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Expression and Purification of *Pseudomonas aeruginosa* Keratinase in *Bacillus subtilis* DB104 Expression System

Hsin-Hung Lin,[†] Li-Jung Yin,[‡] and Shann-Tzong Jiang*,[†,§]

[†]Department of Food Science, National Taiwan Ocean University, No. 2 Beining Road, Keelung 20224, Taiwan, [‡]Department of Sea Food Science, National Kaohsiung Marine University, No. 142 Hai-Chuan Road, Nan-Tzu, Kaohsiung 81143, Taiwan, and [§]Department of Food and Nutrition, Providence University, No. 200 Chung-Chi Road, Shalu, Taichung 43301, Taiwan

The DNA encoding *Pseudomonas aeruginosa* keratinase was ligated into pRPA expression vector and transformed into *Bacillus subtilis* DB104. Recombinant keratinase (rK), secreted by *B. subtilis* after 72 h of incubation, was purified to electrophoretical homogeneity by nickel affinity chromatography and found to have a molecular mass of 33 kDa. The 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. It was strongly inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, ethylene glycol tetraacetic acid, and ethylene diamine tetraacetic acid but activated by Ca^{2+}, Mg^{2+}, Zn^{2+}, dithiothreitol, glutathione, and β-mercaptoethanol. According to substrate specificity, the rK was considered to be a metalloprotease.

KEYWORDS: *Pseudomonas aeruginosa*; *Bacillus subtilis*; expression; keratinase

INTRODUCTION

Keratin, which exists in skin, nail, feather, and wool, is insoluble and highly resistant to proteolysis by pepsin, trypsin, papain, etc., since it consists of tightly packed polypeptide chains in α-helices and β-sheets and a high degree of disulfide bonds (*1*). Although it is rigid and very stable, feathers do not accumulate in nature. This phenomenon suggests that it is degradable by some specific enzyme secreted from a microorganism such as bacteria, actinomycetes, and fungi (*2*). Bacterial keratinases are, in particular, attracting many scientist's concerns because of their hydrolytic ability on a broad range of protein substrates (*3*) and multiple applications in feed, fertilizer, detergent, leather, and pharmaceutical industries (*2*).

Bacillus subtilis has an excellent secretion ability, displays fast growth, and is a nonpathogenic bacterium free of endotoxin (*4*). It can, therefore, be used in food, enzyme, and pharmaceutical industries and can replace *Escherichia coli* for protein expression (*5*). Furthermore, the extracellular heterogeneous proteins secreted from *B. subtilis* are more convenient for recovery and purification in large-scale production during downstream processing (*4*).

In our previous studies, the rK (recombinant keratinase) from *Pseudomonas aeruginosa*, a pathogenic bacterium (*6*), was well expressed in *E. coli* (*7*). This study aimed to clone and express this keratinase in *B. subtilis* and to further purify and characterize its rK.

MATERIALS AND METHODS

Bacterial Strains and Vectors. *E. coli* Top10F′, purchased from Invitrogen Co. (Carlsbad, CA), was used as a DNA manipulation host. *B. subtilis* DB104, the protease-negative mutant, was kindly provided by Yeh et al. (*4*) and used as an expression host. Plasmid pGEM-T Easy Vector, a product of Promega Co. (Madison, WI), was used as a TA cloning vector. Plasmid pRPA, with ampicillin- and tetracycline-resistant genes, constitutive artificial promoter, and Subtilisin YaB signal peptide, which was a shuttle vector for *E. coli* and *Bacillus*, was provided by Dr. Yeh (*8*) and used for the expression of rK.

Amplification of rK and Molecular Cloning with pGEM-T Vector. Cloning was performed according to the guideline of Sambrook et al. (*9*). The forward primer 1 was designed as 5′-(TCC *CAT ATG* AAG AAG GTT TCT ACG CTT GAC CTG TTG)-3′ [with a *Nde*I (*italic*) cutting site at the 5′-terminal and cloning full sequence of keratinase gene (1497 bp)], while the forward primer 2 was designed as 5′-(ATT *GGA TCC* GCC GAC CTG ATC GAC GTG TCC AAA CTC CCC)-3′ [with a *Bam*HI (*italic*) cutting site at the 5′-terminal, removal of the native signal peptide from full sequence of keratinase gene and cloning pro peptide and mature keratinase gene (1428 bp)]. The reverse primers with and without his-tag were 5′-(TAA *CTC GAG* TTA ATG ATG ATG ATG ATG ATG CAA CGC GCT CGG GCA GGT CAC G)-3′ and 5′-(TAA *CTC GAG* TTA CAA CGC GCT CGG GCA GGT CAC G)-3′, respectively [with a *Xho* I (*italic*) cutting site at the 3′-terminal]. Using these primers, the rK was amplified by polymerase chain reaction (PCR) (2720 Thermal Cycler; Applied Biosystems, Foster, CA) and purified by using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), ligated with pGEM-T easy vector, transformed into *E. coli* Top 10F′, and screened, according to Lin et al. (*7*).

Construction of Expression Vector, Transformation, and Screening. To construct these expression vectors (**Figure 1**), rk DNA was digested by restriction enzyme from pGEMT-keratinase vector and then ligated into pRPA using T4 DNA ligase. After transformation into *E. coli* and identification of positive colonies, the precise plasmids, containing keratinase gene and negative control, and pRPA vector were electrotransformed into *B. subtilis* DB104 by electroporation using a MicroPulser (Bio-Rad, Hercules, CA) at 1.75 KV with a 0.2 cm cuvette (*4*). After 16−24 h of incubation at 37 °C on LB agar containing 12.5 μg/mL tetracycline, the correct transformant was confirmed by colony PCR and used for further expression and purification of rK.

*To whom correspondence should be addressed. Tel: +886-4-26328001 ext. 15375. Fax: +886-943207241. E-mail: stjiang@pu.edu.tw.

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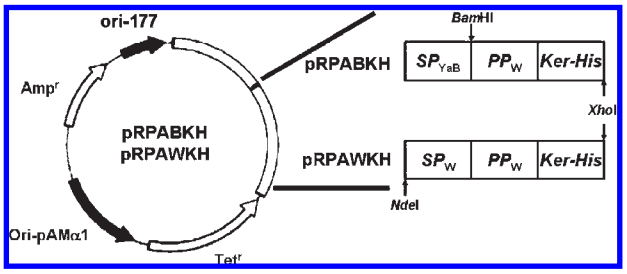

Figure 1. Construction of expression vector and schematic representations of the signal peptide sequences from subtilisin YaB (SP_{YaB}) and wild-type keratinase (SP_W), pro peptide sequences from wild-type keratinase (PP_W), mature keratinase gene (*Ker*), and the six histidine tag gene (*His*).

Expression of rK from *B. subtilis* DB104 Transformant. The transformant, grown on LB agar containing 12.5 μg/mL tetracycline at 37 °C for 12 h, was picked and inoculated into 10 mL of LB broth containing 12.5 μg/mL tetracycline. After 12 h of incubation at 37 °C with constant shaking (180 rpm), 1% (v/v) of the cultures was subinoculated to 500 mL of fresh super-rich broth (2.5% bacto tryptose, 2% yeast extract, and 0.3% K_2HPO_4; pH 7.5) (*10*) at a OD_{600} density of 0.1 for expression and secretion of rK. The cultures were incubated at 37 °C with constant shaking (180 rpm) for 120 h. The optimal expression was monitored by the changes in colony-forming unit (CFU/mL), keratinase activity, and pH of broth. The culture supernatant after 72 h of incubation was collected for further rK purification and characterization.

Purification of rK from *B. subtilis* DB104 Transformant. About 500 mL of culture broth was centrifuged at 5000*g* for 30 min and then filtered through a 0.45 μm membrane (Gelman Sciences, Ann Arbor, MI) to remove bacterial cells. After it was concentrated by an Amicon ultrafiltration (cutoff, 5000 Da) (Amicon Div., W. R. Grace and Co., Beverly, MA) and dialyzed against 25 mM potassium phosphate buffer, pH 7.5 (buffer A), the concentrated enzyme was chromatographed on a nickel affinity column (2.6 cm × 4.7 cm), which was pre-equilibrated with buffer A. After it was washed with the same buffer, the rK was eluted with 4 volumes of buffer A containing 250 mM imidazole and 300 mM NaCl and dialyzed against 20 mM phosphate buffer (pH 7.5) for further characterization.

Assay of Keratinase Activity. The keratinase activity was determined by measuring the azokeratin hydrolysis ability according to Sangali and Brandelli (*11*). Azokeratin was prepared according to 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. The protein concentration was determined by the dye-binding method (*13*). Bovine serum albumin (BSA) was used as a 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 (*14*). After electrophoresis, the gels were stained with Coomassie blue R-250. Ten micrograms of enzyme samples was applied on each well of gels.

N-Terminal Amino Acid Sequence Analysis. Purified rKs were subjected to SDS-PAGE analysis and electrotransferred onto polyvinylidene difluoride (PVDF) membrane, respectively. Proteins were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences of proteins electrotransferred on to PVDF were analyzed by Edman degradation (*15*).

Optimal 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 Tris-HCl buffer; and 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 azokeratin substrate according to Sangali and Brandelli (*11*).

pH and Thermal Stabilities. To determine the pH stability, rK 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 Sangali and Brandelli (*11*).

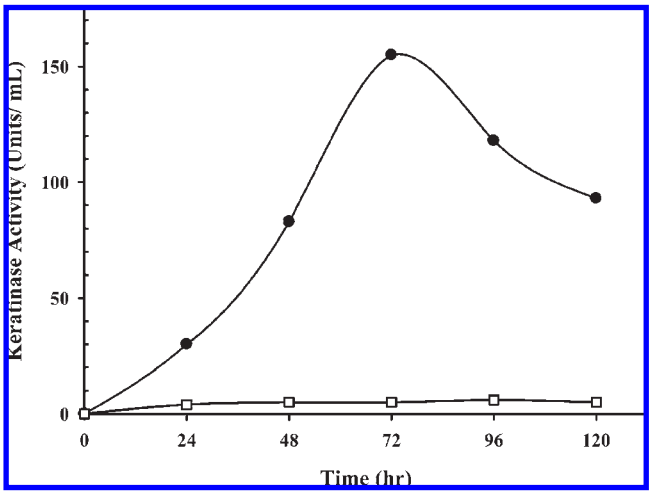

Figure 2. Effect of secretion ability of signal peptides from subtilisin YaB (●) and wild-type keratinase (□) on rK production from *B. subtilis* DB104 transformant during 120 h of incubation at 37 °C.

To determine the thermal stability, the 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 (*11*).

Effects of Metal Ions, Reducing Agents, and Inhibitors. Purified rK 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), ethylene diamine tetraacetic acid (EDTA), glutathione (GSH), N-ethylmaleimide (NEM), β-Me, *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonylfluoride (PMSF), and N-toyl-L-lysine chloromethyl ketone (TLCK) was incubated at 37 °C for 30 min. The residual activity was measured according to Sangali and Brandelli (*11*).

Substrate Specificity. BSA, casein, collagen, elastin, feather, and hemoglobin (Hb) were used as substrates. 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, 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. The absorbance at 507 nm was measured after cooling (*16*). One unit of activity was defined as the amount of enzyme that could cause the release of an equivalent to 1 μmol of tyrosine after 60 min of reaction (*17*).

To 0.1 mM 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. (*18*).

RESULTS AND DISCUSSION

Screening and Expression of rK in *B. subtilis*. Both signal peptides from subtilisin YaB and wild-type keratinase were capable of secreting the rK containing his tag protein in *B. subtilis* expression system (**Figures 1** and **2**). However, as compared with wild-type keratinase of *P. aeruginosa*, a higher production yield of rK by signal peptide from subtilisin YaB was obtained (**Figure 2**). Furthermore, no significant difference in the yield of rK with/without His-tag secreted by signal peptides from subtilisin YaB from *B. subtilis* was observed (data not shown). Therefore, the *B. subtilis* DB104 transformant, containing pRPABKH, was used for further expression and characterization of rK. The keratinase activity, the CFU, and the pH of the broth were measured in the cultural broth for expression of transformant (**Figure 3**). During the period of expression, the maximum rK steadily increased to

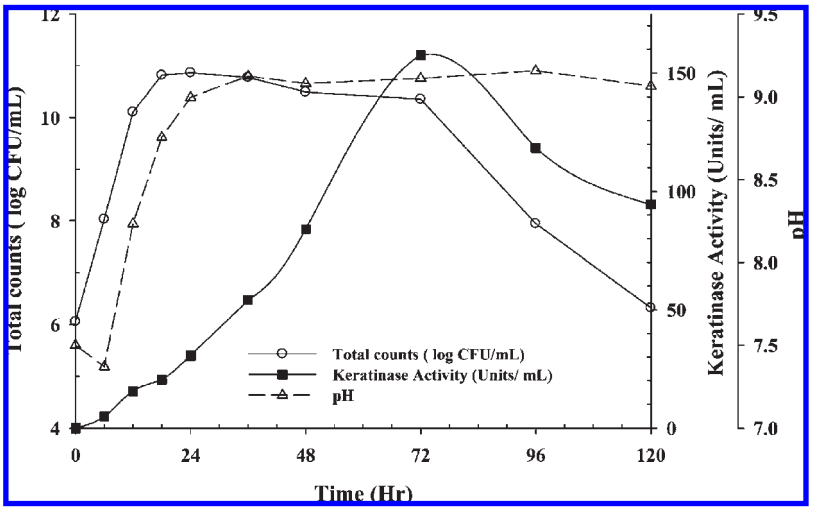

Figure 3. Changes in total count, keratinase activity, and pH of broth during incubation of *B. subtilis* DB104 transformant at 37 °C.

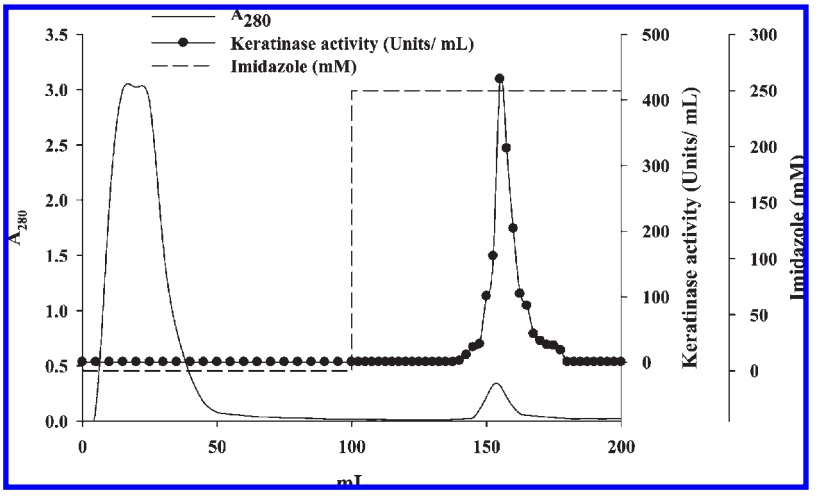

Figure 4. Chromatogram of rK on a nickel affinity column.

157.5 units/mL, while the protein concentration was 1.06 mg/mL (**Figure 3**). The pH of broths decreased during early 6 h of incubation and then increased during elongated incubation at 37 °C. The death phases of transformant were observed after 72 and 120 h of incubation at 37 °C, while the corresponding keratinase activity decreased rapidly.

The data obtained from this study clearly confirmed that *B. subtilis* system could be used for expressing the keratinase from *P. aeruginosa*. As we know, development of an asporogenic strain, integration of keratinase DNA in the chromosome of *B. subtilis*, and modification of promoters were the strategies for rK production in *B. subtilis* (*19*−*21*). An *E. coli*−*B. subtilis* shuttle vector, plasmid pRPA, inserted with high-efficient artificial promoter and *B. subtilis* DB104, a protease negative expression host, were successful in the production of recombinant protein by *B. subtilis* (*4*). According to the data obtained in this study, this transformant could substitute for *P. aeruginosa*, a pathogenic bacterium, and *E. coli*, a non-GRAS strain, to produce rK. Among these two expression systems (pRPABKH and pRPAWKH, **Figure 1**), the signal peptide from subtilisin YaB obtained a higher yield of rK than that from wild-type keratinase. The yield of rK (143.1 unit/mg) obtained from the *B. subtilis* DB104 transformant containing pRPABKH was also higher than those of previous studies by Yin et al. (*6*) and Lin et al. (*7*) using *P. aeruginosa* (41.5 unit/mg) and *E. coli* AD494(DE3)pLysS (12.0 unit/mg) as expression hosts, respectively. During the secretion of mature rK across the cell wall, the signal peptide was digested from the N terminal of pro-peptide. The presence of pro-peptide is necessary for functional expression of recombinant and for protection of this enzyme against autolysis, which was considered as a key for correct folding of the mature keratinase (*7*, *22*). In addition, insertion of his-tag at the C-terminal could protect rK from degradation during expression (*23*) and simplify the purification. As compared with the protein and DNA sequences between *P. aeruginosa* and *Bacillus licheniformis* by Basic Local Alignment Search Tool (BLAST) at GenBank, these sequences of keratinase from *P. aeruginosa* (Gram-negative) were totally different from those from *B. licheniformis* (Gram-positive) in the database of NCBI.

In contrast with Gram-negative *E. coli*, *B. subtilis* was considered as GRAS and more suitable for expression and cloning of recombinant protein (*8*). However, the gradual death of transformant cells and rapid decrease in keratinase activity were observed during incubation. The gradual death of cells might be because of the damage and toxicity of rK to cell membranes (*24*), and the rapid decrease of keratinase activity might be due to the self-keratinolysis (autolysis) and poor stability in alkaline state. To date, only keratinase DNA from *Bacillus licheniformis* was cloned and expressed in a *Bacillus* expression system (*19*, *21*, *25*). Because there are several bottlenecks in the production of recombinant

Table 1. Summary of the Purification of rK from *B. subtilis* DB104 Transformant

procedures	total protein (mg)	total activity (unit)	specific activity (unit/mg)	recovery (%)	purification (fold)
crude enzyme	212.5	30400	143.1	100.0	1.0
ultrafiltration	169.0	27630	163.5	90.9	1.1
nickel affinity column	19.4	19300	994.8	64.0	7.0

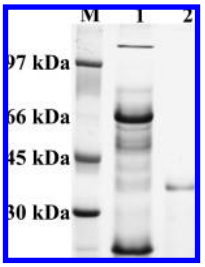

Figure 5. Profile of SDS-PAGE of rK (M, marker; lane 1, crude enzyme from transformant cell; lane 2, purified enzyme obtained after a nickel affinity column).

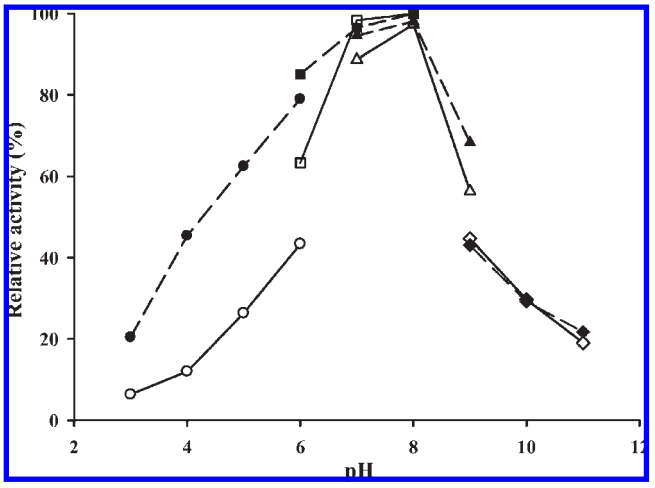

Figure 6. Effect of pH on rKs (– – –, stability; ———, optimal pH; ● and ○, pH 3.0−6.0 in 20 mM citrate buffer; ■ and □, pH 6.0−8.0 in 20 mM sodium phosphate buffer; ▲ and △, pH 7.0−9.0: 20 mM Tris-HCl buffer; and ♦ and ◇, pH 9.0−11.0 in 20 mM sodium carbonate buffer).

proteins from *B. subtilis*, such as protein mal-folding, lack of suitable or stable expression vectors, proteolysis, or poor codon usage (*26*), a previous study indicated that using different protease-deficient *B. subtilis* or vectors containing strong promoters and signal peptides could enhance the recombinant protein yield (*4*). Alternatively, a combination use of nitrogen substrates in cultivation medium such as super-rich broth could also significantly improve the production of recombinant protein from *B. subtilis* (*10*, *20*).

Purification of the rK. A specific activity of 143.1 units/mg was obtained after passing through a 0.45 μm sterilized membrane. After ultrafiltration, rK was purified to electrophoretical homogeneity after being eluted by 250 mM imidazole on nickel affinity chromatography. A specific activity of 994.8 units/mg, 64.0% recovery, and 7.0-fold of purification were achieved at this stage (**Figure 4** and **Table 1**). The molecular mass (*M*) of purified rK without signal peptide and pro-peptide was 33 kDa, estimated by SDS-PAGE (**Figure 5**), which was the same as that of predicted *M*, 33 kDa, and purified mature keratinase from the wild type (33 kDa), estimated by SDS-PAGE (*6*). The N-terminal sequence (AEAGGPGG) of rK was the same with that of the wild type. This study was the first report on being able to constitute the functional keratinase from *P. aeruginosa* in a *B. subtilis* expression system.

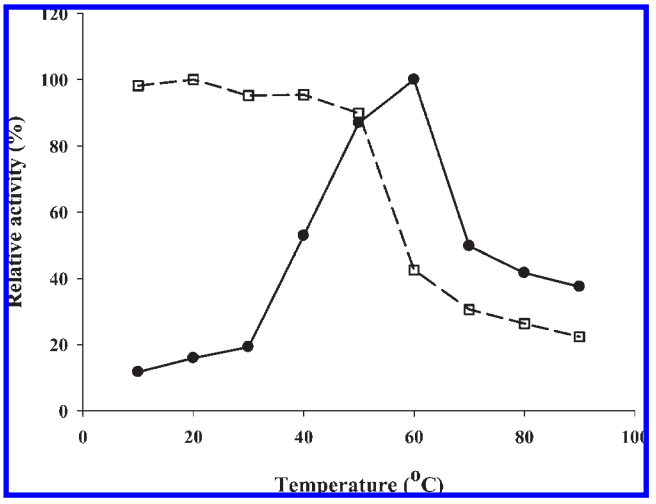

Figure 7. Effect of temperature on rKs (●, optimal; and □, stability).

Table 2. Effects of Metal Ions and Chemicals on rKs

	relative activity (%)		
metals	1 mM	5 mM	10 mM
none[a]		100	
Ag^{+}	105.0	102.5	95.4
K^{+}	102.1	99.2	94.5
Li^{+}	100.9	95.3	93.2
Na^{+}	93.2	94.5	93.4
Ba^{2+}	98.7	94.5	94.0
Ca^{2+}	99.6	103.8	110.7
Cd^{2+}	61.9	55.1	50.4
Co^{2+}	77.9	64.8	61.0
Cu^{2+}	45.3	7.2	3.4
Fe^{2+}	81.3	77.1	22.0
Hg^{2+}	44.1	18.2	9.3
Mg^{2+}	102.5	107.2	110.6
Mn^{2+}	101.7	94.0	75.0
Ni^{2+}	83.3	71.1	50.4
Zn^{2+}	99.2	90.3	72.9
Fe^{3+}	88.1	75.4	25.9
	relative activity (%)		
chemicals	1 mM	2 mM	5 mM
DTT	111.9	115.1	121.4
EGTA	94.4	78.5	56.4
EDTA	88.1	61.9	40.4
GSH	105.6	115.9	123.0
NEM	95.2	92.6	88.9
β-ME	200.1	211.9	226.2
PCMB	92.1	73.0	62.7
PMSF	97.6	94.7	98.4
TLCK	92.9	92.1	96.8

[a] The activity of that without metal ion or chemical added was defined as 100%.

Characterization of the rK. The optimal pH and temperature for rK were 8.0 and 60 °C, respectively. It was stable at pH

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

protein substrate	specific activity (units/mg)
casein	58.4 (100%)[a]
Hb	42.4 (72.6%)
BSA	35.7 (61.2%)
elastin	28.8 (49.2%)
feather	27.6 (47.2%)
collagen	14.9 (25.4%)
synthetic substrates	**specific activity (nmol/min/mg)**
N-succinyl-Ala-Ala-Pro-Phe-*p*NA	61.7
Suc-Ala-Ala-Ala-*p*NA	27.4

[a] Percentage ratios of specific activity of tested substrates related to that of casein.

6.0−9.0 and 10−50 °C (**Figures 6** and **7**). Purified rK was moderately inhibited by Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, NEM, and *p*CMB but markedly inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+}, Fe^{3+}, EGTA, and EDTA. However, it was activated by Ca^{2+}, Mg^{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 61.7 and 27.4 nmol/mg/min, respectively (**Table 3**).

The pH and temperature stabilities of rK from *B. subtilis* transformant were close to those of *E. coli* transformant (pH 6.0−9.0 and 10−60 °C) (*7*), *P. aeruginosa* (pH 5.5−9.0 and 10−50 °C) (*6*), and *Streptomyces albidoflavus* (pH 6.0−9.5 and 40−70 °C) (*27*). In this study, the optimal temperature of rK was similar to those of wild type, *P. aeruginosa* (60 °C) (*6*) and *B. licheniformis* (60 and 52 °C) (*17*, *28*), and transformant, *E. coli* (50 °C) (*7*). In addition, its optimal pH at neutrality was similar to those from other studies (pH 8.5−9.0) (*2*, *6*, *7*, *17*).

Because the rK from G(+) transformant, *B. subtilis* was strongly inhibited by EDTA and EGTA and activated in the presence of divalent metal ions such as Ca^{2+} and Mg^{2+}, it seemed to be a metalloprotease (*12*, *29*). It was similar to those from wild type, *P. aeruginosa* (*6*), and G(−) transformant, *E. coli* (*7*). However, the inhibition of Zn^{2+} corresponded to a previous study (*12*), which indicated that some metallopeptidases were inhibited by excess metals, particularly at pH from neutral to alkaline. Additionally, keratinases from *Vibrio* kr2 (*30*) and *Bacillus* sp. SCB-3 (*31*) also had similar keratinolytic characteristics, including activation by Ca^{2+} and inhibition by a high concentration of Zn^{2+}. Heavy metals, such as Cu^{2+} and Hg^{2+} or Co^{2+} (*31*), could also inhibit keratinase activity. Hence, Hg^{2+} could not only bind the thiol groups but also interact with tryptophan residues or carboxyl group of amino acids (*32*). It was, therefore, also considered to be an inhibitor. The activation of keratinase by reducing agents might be due to the reduction of disulfide bonds, which consequently increased the contact with substrates for keratinolysis (*2*, *12*, *31*).

Keratinase could hydrolyze a broad range of protein substrates including soluble proteins and a few insoluble proteins such as elastin and feather meals (*2*, *17*). This phenomenon was also observed in this study. For the complete degradation of insoluble substrates, the activation of keratinase by increasing metal salts or pretreatments of substrates with physical methods, and addition of reducing agents or detergents were required (*17*). According to the results of synthetic substrates specificity, the purified rK exhibited specificity with aromatic and hydrophobic amino acid residues at the carboxyl side of the splitting point in P1 substrate position (*2*). This phenomenon may play a key point in degradation of feather since keratin molecules contained approximately 50% of aromatic and hydrophobic amino acids (*33*). Therefore, those two representative synthetic substrates were suitable for clearly confirmed the specificity of rK. Briefly, the enzymatic character of wild-type keratinase from *P. aeruginosa* (*6*) was similar to that of rK in this study.

In summary, keratinase gene from *P. aeruginosa* was successfully expressed and secreted by the *B. subtilis* expression system. This rK behaved as a native bacterial keratinase. Improvement of rK yield is worthy of further study for enhancing the production by this expression system or by other expression systems such as *Pichia pastoris*. The domain structure for rK is also another topic for further studying the pH and thermal stabilities and functional amino acid residues.

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