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Isolation and characterization of a feather-degrading enzyme from *Bacillus pseudofirmus* FA30-01

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Abstract We isolated the feather-degrading *Bacillus pseudofirmus* FA30-01 from the soil sample of poultry farm. The isolate completely degraded feather pieces after liquid culture at 30°C (pH 10.5) for 3 days. Strain FA30-01 is a Gram-positive, spore-forming, rod-shaped bacterium and was identified with *B. pseudofirmus* based on 16S rDNA analysis. The keratinase enzyme produced by strain FA30-01 was refined using ammonium sulfate precipitation, negative-ion DEAE Toyopearl exchange chromatography, and hydroxyapatite chromatography. The refinement level was 14.5-fold. The molecular weight of this enzyme was 27.5 kDa and it had an isoelectric point of 5.9. The enzyme exhibited activity at pH 5.1–11.5 and 30–80°C with azokeratin as a substrate, although the optimum pH and temperature for keratinase activity were pH 8.8–10.3 and 60°C, respectively. This enzyme is one of the serine-type proteases. Subtilisin ALP I and this enzyme had 90% homology in the N-terminal amino acid sequence. Since this enzyme differed from ALP I in molecular weight, heat resistance and isoelectric point, they are suggested to be different enzymes.

Keywords Extremophiles · Alkaliphile · Keratinase · Feather-degrading enzyme · *Bacillus*

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Introduction

Some 50,000 tons of feathers are generated annually as a by-product in the domestic poultry industry in Japan. There have been few trials on the use of waste feathers from the poultry-raising sector, and currently most such feathers are simply incinerated. Feathers are composed mainly of almost pure keratins, which are not easily degradable by common proteases. Because keratins contain many useful amino acids, processing by keratinases from microorganisms could be an efficient, environment-friendly method of feather degradation.

Among microorganisms reported to have the ability to degrade feathers are some *Bacillus* sp. (Williams et al. 1990; Atalo and Gashe 1993; Zaghloul et al. 1998; Lin et al. 1999; Suntornsuk and Suntornsuk 2003; Werlang and Brandelli 2005), *Chrysosporium* sp. (El-Naghy et al. 1998), *Flavobacterium* sp. (Riffel and Brandelli 2002), *Streptomyces* sp. (Böckle et al. 1995; Letourneau et al. 1998; Bressollier et al. 1999; Chitte et al. 1999), *Vibrio* sp. (Sangali and Brandelli 2000), *Xanthomonas* sp. (De Toni et al. 2002), *Fervidobacterium* sp. (Friedrich and Antranikian 1996; Nam et al. 2002), *Thermoanaerobacter* sp. (Riessen and Antranikian 2001), etc. There have been numerous reports on the feather-degrading activity of *Bacillus* species, for example, *Bacillus licheniformis* PWD-1 (Williams et al. 1990), *Bacillus* sp. strain P-001A (Atalo and Gashe 1993), *Bacillus subtilis* strain DB100 (Zaghloul et al. 1998), *Bacillus* sp. strain FK46 (Suntornsuk and Suntornsuk 2003), and others, at temperatures of 37–55°C and at pH values of 7.0–9.0.

We attempted to isolate feather-degrading alkaliphilic *Bacillus* sp. cultured under alkaliphilic and thermophilic conditions. Four alkaliphilic *Bacillus* sp. able to degrade feathers were isolated. Among them, strain FA30-01 showed the highest level of keratinase activity. This paper describes the screening of alkaliphilic bacteria and the purification and characterization of the feather-degrading enzyme of *Bacillus pseudofirmus* FA30-01.

Material and methods

Culture media, culture conditions, and isolation of bacteria

Soil samples were collected from several commercial poultry farms. Each sample was suspended in sterilized water and inoculated into liquid modified Horikoshi medium containing (in grams per liter, pH 10.5): soluble starch, 5.0; glucose, 5.0; polypeptone (Nihon Pharmaceutical Co., Ltd, Japan), 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2CO_3, 10; and feathers. Feathers were washed with tap water, cut into sections of approximately 5 mm^2, and sterilized by autoclaving at 121°C, for 15 min.

Cultures were grown in the liquid feather medium with shaking for 1 week (30°C, 160 rev/min^{-1}). Feather degradation in the liquid medium inside was confirmed visually. The culture broth in which feather degradation was confirmed was spread on modified Horikoshi agar medium containing feather meal 10 g/l.

Colonies that formed halos were subjected to single-colony isolation. The colonies isolated were then reinoculated into liquid feather medium. The single colonies in which the degradation of feathers was confirmed were used to determine the bacteria responsible for feather degradation.

Characterization of the isolates

Biochemical and morphological characterizations and identification of the bacterium were performed by measuring API50CHB. 16S rDNA analysis was performed using the extracted DNA of strain FA30-01 as the template and PCR amplification of the 16S rDNA gene portion was carried out. The sequence reaction was performed using DYEnamic ET terminator cycle sequencing Kit (Amersham Biosciences Co., Tokyo, Japan). The 16S rDNA sequence of FA30-01 was determined with ABI PRISM(R) 377 DNA Sequencer (Applied Biosystems Japan Ltd, Tokyo, Japan).

Cultivation condition for large-scale culture

The optimal culture conditions for enzyme production were investigated. The optimal concentration of the carbon source (glucose or soluble starch) in modified Horikoshi culture medium used for screening was examined. The concentration of carbon source and polypeptone was changed from 0.5 to 5%, respectively, and the yeast extract concentration from 0.1 to 2.0%. Then the enzyme activity of the culture supernatant was measured and the optimal culture medium conditions for the enzyme production were determined.

Enzyme purification

To refine the enzyme, large-scale culture was carried out using the optimal medium at 30°C for 24 h and cells were harvested by centrifugation (7,000×*g*, 4°C, 15 min). The composition of the optimal medium was the modified Horikoshi medium containing the soluble starch 10 g/l. The addition of 80% ammonium sulfate precipitated the enzyme in this culture broth. The resulting precipitate was collected by centrifugation (7,000×*g*, 4°C, 30 min) and dissolved in a minimal volume of Tris–HCl buffer 10 mM (pH 9.0) and dialyzed overnight against the same buffer. The enzyme solution was passed through a DEAE-Toyopearl 650s column (Tosho Co., Tokyo, Japan) equilibrated with Tris–HCl buffer 10 mM (pH 9.0) at 4°C. After washing with the same buffer, adsorbed proteins were eluted with a linear gradient of NaCl 0–0.5 M. We determined the NaCl concentration in each fraction on DEAE-Toyopearl column using a NaCl densitometer (Atago Co., Ltd, Tokyo, Japan). The enzyme activity was assayed with azokeratin as the substrate. The majority of enzyme activity was found in the flow-through. The fractions with high enzyme revitalization were subjected to hydroxyapatite chromatography (Seikagaku Co., Tokyo, Japan) with liquid phosphoric acid buffer 10–100 mM (pH 7.6) containing NaCl 0.1 M. Protein concentrations were measured photometrically at 280 nm with Bradford dye reagent (Bradford 1976). Bovine serum albumin was used as the standard protein.

Determination of enzyme activity

Keratinase activity was measured using azokeratin as a substrate (Tomarelli et al. 1949; Lin et al. 1992). The enzyme preparation (0.2 ml) was incubated with azokeratin 5 mg in 0.8 ml of glycine–NaCl–NaOH buffer 50 mM (pH 10.5) for 15 min at 50°C with constant agitation at 1,200/min using a Bio Shaker (Taitec Co., Saitama, Japan). A 10% TCA solution was added, and the reaction was stopped. One unit of keratinase activity was represented by an increase in absorbance at 450 nm (A_{450}) of 0.01 after 15 min in the test reaction compared with the control reaction. Azokeratin staining at different pH values and substrate temperatures was examined, 0.2 ml of buffer (Tris–HCl 10 mM, pH 9.0) was added to the change of enzyme, and the A_{450} value was measured. The heat reaction was determined similarly. The actual A_{450} values were used and uncorrected. The reaction buffer, the addition of 10% TCA solution, was used as the control.

Protease activity was assayed using the modified method of Anson (Anson 1938; Takami et al. 1990). The reaction mixture contained 0.5 ml of enzyme preparation and 2.5 ml of glycine–NaCl–NaOH buffer 50 mM, pH 10.5 with Hammerstan casein 6 g/l (Merck Ltd, Tokyo, Japan). After incubation for 20 min at 50°C, the reaction was stopped by the addition of TCA solution

(CH_3COONa 0.22 M, CH_3COOH 0.33 M, TCA 0.11 M). Diluted phenol reagent was added to the filtrate, and A_{660} was measured after 30 min. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tryrosine in 1 min at 50°C.

NH_2-terminal amino acid sequence of keratinase

After electrophoresis, the purified enzyme was transferred in Immobilon-p transfer membrane (Nihon Millipore Co., Tokyo, Japan) using the electoroblotting method. The N-terminal amino acid sequence of the purified keratinase was determined with a protein sequencer (Model 476A, Applied Biosystems, Foster City, CA, USA).

Effects of pH and temperature on the enzyme activity

The effects of pH and temperature on enzyme activity were measured. Keratinase activity was measured in the pH range from 3.0 to 13.0 using the following buffers: citric acid–sodium citrate 50 mM (pH 3.0–4.0), acetic acid–sodium acetate 50 mM (pH 5.0–6.0), 3-(*n*-morpholino) propanesulphonic acid (MOPS)–sodium MOPS 50 mM (pH 7.0–8.0), glycine–NaCl–NaOH 50 mM (pH 9.0–10.5), and KCl–NaCl 50 mM (pH 11–13). The optimum temperature for keratinase activity was determined by varying the incubation temperature between 30°C and 80°C. The actual pH value at each temperature was measured. To investigate changes in heat stability upon Ca^{2+} addition, Ca^{2+} (0, 5 mM) was added and the residual activity after 1 h was measured after heating at 40°C to 90°C. In addition the residual protease activity was detected by a method of Tsuchida (Tsuchida et al. 1986).

Effects of proteinase inhibitors, metal ions, and reducing agents on keratinase activity

To investigate the effects of different inhibitors, metal ions, and reducing agents on keratinase activity, the residual activity after allowing the enzyme to stand with the reagent for 30 min at room temperature was measured. The following proteinase inhibitors were added to the enzyme: pepstatin A (3 μg/ml), EDTA (1 mM), phenylmethylsulfonyl fluoride (PMSF) (1 mM), dithiothreitol (DTT) (1 mM), tosyl-L-alanylchloromethylketone (TPCK) (0.1 mM), β-mercaptoethanol (1.0%), Triton-X100 (0.6%), SDS (0.5%), and Ca^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Hg^{2+}, Fe^{2+} (1 mM each).

Zymogram with feather meal

SDS-PAGE was performed in 12% gel containing 1.0% feather meal (Riessen and Antranikian 2001). The gel was washed with 0.25% Triton-X for 30 min, stained with CBB for 30 min, and then decolorized. The band of protein exhibiting keratinase activity appeared on the gel as a clear band.

Results

Isolation and characterization of *B. pseudofirmus* FA30-01

We attempted to isolate feather-degrading bacteria from about 40 soil samples. Four of the bacteria isolated from soil from poultry farms were to degrade chicken feathers. Among the four, strain FA30-01 showed stable ability to degrade chicken feathers. The crude enzyme of FA30-01 (0.1 mg/ml) degraded feathers in the buffer solution (glycine–NaCl–NaOH 50 mM, pH 10.5) at temperatures of 30–60°C conditions after 24 h incubation. The enzyme activities of three other strains were unstable. Therefore, the strain of FA30-01 was selected for further study.

Strain FA30-01 is a Gram-positive, spore-forming, rod-shaped bacterium. This strain hydrolyzed casein, gelatin, starch, and Tween 60; exhibited no ability to reduce nitric acid; and grew at 10°C. In addition, analysis of 16S rDNA of this strain showed a high sequence identity to *B. pseudofirmus* of 98%. Therefore, on the basis of these biochemical, physiological and 16S rDNA analyses, the isolate was identified as *B. pseudofirmus* FA30-01.

Optimal medium for enzyme production

The results of the investigation of the optimal culture medium for enzyme production are determined (data not shown). High enzyme production was seen at soluble starch concentrations of 0.5 and 1.0%. However, enzyme production was not affected by the glucose concentration even when excess amounts were added. Based on these results, the soluble starch concentration of 1.0% in the culture medium was determined to be the optimum for enzyme production.

The results of large-scale culture in 5 l flasks using the optimal culture medium conditions are shown in Fig. 1. Strain FA30-01 produced the keratinase enzyme after it entered the log phase of growth. Moreover, the enzyme production was maintained even after entering the stationary phase. Although the pH of the culture medium decreased at the beginning of cultivation, it increased again after 24 h.

Purification of keratinase

The results on the purification of the keratinase from FA30-01 are summarized in Table 1. The feather-degrading enzyme was purified using the ammonium

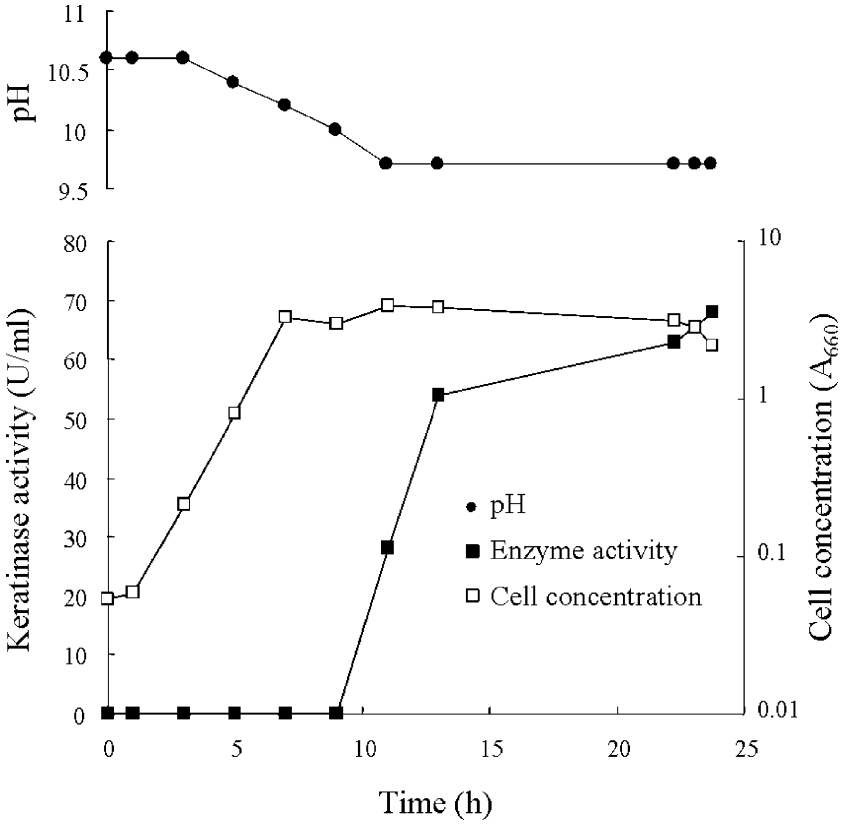

Fig. 1 Time course of growth and keratinase production by FA30-01 in a 5-l fermenter. Culture solution was extracted every 2 h and the culture medium pH, cell concentration (A_{660}), and enzyme activity were measured. Culture conditions were: temperature 30°C, aeration rate 0.5 v/min, agitator speed 250 rpm, and liquid volume 3 l

sulfate precipitation method and DEAE-Toyopearl 650s and hydroxyapatite column chromatography. The keratinase was purified 14.5-fold, with a yield of 7.3%. The purified enzyme had a molecular mass of 27.5 kDa and the isoelectric point was pI 5.9. On the gel with powdered feathers, a clear band was noted near 27 kDa, and it was thus confirmed that the purified enzyme was a feather-degrading enzyme (Fig. 2).

NH_2-terminal amino acid sequence

About 20 amino acids were identified in the analysis of the NH_2-terminal amino acid arrangement. This arrangement had high homology (90%) with that of subtilisin ALP I. When alignment was performed with the enzyme ALP I, keratinases (*kerA* protease, heat-resistant alkalinity protease AH-101) and alkaline proteases (subtilisin Carlsberg, M-protease, and subtilisin BPN′) (Fig. 3.) were the same as reported previously (Wells et al. 1983; Jacobs et al. 1985; Tsuchida et al. 1986; Takami et al. 1992; Hakamada et al. 1994; Lin et al. 1995; Yamada et al. 1995). Both alkaline protease and alkaline keratinase were compared, and the homology of the NH_2-terminal amino acid arrangement was high.

Influence of pH and temperature on enzyme activity

The optimum pH and temperature for the keratinase activity of the purified enzyme were determined (Fig. 4). The reaction pH was indicated by the actual pH value. The enzyme exhibited keratinase activity in the range from pH 6 to 11.5 and 30–80°C. The optimum pH range and temperature were pH 8.9–10.3 and 60°C, respectively. High keratinase activities were also observed at 70 and 80°C. A tendency for the optimal pH to shift to neutral values was noticed as the reaction temperature increased. At a temperature of 40°C, optimal activity

Table 1 Purification of the feather-degrading enzyme produced by strain FA30-01

Purification step	Protein (mg)	Total activity (U[a])	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	185	57,500	310.8	100	1
Ammonium sulfate precipitation	108	55,680	515.6	96.8	1.7
DEAE-Toyopearl	1.75	5,730	3274.3	10.0	10.5
Hydroxyapataite	0.93	4,200	4516.1	7.3	14.5

[a]One unit is defined as an increase in the A_{450} of 0.01 after reaction with azokeratin for 15 min, at 50°C and pH 10

generally occurred at alkaline pH 11.5. The effect of Ca^{2+} addition on heat stability of the enzyme was determined. It hardly required Ca^{2+} for heat stability. This enzyme maintained residual activity of about 20% after incubation for 1 h at 80°C (data not shown).

Effects of proteinase inhibitors, metal ions, and reducing agents on keratinase activity

The enzyme was completely inhibited by the serine protease inhibitor PMSF. It was not inhibited by the same serine protease inhibitor in TPCK (0.1 mM). In EDTA, E-64, and pepstatin A, little inhibition of enzyme activity was seen. When Hg^{2+} and Fe^{2+} were added, the enzyme activity was completely inhibited. However, the keratinase activity was slightly enhanced by the addition of Mg^{2+}, Co^{2+}, and Zn^{2+} 1 mM (Table 2).

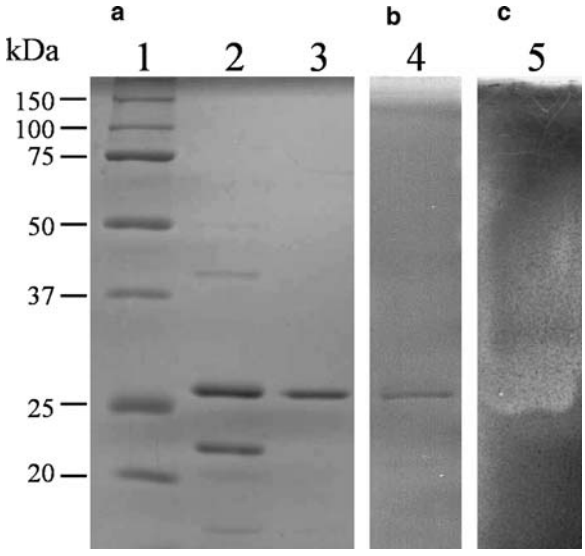

Fig. 2 SDS-PAGE of keratinase obtained from strain FA30-01. **a** SDS-PAGE. *Lane 1* molecular mass standards (Precision Plus Protein standards, dual color; Bio-Rad Laboratories, Inc. Tokyo, Japan); *2* crude enzyme; *3* purified enzyme. **b** SDS-PAGE with silver staining. *Lane 4* purified enzyme. **c** SDS-PAGE containing feather meal. *Lane 5* crude enzyme

Comparison of keratinase and protease activities with those of commercial proteases

To compare various commercially available proteases [Subtilisine Carlsberg (Sigma-Aldrich Co., Tokyo, Japan), Pronase (Wako Pure Chemical Industries, Ltd, Osaka, Japan), Proteinase K (Nacalai Tesque, Inc., Kyoto, Japan), trypsin 250 (BD Difco Co., Tokyo, Japan), papain (Nacalai Tesque, Inc., Kyoto, Japan)] and the enzyme from FA30-01, their protease activity and keratinase activity were measured using the methods described above. Both enzyme reactions were performed at 50°C and pH 10.5. Each specific activity was measured and the ratio of keratinase activity/protease activity compared. The ratio of keratinase/protease activity of the enzyme from FA30-01 was 1.58, and since all the commercially available proteases had a ratio of ≤ 1, the strain had high feather keratin-degrading activity.

Discussion

Previously reported aerobic bacteria with feather-degrading ability were grown at alkaline pH, such as *Streptomyces thermoviolaceus* (Chitte et al. 1999) and *Bacillus* sp. P-001A (Atalo and Gashe 1993), which both grew at pH 8 and were isolated from an alkaline lake or alkaline hotsprings, respectively. We therefore attempted to find alkaliphilic feather-degrading bacteria that could grow at pH 10.5 to isolate feather-

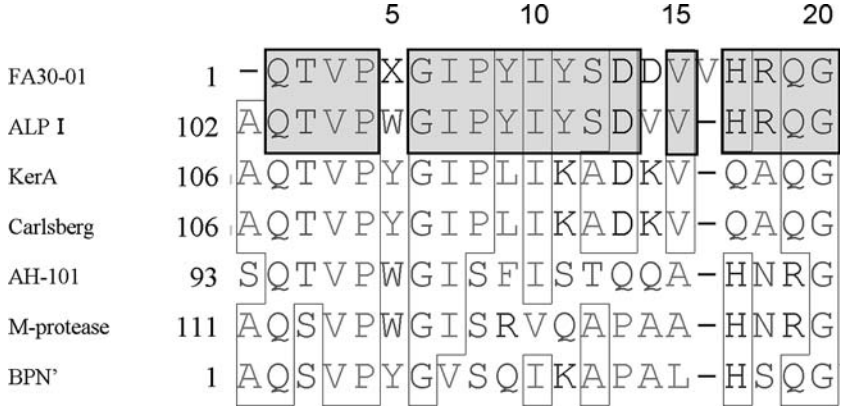

Fig. 3 Alignment of the N-terminal sequence of keratinase of FA30-01 (FA30-01), subtilisin ALP I (ALP I), *kerA* protease (KerA), subtilisin Carlsberg (Carlsberg), thermostable alkaline protease (AH-101), alkaline protease (M-protease), and subtilisin BPN′ (BPN′). The data for ALP I (Tsuchida et al. 1986; Yamada et al. 1995), kerA (Lin et al. 1995), Carlsberg (Jacobs et al. 1985), AH-101 (Takami et al. 1992), M-protease (Hakamada et al. 1994), and BPN′ (Wells et al. 1983) were obtained from the literature

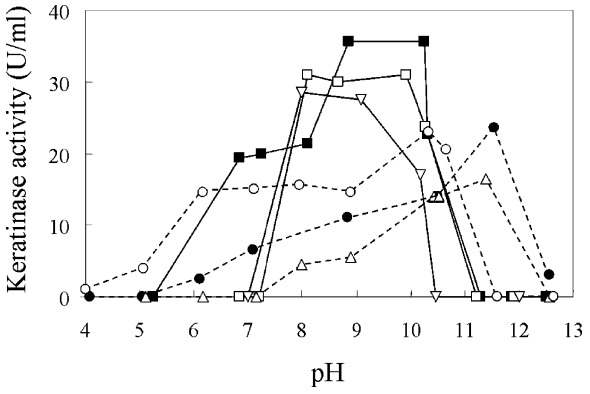

Fig. 4 Effects of pH and temperature on keratinase activity with azokeratin as a substrate. *Open tirangles* 30°C, *filled circles* 40°C, *open circles* 50°C, *filled squares* 60°C, *open squares* 70°C, *open inverted triangles* 80°C. Keratinase activity was measured at pH 4–13. Activity was measured as described in Materials and methods

degrading enzymes exhibiting optimum activity at higher pH values.

Strain FA30-01 was isolated from the soil of a poultry farm. We inoculated the soil in liquid culture medium to which feather pieces had been added. A previous study (Lin et al. 1999) investigated whether feather degradation occurred after adding the enzyme of the isolate to milk agar plates. Feather-degrading bacteria were efficiently isolated from liquid culture medium. We used the same method to isolate feather-degrading bacteria efficiently from 40 soil samples.

The feather-degrading enzyme from *Bacillus* sp. P-001A, which exhibited activity at 55°C and pH 9.5, was previously reported (Atalo and Gashe 1993). The keratinase from *Bacillus* sp. AH-101 was reported to show activity at highly alkaline pH 11.0, although it was not reported to degrade feather keratin (Takami et al. 1990). The enzyme from FA30-1 exhibited the optimum activity at pH 8.8–10.3 and 60°C. Moreover this enzyme also maintained residual activity (about 20%) after incubation at 80°C for 1 h and thus had thermostability.

Since the activity of the enzyme from FA30-01 was completely inhibited by 1 mM PMSF, it is a serine protease. However, it was not inhibited in TPCK, which is the same serine protease inhibitor. This was also noted in the enzyme from *Streptomyces albidoflavus* (Bressollier et al. 1995). However, the isoelectric point of this enzyme was 5.9, which differed from the point of typical serine alkaline proteases. Their isoelectric point is around pH 9 (Rao et al. 1998). It may therefore be possible that it represents a new type of enzyme.

Although the enzyme from FA30-01 degraded feathers, which are composed mainly of β-keratin, it degraded neither wool nor hair, which is mainly composed of α-keratin. Enzymes that degraded both feathers and wool were reported from *Thermoanaerobacter keratinophilus* (Riessen and Antranikian 2001), *Bacillus* sp. P-001A (Atalo and Gashe 1993), etc. Enzymes from *Thermoactinomyces candidus* (Ignatova et al. 1999) and *B. subtilis* II QDB32 (Varela et al. 1997) degraded wool but not feathers. A comparison of enzymes that can degrade only β-keratin with those that can degrade both types of keratin might shed light on the mechanism involved in feather degradation.

The NH_2-terminal amino acid arrangement of the enzyme from FA30-01 had high homology with the alkali protease and keratinase produced by *Bacillus* sp. (Wells et al. 1983; Jacobs et al. 1985; Tsuchida et al. 1986; Takami et al. 1992; Lin et al. 1995; Yamada et al. 1995; Hakamada et al. 1994). However, based on the report of Bressollier et al. (1999), there was little homology in the NH_2-terminal amino acid arrangement between our enzyme and the serine protease produced by *S. albidoflavus*. Such arrangements are thought to be specific to the *Bacillus* sp. that produces alkali proteases.

Subtilisin ALP I, which has high homology of its NH_2-terminal amino acid sequences with the enzyme from FA30-01, shares other common features, such as activity toward serine protease and activity under alkaline conditions. The ALP I had a molecular weight of 31 kDa by SDS-PAGE and isoelectric point of 8.2

Table 2 Effects of detergents, reducing agents, inhibitors, and metal ions on the keratinase activity of purified FA30-01 keratinase

Group	Compound	Concentration (mM)	Residual keratinase activity (%)
Control without additives			100
Detergent	SDS	0.5 (%)	79
	Triton X-100	0.6 (%)	110.5
Reducing agent	DTT	1	0
	β-Mercaptoethanol	1.0 (%)	0
Inhibitor	PMSF	1	0
	EDTA	1	85.2
	Pepstatin A	3 (mg/ml)	87
	TPCK	0.1	108
Metal ion	Ca^{2+}	1	74
	Mg^{2+}	1	113.5
	Zn^{2+}	1	113.5
	Co^{2+}	1	121.6
	Hg^{2+}	1	0
	Fe^{2+}	1	0

(Tsuchida et al. 1986). The isoelectric point and molecular weight of the enzyme from FA30-01 also differed from that of the ALP I enzyme. The enzyme from FA30-01 maintained its feather-degrading activity even after heating at 60°C for 10 min, although it was reported that ALP I no longer exhibited activity at pH 10 after heating at 50°C for 10 min. Thus, the reaction of the two enzymes to heat appears to differ. To the best of our knowledge, there has been no report that ALP I can degrade feathers. It would be useful in future studies to compare the amino acid sequence and gene arrangement of the ALP I enzyme and those of FA30-01 to determine the mechanisms of feather degradation and thermostability.

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