformed into *Escherichia coli* AD494(DE3)pLysS. After isopropyl β-D-thiogalactopyranoside induction, the soluble recombinant keratinase was expressed in *E. coli*. The keratinase with a molecular mass of 33 kDa was purified to electrophoretical homogeneity after nickel affinity chromatography. It had an optimal pH and temperature of 8.0 and 50 °C, respectively, and was stable at pH 6.0−9.0 and 10−60 °C. It was highly inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Fe^{3+}, ethylene glycol tetraacetic acid, ethylenediaminetetraacetic acid, and *p*-chloromercuribenzoate, but activated by Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, dithiothreitol, glutathione, and β-mercaptoethanol. According to substrate specificity results, the purified keratinase was considered to be a metalloprotease.

KEYWORDS: ***Pseudomonas aeruginosa*; *E. coli*; cloning; expression; keratinase**

INTRODUCTION

Keratinases (EC 3.4.99.11) are of particular interest because of their action on insoluble keratin and wide protein substrates (*1*). Keratin is insoluble and not degradable by proteolytic enzymes such as trypsin, pepsin, and papain, because it is rich in disulfide bonds (*2*). These mentioned enzymes have long been studied in the dehairing process in the leather industry (*3*) and for hydrolysis of feather keratin (*1*). Although keratin is insoluble and very stable, feathers do not accumulate in nature. This phenomenon suggested that it is degradable by some specific bacteria. Because keratin hydrolysates can be good organic fertilizers, edible films, and resources of some rare amino acids (*3*), enzymes such as keratinase that can be used in the hydrolysis of keratin are worth investigating.

Many bacteria, actinomycetes, and fungi have been found to have keratin digestion abilities (*3*). A keratinase-producing bacterium, *Pseudomonas aeruginosa*, has been isolated and found to have high keratin hydrolysis ability (*4*), but it is a pathogenic strain and easily cultured on any nutrient resource to cause fatal infections in vulnerable hosts (*5*). Therefore, it might belong to a high pathogenic risk group of this species. This novel keratinase from *P. aeruginosa* has been studied and found to be able to hydrolyze a broad range of protein substrates including soluble proteins and a few insoluble proteins such as elastin and feather (*4*). Some studies had been done on isolating and expressing the keratinase gene from Gram-positive bacteria (*Bacillus* sp.) and actinomycete (*Nocardiopsis* sp.) (*3*). However, cloning and expressing of keratinase from pathogenic bacteria are still limited. They are very good alternatives to increase enzyme yields and to ensure their safety (*6*). For improving the utilization of this novel keratinase from *P. aeruginosa*, its DNA sequence was cloned in an *Escherichia coli* expression system. The recombinant keratinase was purified and characterized. This study is the first report of constituting the functional keratinase from *P. aeruginosa* in an *E. coli* expression system. The results will provide good references or clues for the further studies on expression in *Bacillus* and *Pichia* systems and for the application of this enzyme.

MATERIALS AND METHODS

Bacterial Strains and Vectors. *P. aeruginosa*, a keratinase-producing bacterium isolated in our laboratory (*4*), was used in this study. *E. coli* Top10F′ and *E. coli* AD494(DE3)pLysS were purchased from Invitrogen Co. (Carlsbad, CA) and Novagen Inc. (Darmstadt, Germany), respectively. All media for bacteria cultivation were from Difco Laboratories (BD Co.). ExSel, high-fidelity DNA polymerase, was the product of JMR Holdings, Inc. (London, U.K.). T4 DNA ligase, pGEM-T Easy Vector, protein markers, and all restriction enzymes were the products of Promega Co. (Madison, WI). The pET-43b(+) vector was from Novagen Inc. (Darmstadt, Germany). X-Gal, isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin, chloramphenicol, and other chemicals were the products of Sigma-Aldrich Inc. (St. Louis, MO). Ni 6 Sepharose Fast Flow (nickel affinity column) was obtained from Amersham Biosciences (GE Healthcare BioSciences Corp.).

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Purification and N-Terminal Amino Acid Sequencing. Keratinase, purified according to the method of Yin et al. (*4*), was subjected to SDS-PAGE analysis and electrotransferred onto polyvinylidene difluoride (PVDF). Proteins were stained with Coomassie blue R-250 after electrophoresis. N-Terminal amino acid sequence of proteins was analyzed by Edman degradation (*7*).

Molecular Cloning with pGEM-T Vector. Total genomic DNA was extracted from *P. aeruginosa* according to the method of Ausubel et al. (*8*). Cloning was performed according to the guidelines of Sambrook et al. (*9*). Polymerase Chain Reaction (PCR) was used to amplify keratinase DNA with 28 cycles (30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 90 s extension at 70 °C) and then 10 min of DNA thermal cycler at 70 °C (2720 Thermal Cycler; Applied Biosystems, Foster, CA). Primer was designed according to the alignment between N-terminal amino acid and functional sequences from *Pseudomonas* Genome Database (http://www.pseudomonas.com) and used for PCR and construction of pET-43b(+) expression vectors. The forward primer was 5′-(ACC CAT ATG AAG AAG GTT TCT ACG CTT GAC CTG T)-3′, whereas the reverse primers with and without His-tag were 5′-(ATT CTC GAG CGC GCT CGG GCA GGT CAC)-3′ and 5′-(TAA CTC GAG *TTA* CAA CGC GCT CGG GCA G)-3′, respectively. The underlined sequences contained in the sense and antisense primers were restriction enzyme recognition sites (*Nde*I and *Xho*I, respectively), whereas the *italic* sequences were stop codons. After amplification, the PCR product was ligated with pGEM-T easy vector and transformed into cloning host, *E. coli* Top 10F′, according to the procedure of Hanahan and Meselson (*10*). After blue/white selection, plasmid from transformed *E. coli* was extracted and subjected to *Nde*I/*Xho*I digestion and DNA sequencing using T7 and SP6 as sequencing primers.

Construction of pET-43b(+)-Keratinase Expression Vector and Transformation into *E. coli* AD494(DE3)pLysS. DNA of keratinase was ligated into pET-43b(+) expression vector using T4 DNA ligase. The resulting plasmid was transformed into *E. coli* AD494(DE3)pLysS using a heat-shock procedure (*9*). Because pET-43b(+) vector, pLysS plasmid, and *E. coli* AD494(DE3)pLysS are ampicillin-, chloramphenicol- and kanamycin-resistant, *E. coli* AD494(DE2)pLysS with pET-43b (+) can be screened with LB agar (0.5% yeast extract, 1% of tryptone and NaCl, and 1.5% agar) containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol. The selected single colony was further confirmed by PCR using keratinase primers to ensure the transformants with correct pET-43b(+)-keratinase.

Cultivation of *E. coli* Transformant and Isolation of Recombinant Keratinase. *E. coli* AD494(DE3)pLysS with pET-43b(+)-keratinase was cultivated in 50 mL of LB broth containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol at 37 °C overnight with 200 rpm shaking. Fifty milliliters of activated culture was transferred into 1.0 L of fresh LB broth with the three mentioned antibiotics under the same condition for 3 h. When OD_{600} reached 0.6, IPTG (final concentration = 0.1 mM) was added to induce the synthesis of keratinase. After 4 h of induction at 30 °C, *E. coli* was harvested by 30 min of centrifugation at 4000*g* and resuspended in 20 mL of 20 mM phosphate buffer (pH 8.0, buffer A). The suspended cells were sonicated (200 cycles with 5 s on/15 s off) in ice using a sonicator XL 2020 system (HEAT Systems Inc., Farmingdale, NY) and then centrifuged at 13000*g* for 30 min.

Purification of Recombinant Keratinase from *E. coli* AD494 (DE3)pLysS. The isolated recombinant keratinase in buffer A was filtered through a 0.45 μm sterilized membrane (Gelman Sciences, Ann Arbor, MI). The filtrate was chromatographed on a nickel affinity column (2.6 × 4.7 cm), which was pre-equilibrated with buffer A. After a washing with the same buffer, the recombinant keratinase was eluted with buffer A containing 250 mM imidazole and 300 mM NaCl. The resulting samples were dialyzed against 20 mM phosphate buffer (pH 7.5) for further analyses.

Assay of Keratinase Activity. Keratinase activity was determined by measuring the azokeratin hydrolysis ability according to the method of Sangali and Brandelli (*11*). Azokeratin was prepared according to the procedure of Riffel et al. (*12*). 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 using the dye binding method (*13*). Bovine serum albumin was used as standard protein.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol, β-Me) were heated at 95 °C for 3 min and then subjected to SDS-PAGE analysis with 12.5% polyacrylamide gel. After electrophoresis, gels were stained with Coomassie blue R-250. The quantity of enzyme sample on each lane of gel was 10 μg (*14*).

Optimal pH and Temperature. The optimal pH was determined by measuring the activity of purified keratinase 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 Tris-HCl buffer; pH 9.0−11.0, 20 mM sodium carbonate buffer), whereas the optimal temperature of purified keratinases in 20 mM sodium phosphate buffer (pH 7.5) was measured at 10−90 °C using azokeratin substrate according to the method of Sangali and Brandelli (*11*).

Effects of pH and Temperature. To determine the pH stability, recombinant keratinase in various pH values of buffer (as shown above) was incubated at 37 °C for 30 min. An 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 the method of Sangali and Brandelli (*11*).

To determine the thermal stability, the recombinant keratinase 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 the method of Sangali and Brandelli (*11*).

Effects of Metals, Reducing Agents, and Inhibitors. Purified recombinant keratinases in 20 mM phosphate buffer (pH 7.5) with 1, 5, and 10 mM 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 dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), glutathione (GSH), *N*-ethylmaleimide (NEM), β-mercaptoethanol (β-Me), *p*-chloromercuribenzoate (*p*CMB), phenylmethanesulfonyl fluoride (PMSF), and *N*-toyl-L-lysine chloromethyl ketone (TLCK) were incubated at 37 °C for 30 min. The residual activity was measured according to the method of Sangali and Brandelli (*11*).

Substrate Specificity. Bovine serum albumin (BSA), casein, collagen, elastin, feather, 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 kertatinase. After 60 min of reaction, an equal volume of 15% trichloroacetic acid (TCA) was added to stop the reaction and then centrifuged at 10000*g* and 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 (*15*). One unit of activity was defined as the amount of enzyme that could cause the release equivalent to 1 μmol of tyrosine after 60 min of reaction (*16*).

To 0.1 mM synthetic substrates in 20 mM phosphate buffer (pH 7.5) was added 10 μg of purified keratinase. After 4 min of incubation at 25 °C, absorbance at 405 nm was measured to determine the hydrolysis ability against synthetic substrates according to the method of Lottenberg et al. (*17*).

RESULTS AND DISCUSSION

Molecular Cloning of Kertainase in *E. coli* AD494(DE3) pLysS Transformant. The homology of the N-terminal amino acid sequence of keratinase (AEAGGPGG) with the genome of functional proteins from *P. aeruginosa* (*5*) was used to design the primers. The DNA fragment encoded keratinase was amplified (**Figure 1**) from genomic DNA by PCR and cloned into pGEM-T Easy cloning vector. The full DNA of kertainase was confirmed by screening and sequencing and compared with those from different microorganisms in GenBank using the Basic Local Alignment Search Tool (BLAST). It was different from those produced by *Bacillus* sp. and *Streptomyces* sp. (data not shown). The resulting vector containing the correct DNA sequence was used to construct keratinase expression vector. After the full sequence for keratinase (1497 bp; **Figure 2**) was digested, the keratinase gene was ligated with pET-43b(+) to form pET-43b(+)−keratinase expression vector (**Figure 3**). It was then transformed into *E. coli* AD494(DE3)pLysS. To enhance the expression of soluble keratinase, the pre and pro gene was also cloned into 5′ flanking of mature keratinase, which was expected to yield higher expression of recombinant keratinase (*18*). As we know, pET-43b(+), a powerful expression vector, carries a strong T7 promoter for target gene transcription, whereas *E. coli* AD494(DE3)pLysS is a thioredoxin reductase mutant strain and can express target proteins with accurate disulfide bonds. This mutant is, therefore, considered to have high potential for the production of properly folded active proteins (*19*).

Expression of Recombinant Keratinase from *E. coli* AD494 (DE3)pLysS Transformant. After 4 h of IPTG induction at 30 °C, the highest keratinase activity was observed in the cytoplasm of *E. coli* AD494(DE3)pLysS transformant. However, the yield of recombinant keratinase with/without His-tag from *E. coli* was similar (data not shown). In this study, keratinase gene was cloned in pET-43b(+) plasmid under the control of T7 promoter and expressed in *E. coli* AD494(DE3)pLysS by the induction of T7 RNA polymerase. According to Sambrook et al. (*9*), the most important variables in the overexpression of any target protein in *E. coli* were considered to be temperature and IPTG concentration. Other factors such as intracellular folding of expression protein, activity of endogenous proteases, and growth rate of cells, etc., can also affect the expression level. Defects such as low yield and formation of inclusion body during expression in *E. coli* were generally caused by a hydrophobic form

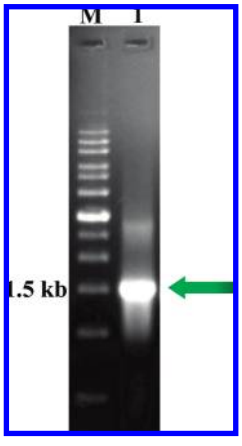

Figure 1. DNA fragment encoded keratinase amplified from genomic DNA of *Pseudomonas aeruginosa*.

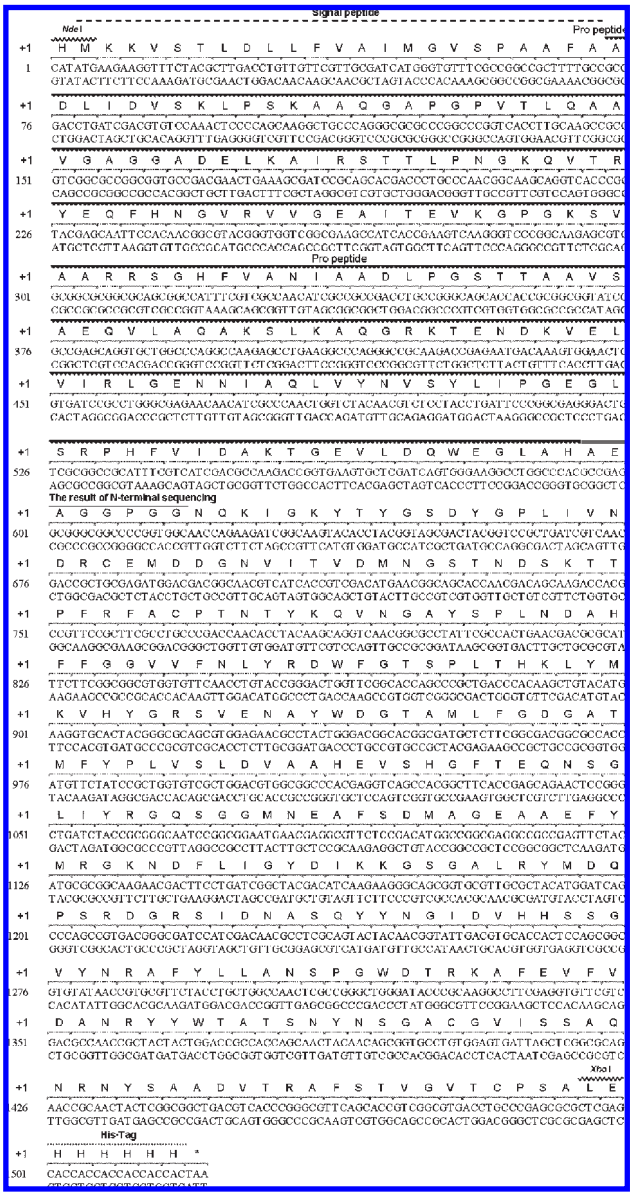

Figure 2. Full sequence of keratinase amplified from *Pseudomonas aeruginosa*. (∼∼∼, cutting site of restriction enzyme; − − −, signal peptide; —, pro-peptide; ===, result of N-terminal sequencing from mature keartinase; · · ·, His·Tag; ∗, stop codon.)

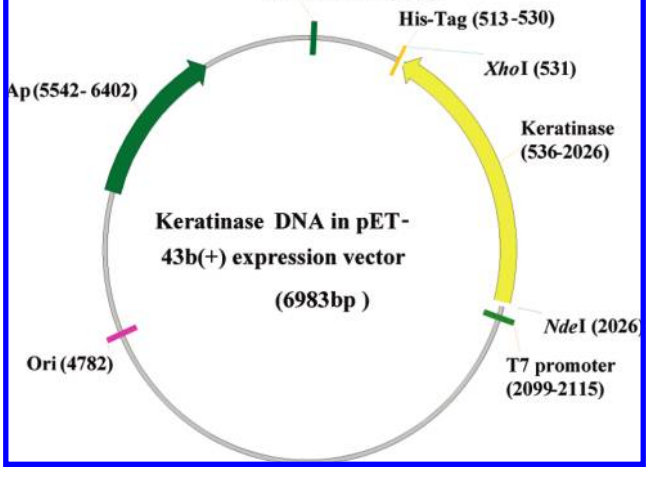

Figure 3. Construction of pET-43b(+)-keratinase expression vector. The start codon contains the cutting site *Nde*I (2026).

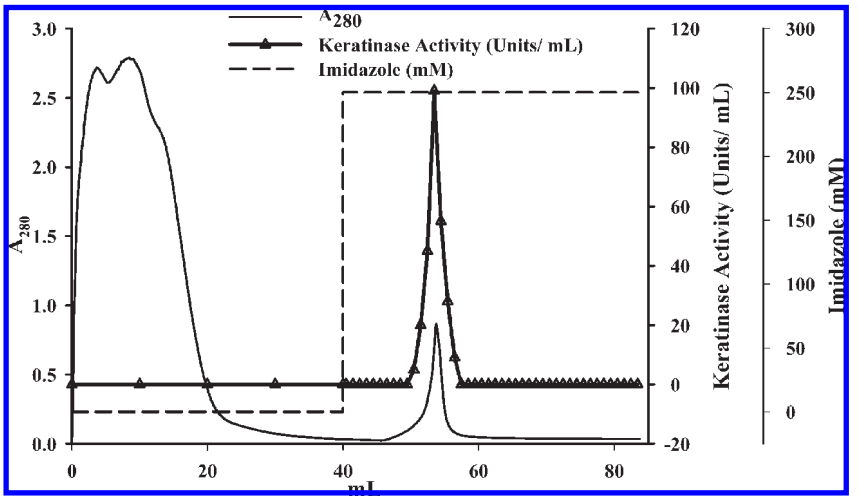

Figure 4. Chromatogram of recombinant keratinase on nickel affinity column.

Table 1. Summary of the Purification of Recombinant Keratinase from *E. coli* AD494(DE3)pLysS

procedure	total protein (mg)	total activity (units)	specific activity (units/mg)	recovery (%)	purification (-fold)
crude enzyme[a]	360.9	4340	12	100.0	1.0
nickel affinity column	21.0	3057	146	70.5	12.1

[a] The crude enzyme was extracted from the transformant cell incubated in 2.0 L of LB broth.

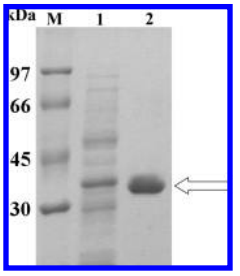

Figure 5. Profile of SDS-PAGE of recombinant keratinase: M, marker; lane 1, crude enzyme from transformant cell; lane 2, purified enzyme obtained after nickel affinity column.

of the signal peptide (*20*). Because this recombinant keratinase could be expressed in the cytoplasm of *E. coli*, it would be very easy to recover, time-saving, and beneficial to further industrial applications.

Purification of Recombinant Keratinase. After 3 h of cultivation at 37 °C and IPTG induction, the cells were harvested and sonicated. Crude enzyme in *E. coli* AD494 (DE3)pLysS was collected after 30 min pf centrifugation at 4000*g*. A protein peak with keratinase activity was eluted by 250 mM imidazole on nickel affinity chromatography. At this stage, the sample was purified to electrophoretical homogeneity with a specific activity of 146 units/mg, 70.5% recovery, and 12.1-fold purification (**Figure 4** and **Table 1**). The molecular mass (*M*) of purified recombinant keratinase was 33 kDa, estimated by SDS-PAGE (**Figure 5**, lane 2), which was similar to the predicted *M* of 33 kDa. The N-terminal sequence (AEAGGPGG) of the recombinant keratinase was the same as that of wild type (AEAGGPGG; **Figure 2**). Many studies on the cloning and expression of keratinase indicated that the plasmid-based expression was very unstable and easily formed inclusion body or incorrect folding of mature keratinase in *E. coli* or *B. subtilis* (*3*). This study is the first report of the constitution of the functional keratinase from *P. aeruginosa* in an *E. coli* expression system.

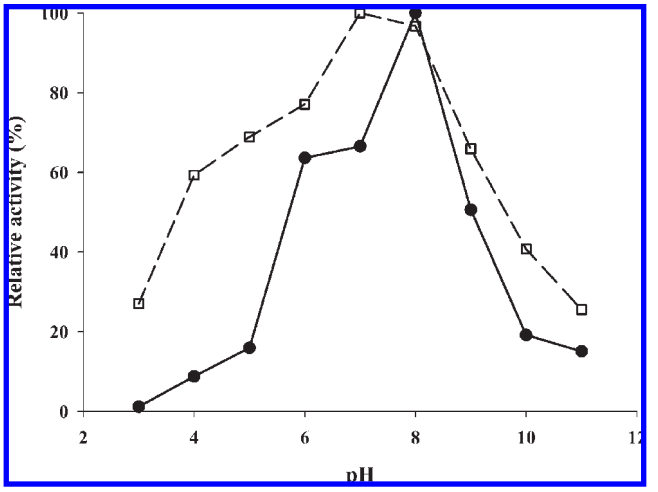

Figure 6. Effect of pH on recombinant keratinases: ●, optimal; □, stability.

Effect of pH and Temperature. The optimal pH and temperature for the recombinant keratinase were 8.0 and 50 °C, respectively, and the keratinase was stable at pH 6.0−9.0 and 10−60 °C (**Figures 6** and **7**). These characteristics were similar to those of wild type (*4*). Previous studies indicated the optimal pH and temperature were from neutral to alkaline and from 30 to 80 °C, respectively, for keratinase from different species (*3*). However, a few keratinases had an extreme optimum at pH 12 (from *Bacillus halodurans*) (*21*) or at 100 °C (from *Fervidobacterium islandicum* AW-1) (*22*). According to pH and thermal stabilities, it was similar to those from other studies on *Streptomyces albidoflavus* (*23*) and *Chrysosporium keratinophilum* (*24*). However, exceptional stability of keratinase was also observed at pH 4.0 (from *Streptomyces pactum*) (*25*), at pH 13.0 (from *B. halodurans*) (*21*), and at 100 °C (from *F. islandicum* AW-1) (*22*).

Effects of Metal Ions, Reducing Reagents, and Inhibitors. Purified recombinant keratinase was moderately inhibited by Ag^{+}, Co^{2+}, Fe^{2+}, NEM, and high concentration of

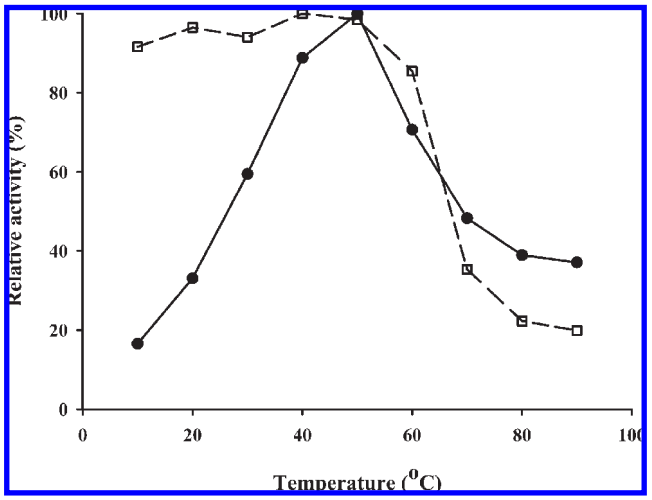

Figure 7. Effect of temperature on recombinant keratinases: ●, optimal; □, stability.

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

	relative activity (%)		
metal	1 mM	5 mM	10 mM
none[b]		100	
Ag^{+}	88.7	83.9	75.1
K^{+}	104.5	95.4	94.8
Li^{+}	101.1	92.9	87.1
Na^{+}	100.1	95.3	90.5
Ba^{2+}	96.6	120.8	125.6
Ca^{2+}	101.4	120.3	124.2
Cd^{2+}	70.6	62.6	55.1
Co^{2+}	74.9	73.1	73.5
Cu^{2+}	49.5	32.8	8.5
Fe^{2+}	86.4	70.6	18.1
Hg^{2+}	49.2	11.3	1.1
Mg^{2+}	95.4	115.3	121.3
Mn^{2+}	106.1	114.4	126.5
Ni^{2+}	50.3	29.4	26.5
Zn^{2+}	94.5	120.3	70.3
Fe^{3+}	85.9	79.4	22.0

	relative activity (%)		
chemical	1 mM	2 mM	5 mM
DTT	90.7	111.68	128.9
EGTA	68.2	49.6	9.8
EDTA	41.5	27.1	7.9
GSH	92.4	107.3	116.9
NEM	82.2	79.1	84.0
β-ME	288.1	309.8	327.3
*p*CMB	61.3	47.7	42.8
PMSF	97.5	92.4	90.0
TLCK	98.1	98.6	96.4

[a] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; NEM, *N*-ethylmaleimide; β-ME, β-mercaptoethanol; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-toyl-L-lysine chloromethyl ketone. [b] The activity of that without metal ion or chemical added was defined as 100%.

Zn^{2+} and highly inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Fe^{3+}, EGTA, EDTA, and *p*CMB. However, it was activated by Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, DTT, GSH, and β-Me (**Table 2**). The activation of keratinase by reducing agents might be due to the reduction of disulfide bonds, which consequently increased the contact with substrate for keratinolysis (*12*, *24*, *26*). Heavy metals, such as Cu^{2+}, Hg^{2+} (*12*), or Co^{2+} (*26*), could inhibit keratinase activity. Among these metals, Hg^{2+} can bind the thiol groups and also interacts with tryptophan residues or the carboxyl group of amino acids (*27*). It was, therefore, considered to be an inhibitor.

Table 3. Effect of Various Substrates on Recombinant Keratinase: (**A**) Protein Substrates; (**B**) Synthetic Substrates

(**A**) protein substrate	specific activity (units[a]/mg)
casein	43.7 (100%)[b]
hemoglobin	38.9 (88.0%)
bovine serum albumin	29.5 (65.0%)
elastin	26.8 (58.3%)
feather	22.1 (46.8%)
collagen	8.9 (14.1%)

(**B**) synthetic substrate	specific activity (nmol/min/mg)
N-Succinyl-Ala-Ala-Pro-Phe-*p*NA	74.3
Suc-Ala-Ala-Ala-*p*NA	5.7

[a] One unit of activity was defined as the amount of enzyme that could cause the release equivalent to 1 μmol of tyrosine after 60 min of reaction. [b] Percentage ratios of specific activity of tested substrates related to that of casein.

From the data obtained, the recombinant keratinase was considered to be a metalloprotease. According to Riffel et al. (*12*) and Rissen et al. (*28*), metalloproteases could be inhibited by EDTA and EGTA and activated in the presence of divalent metals such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. However, the inhibition of a high concentration of Zn^{2+} (10 mM) was similar to a previous study (*12*), which showed that some metallopeptidases were inhibited by excess metals, particularly at pH from neutral to alkaline. The keratinolytic characteristics, including activation by Ca^{2+} and inhibition by a high concentration of Zn^{2+}, were also observed at *Vibrio* kr2 (*29*) and *Bacillus* sp. SCB-3 (*26*). The activation effect of Mg^{2+} suggested the key role of Mg^{2+} in keratinase stability (*30*).

Substrate Specificity. Purified recombinant keratinase from *E. coli* AD494(DE3)pLysS could hydrolyze a broad range of protein substrates including soluble proteins and a few insoluble proteins, such as elastin and feather (**Table 3**). This phenomenon is similar to that reported in some previous studies (*3*). However, the hydrolytic ability of the keratinases from *Bacillus licheniformis* (*16*) and *Bacillus* sp. SCB-3 (*26*) against elastin was < 20% compared with other substrates. Recombinant keratinase from this study had a much higher hydrolytic ability against elastin. To completely digest these insoluble substrates, the activation of keratinase by increasing metal salts or pretreatments of substrates with physical methods and the addition of reducing agents or detergents were required (*16*). The specific activities of this recombinant keratinase on *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and Suc-Ala-Ala-Ala-*p*NA were 74.3 and 5.7 nmol/min/mg, respectively. According to synthetic substrate specificity, the purified keratinase has high specificity to aromatic and hydrophobic amino acids at the P1 position of the substrate. This phenomenon was similar to that obtained from *Streptomyces albidoflavus* (*23*), which also had high specificity to aromatic and hydrophobic amino acids at the P1 position of the substrate.

The keratinase gene from *P. aeruginosa* was successfully expressed in *E. coli* AD494(DE3)pLysS. The recombinant

keratinase behaved as that from wild type and was considered to be a metalloprotease. These data will be helpful for further applications and studies on expression in other systems such as *B. subtilus* and *P. pastoris*.

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