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Characterization of a Keratinolytic Metalloprotease from *Bacillus* sp. SCB-3

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Abstract

A keratinolytic protease–producing microorganism was isolated from soybean paste waste and was identified as a strain of *Bacillus* sp. The keratinase was purified by polyethylene glycol precipitation and two successive column chromatographies with DEAE-Toyopearl 650C and Sephacryl S-200 HR. The purified enzyme had overall 11 purification folds with an 18% yield. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration on Sephacryl G-200 indicated that the purified enzyme was monomeric and had a molecular weight of 134 kDa. The optimum temperature and pH were 40°C and 7.0, respectively. This enzyme was completely inhibited by EDTA and EGTA, and it was restored by the addition of Ca^{+2} and Mg^{+2}. These results suggested that it is a metalloprotease. The stimulated enzyme activity by reducing agents indicated that the reducing condition was important in the expression of the activity.

Index Entries: *Bacillus* sp.; metalloprotease; keratinolytic activity.

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Introduction

Feathers represent 5–7% of the body wt of the domestic fowl. Poultry feather accumulates as a waste after processing the chickens for human consumption; thus, the waste carries potent polluting implications, especially with burgeoning global poultry production. Feather wastes, generated in large quantities as a byproduct of commercial poultry processing, are made up of nearly pure keratin protein *(1)*. Keratin in the native state is not degradable by common proteolytic enzymes such as trypsin, pepsin, or papain. However, keratin does not accumulate in nature, and keratinolytic activities have been reported for species of saprophytic and parasitic fungi *(2,3)*, *Streptomyces (4)*. Molyneaux *(5)* reported the digestion of wool keratin by a *Bacillus* species.

Currently, feather wastes are utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Prior to use, the feather is steam pressure cooked or chemically treated to make it more digestible. These treatment processes require tremendous energy and also destroy certain amino acids *(6,7)*. The degradation by microorganisms with a keratinolytic activity represents an alternative method to improve the nutritional value of feather waste. A number of keratinolytic microorganisms have been reported to degrade feather *(8–13)*. Williams et al. *(10)* and Williams and Shih *(11)* isolated a feather-degrading bacterium, *Bacillus licheniformis* PWD-1, from a poultry waste digester. They subsequently used the PWD-1 strain to convert feathers into a digestible protein, feather-lysate *(12)*. When cell-free extract or a crude preparation of keratinolytic protease of *B. licheniformis* PWD-1 was used as an additive to chicken feed with ground feather, a positive growth response was observed *(13)*. These results suggested that feather-degrading microorganisms and their keratinolytic enzymes could be used to enhance the digestibility of feather keratin.

Although a bacterial keratinolytic protease shows a potential to be utilized in the application of feather bioconversion, enzyme activities and yields must be improved in order to make it possible for industrial applications. The present study presents the purification and characterization of the keratinolytic enzyme secreted by feather-degrading *Bacillus* sp. SCB-3.

Materials and Methods

Materials

Sephacryl S-200 HR was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), EDTA, EGTA, 1,10-phenanthroline, bovine serum albumin (BSA), and polyethylene glycol (PEG) were obtained from Sigma (St. Louis, MO). DEAE-Toyopearl was purchased from Tosohass (Montgomeryville, PA). All other reagents were of analytical grade.

Preparation of Crude Enzyme

From soybean paste waste, 23 µg were isolated. One strain was shown to provide a high keratinolytic activity in isolated microorganisms. It was identified as rod, aerobic, Gram positive, and endospore forming. In addition, it grew well at an NaCl concentration up to 7%. Since the preliminary morphologic and biochemical characteristics of this bacterium coincided with those of *Bacillus* species, we designated it *Bacillus* sp. SCB-3 *(14)*.

The feather medium used for isolation, maintenance, and growth of the feather-degrading microorganism contained 0.5 g/L of NH_4Cl, 0.5 g/L of NaCl, 0.3 g/L of K_2HPO_4, 0.4 g/L of KH_2PO_4, 0.1 g/L of $MgCl{\cdot}6H_2O$, 0.1 g/L of yeast extract, and 10 g/L of hammer-milled chicken feathers. The pH was adjusted to 7.5. Feathers were washed, dried, and hammer milled prior to being added to the medium. The medium was sterilized by autoclaving. After 3 d of incubation at 30°C, the culture was centrifuged at 10,000*g* for 20 min. The supernatant was used as the crude enzyme.

Assay of Keratinolytic Protease Activity

Azokeratin was prepared by reacting ball-milled feather powder with sulfanilic acid and $NaNO_2$ by using a method similar to that described by Tomarelli et al. *(15)* for azoalbumin. For a standard assay, 5 mg of azokeratin was added to a 1.5-mL centrifuge tube along with 0.8 mL of 50 m*M* potassium phosphate buffer (pH 7.5). This mixture was agitated until the azokeratin was completely suspended. A 0.2-mL aliquot of an appropriately diluted enzyme solution was mixed with azokeratin, and the mixture was incubated for 1 h in a 30°C water bath. The reaction was stopped by adding 0.2 mL of 10% trichloroacetic acid (TCA), and the mixture was filtered. The A_{450} of the filtrate was measured with a UV-160 spectrophotometer (Shimadzu, Tokyo, Japan). A control was prepared by adding TCA to a reaction mixture before adding the enzyme solution. One unit of keratinolytic activity was defined as an increase in the A_{450} of 0.01 after 1 h in the test reaction. Protein concentration was measured by the absorbance at 280 nm or by following the method of Bradford *(16)* in using BSA as a standard.

Purification of Keratinolytic Protease

The culture medium was centrifuged at 3000*g* for 20 min, and the supernatant was used as crude enzyme. PEG was added to the crude enzyme to 30% saturation. The resulting precipitate was removed by centrifugation (10,000*g* for 20 min), and the supernatant was collected for further purification. The supernatant was dialyzed against 20 m*M* Tris-HCl (pH 7.0). The dialysate was loaded onto a column of DEAE-Toyopearl 650C (2.5 × 28 cm) equilibrated with 20 m*M* Tris-HCl buffer (pH 7.0). After being washed with 20 m*M* Tris-HCl buffer (pH 7.0), the column was eluted with a gradient of 0–500 m*M* NaCl in the same buffer. The active fractions were combined and then concentrated in an ultrafiltration cell (Amicon, Beverly,

MA) using an ultrafilter (Diaflo YM 10) that removes substances smaller than about 10 kDa. The obtained concentrates were chromatographed on a Sephacryl S-200 HR column (1.8×50 cm) equilibrated with 20 m*M* Tris-HCl buffer (pH 7.0), and the column was eluted at a flow rate of 6 mL/h. The active fractions were collected and then concentrated to 2 mL. The purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted by the method of Laemmli *(17)* using a 10% polyacrylamide gel. Gel was stained with Coomassie Brilliant Blue R250.

Effects of Protease Inhibitors and Metal Ions on Keratinolytic Activity

To study the effects of protease inhibitors and metal ions on enzyme activity, the following inhibitors were added to the enzyme solution: EDTA, EGTA, 1,10-phenanthroline, PMSF, leupeptin, pepstatin A, and iodoacetamide. The metal ions investigated were $ZnCl_2$, $CuSO_4$, $CoCl_2$, $CaCl_2$, $HgCl_2$, $MgSO_4$, and $FeCl_2$. Azokeratin was added after incubating the enzyme solution with inhibitor at room temperature for 30 min, and the enzyme activity was measured. To reactivate EDTA-inhibited enzyme, after the purified enzyme was incubated with 0.1 m*M* EDTA in 50 m*M* potassium phosphate buffer (pH 7.5) at 30°C for 30 min, the enzyme solution was dialyzed twice against the buffer to remove the EDTA. The restored activity was measured after incubation of the enzyme with metal ion at 30°C for 30 min.

Effects of Reducing Agents on Keratinolytic Activity

Reducing agents such as L-cysteine, reduced glutathione, 2-mercaptoethanol, dithiothreitol (DTT), and sodium sulfite were used. These compounds were added to the incubation mixture to reach the final concentrations of 0.1, 0.5, 1.0, and 5.0 m*M*. Activities of keratinolytic proteases were always measured against a blank containing the same concentration of reducing agents. All data presented are the average of triplicate experiments.

Assay of Protease Activity with Insoluble and Soluble Substrates

Proteolytic activities were also determined with the following substrates: feather, casein, elastin, collagen, keratin, and BSA (all from Sigma). Purified keratinolytic protease (10 µg) was incubated with 0.5% (w/v) substrate in 50 m*M* potassium phosphate buffer (pH 7.5), and the final volume was adjusted to 1 mL. Assays were carried out at 30°C with constant agitation at 900 rpm for 20–120 min. The reaction was stopped by adding 0.2 mL of 10% TCA. After centrifugation at 4°C and 10,000*g* for 10 min, 0.5 mL of each reaction mixture was added to 0.5 mL of 0.2 *M* sodium acetate buffer. After 1 mL of ninhydrin reagent (Sigma) was added, the free amino groups were measured by the procedure of Moore *(18)* at 570 nm. One proteolytic unit was defined as the amount of enzyme that released 1 mmol of glycine after 1 h.

Table 1
Effect of PEG, Ammonium Sulfate, Ethanol, and Acetone on Protease Activity from *Bacillus* sp. SCB-3

Reagent	Fraction	Residual activity
Ammonium sulfate (0–80%)	Precipitate	Not precipitated
	Supernatant	95.2%
PEG 4000 (0–30%)	Precipitate	Not detected
	Supernatant	97.2%
Ethanol (0–80%)	Precipitate	Not precipitated
	Supernatant	81.2%
Acetone (0–80%)	Precipitate	42.1%
	Supernatant	Not detected

Results and Discussion

Purification of Keratinolytic Protease

Precipitation is a valuable method for protein purification, especially at an initial step for an isolation process. Important reagents for the precipitation are inorganic salts ($[NH_4]_2SO_4$), organic solvents (acetone or alcohols), and nonionic hydrophilic polymers (PEG). Table 1 shows the results of protease fractionation with ammonium sulfate, PEG, ethanol, and acetone. When the crude enzyme was saturated to 80% with ammonium sulfate or ethanol, there was no precipitation. However, the precipitation occurred when acetone was added into the crude enzyme. The protease activity of the acetone precipitate was reduced to 42% of the crude enzyme. When the crude enzyme was saturated with PEG 4000, the precipitate had no protease activity, but the supernatant had some. Polson et al. *(19)* introduced the use of nonionic water-soluble polymers, in particular PEG, for fractional precipitation of proteins. The primary advantage of PEG as a fractional precipitating agent is its well-known benign chemical properties. Unlike ethanol and other organic precipitating agents, PEG has little tendency to denature or otherwise interact with protein even when present at high concentrations and elevated temperatures. PEG was also used for protein extraction in aqueous two-phase systems. Protein extraction in aqueous two-phase systems is a rapid procedure that avoids most of the problems of denaturing fragile molecules in chromatographic beads. Therefore, aqueous two-phase systems provide a gentle environment for biologically active proteins and may be employed on large scale *(20)*.

The results of the purification procedure are summarized in Table 2. The keratinolytic protease was purified in two steps of chromatography by, first, DEAE-Toyopearl 650C and, second, Sephacryl S-200 HR. The first of two protein peaks obtained from DEAE-Toyopearl 650C chromatography showed the keratinolytic activity. The active fractions were combined and then concentrated in an ultrafiltration. The obtained concentrates were chromatographed on a Sephacryl S-200 HR column. Small and large pro-

Table 2
Purification of Protease from *Bacillus* sp. SCB-3

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	649.3	5714.5	8.8	1.0	100.0
PEG fraction	60.0	3543.4	59.0	6.7	62.0
DEAE-Toyopearl 650C	17.2	1454.9	84.5	9.6	25.5
Sephacryl S-200 HR	10.6	1030.3	97.2	11.0	18.0

tein peaks were obtained from Sephacryl S-200 chromatography. A large peak showed a symmetric peak of the keratinolytic activity. The sequences of chromatography steps were effective, resulting in a purification of 11-fold. The final amount of the purified protease was 10.6 mg, and the overall yield was 18%. The specific activity was 97.2 U/mg of protein.

The optimum temperature and pH were 40°C and pH 7.0, respectively. The enzyme was quite stable in a pH range of 5.0–8.0 and maintained >90% activity after 24 h at 25°C. Thermal stability was investigated by incubating the enzyme in a buffer at a designated temperature for 30 min. The protease activity up to 40°C retained >90% of its initial activity, but it was gradually inactivated at a higher temperature (data not shown).

Molecular Weight of Enzyme

The molecular weight of purified protease, which is 133.8 kDa, was calculated on the basis of semilogarithmic plots of the mobility of the band on SDS-PAGE by using a standard curve that was established with proteins of a known molecular weight (Fig. 1). Gel filtration on a Sephadex G-200 column resulted in mol wts of 134 kDa (data not shown). This keratinolytic enzyme was estimated as a single polypeptide with a mol wt of about 133.8 kDa, which was larger than those of *B. licheniformis* (33 kDa) *(21)*, *Streptomyces pactum* (30 kDa) *(22)*, and *Graphium penicolloideus* (85 kDa) *(23)*.

Effects of Inhibitors on Keratinolytic Activity

The effect of a series of protease inhibitors on enzyme activity against azokeratin was studied (Table 3). In these experiments, each compound at an indicated concentration level was tested after a 10-min preincubation with the enzyme. Iodoacetamide had no significant inhibitory effect on the activity. In addition, the serine-type protease (leupeptin, PMSF) and aspartic protease (pepstatin A) inhibitors had no effect on enzyme activity. A specific chelator for Zn^{+2} *(24)*, 1,10-phenanthroline, also did not inhibit enzyme activity. Enzyme activity was strongly inhibited by metal ion chelators, such as EDTA and EGTA. Therefore, the keratinolytic protease from *Bacillus* sp. SCB-3 turns out to be a metalloprotease.

The most commonly used chelating agents are EDTA and EGTA. While EDTA displays strong and nonspecific affinity for a variety of

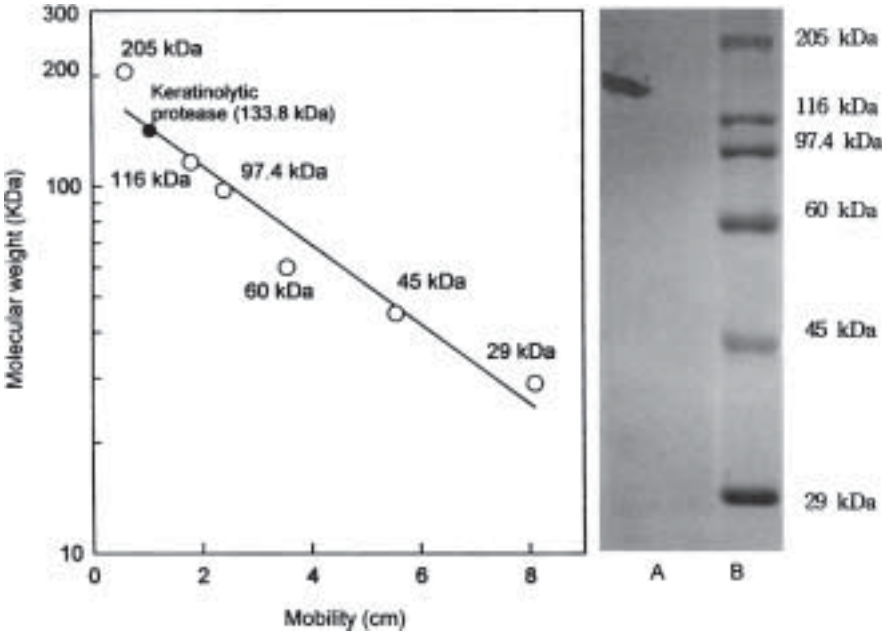

Fig. 1. SDS-PAGE of protease from *Bacillus* sp. SCB-3. Lane A, purified keratinolytic protease; lane B, molecular mass markers (myosine, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 60 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa).

Table 3
Effect of Inhibitors on Protease Activity from *Bacillus* sp. SCB-3

Inhibitor	Concentration (m*M*)	Relative activity (%)
None		100
PMSF	0.1	99.2
	0.5	97.8
Leupeptin	0.1	95.9
	0.5	92.1
Pepstatin A	0.1	98.4
	0.5	96.7
Iodoacetamide	0.1	102.1
	0.5	98.8
EDTA	0.05	43.6
	0.1	12.3
	0.5	0
EGTA	0.05	42.9
	0.1	18.4
	0.5	0
1,10-Phenanthroline	0.1	96.3
	0.5	94.8

Table 4
Effect of Metal Ions on Protease Activity from *Bacillus* sp. SCB-3

Metal ion	Concentration (m*M*)	Relative activity (%)
No addition		100
Zn^{+2}	0.1	60.3
	1.0	0
Cu^{+2}	0.1	10.8
	1.0	42.7
Co^{+2}	0.1	19.4
	1.0	84.7
Ca^{+2}	0.1	255.0
	1.0	129.2
Hg^{+2}	0.1	37.5
	1.0	37.5
Mg^{+2}	0.1	123.3
	1.0	106.9
Fe^{+2}	0.1	98.2
	1.0	102.7
EDTA + Ca^{+2}	0.5 ± 1.0	39.2
EDTA + Mg^{+2}	0.5 ± 1.0	28.3

metals, the EGTA's affinity for calcium is significantly higher than its affinity for magnesium. It permits the preferential sequestering of calcium in solutions with EGTA *(25)*.

It has been reported that there are two types of keratinolytic proteases: serine and cysteine. Serine proteases require an essential serine residue at their active site in keratinolytic proteases of *B. licheniformis*, *S. pactum*, *Streptomyces brevicaulis*, and *Streptomyces* sp. Complete irreversible loss of its keratinolytic activity in the presence of PMSF indicates the involvement of serine in catalytic activity *(11,22,23)*. However, a keratinolytic protease from *G. penicolloideus* required cysteine for catalytic activity *(23)*.

Effects of Metal Ions on Keratinolytic Activity

The effect of different metal ions on enzyme activity was investigated by adding the indicated concentrations of metals to the reaction mixture. As shown in Table 4, the addition of Zn^{+2}, Cu^{+2}, Co^{+2}, and Hg^{+2} resulted in decreased enzyme activity, whereas the addition of 0.1 m*M* Ca^{+2} and Mg^{+2} increased the protease activity to 255.0 and 123.3%, respectively. After the treatment with 0.5 m*M* EDTA and dialysis as described in Materials and Methods, the addition of 1.0 m*M* Ca^{+2} or Mg^{+2} restored the enzyme activity to 39.2 and 28.3%, respectively, of an untreated control. The enzyme was completely inhibited by 0.5 m*M* EGTA (Table 3), suggesting that Ca^{+2} rather than Mg^{+2} ions are the active ions in the native enzyme. These results may suggest that Ca^{+2} ion is required for the enzyme activity or stability, and/or both. The effect of Ca^{+2} on the metalloprotease under investigation may

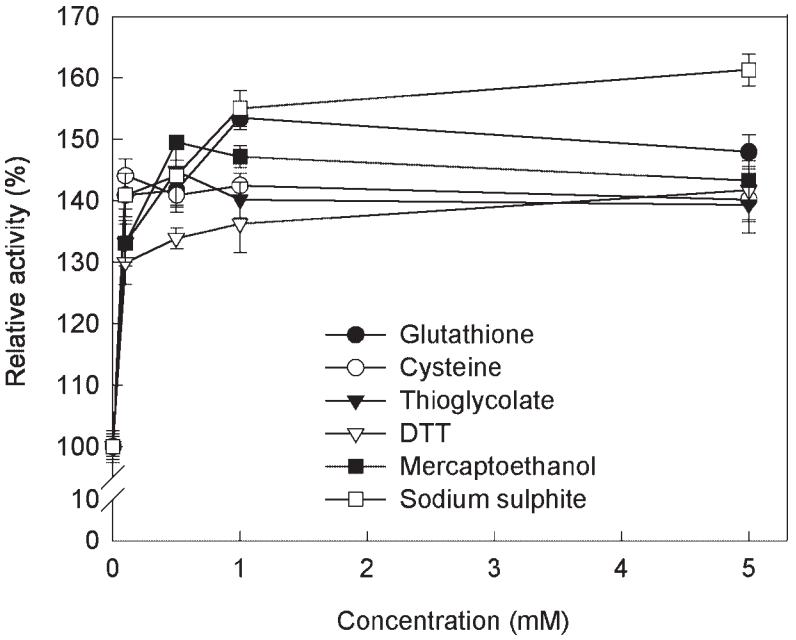

Fig. 2. Effect of reducing agents on keratinolytic activity of *Bacillus* sp. SCB-3. Reducing agents were added to the incubation mixtures as the solutions in buffer to reach the final concentrations of 0.1, 0.5, 1.0, and 5.0 m*M*.

involve the stabilization of the enzyme's structure. A stabilizing effect of calcium cations has already been reported for the *Streptomyces fradiae* protease *(26)*. Specific Ca^{+2}-binding sites that influence protease activity and stability apart from the catalytic site are described in several serine proteases, especially subtilisin-like protease, such as the commercially available keratinolytic protease K *(27)*.

Effects of Reducing Agents on Keratinolytic Activity

Figure 2 shows that reducing agents stimulated hydrolysis of feather by keratinolytic protease. Sodium sulfite enhanced keratinolytic activity maximally at a concentration of 5 m*M*, namely 1.6 times. Glutathione, mercaptoethanol, thioglycolate, cysteine, and DTT were, in this order, less stimulatory. Reducing agents except sodium sulfite stimulated hydrolysis of keratin at a concentration of <1 m*M*. The keratin degradation by hydrolytic enzymes in vitro should be accompanied by a simultaneous reduction of cystine bonds. Kunert *(28)* compared the effects of five reducing agents—sodium sulfite, cysteine, glutathione, mercaptoethanol, and DTT—on the activity of keratinolytic protease of *Microsporium gypseum* to confirm keratin degradation by sulfite excretion prior to being attacked by fungal protease. The results showed that sodium sulfite enhanced keratinolytic protease activity maximally at a concentration of 30 m*M*. Thioglycolate is a strong disulfide-reducing agent and has been applied for degradation of

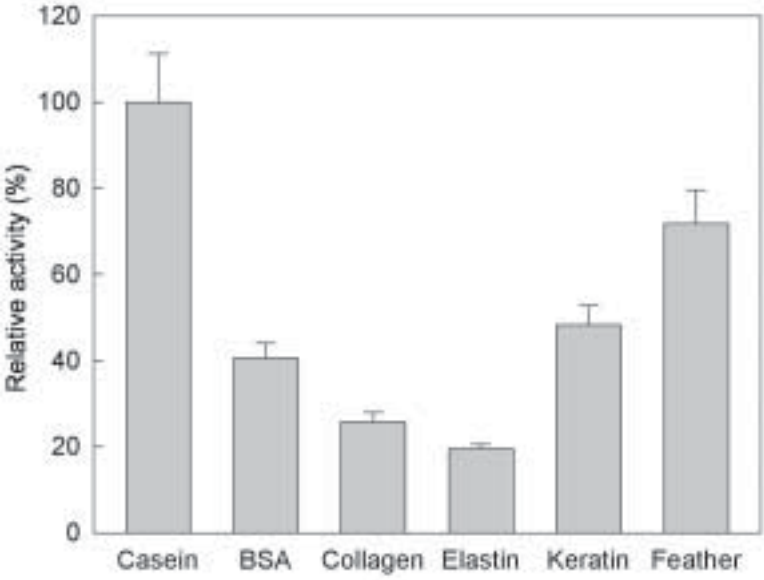

Fig. 3. Hydrolysis of various proteins with keratinolytic protease. The enzyme was incubated with 5 mg of substrate in 1 mL of 50 m*M* phosphate buffer (pH 7.5) for 20–120 min at 30°C.

hair keratin by alkaline proteinase from thermophilic *Bacillus* sp. AH-101 *(29)*. These findings suggest that a differential response of the microbial enzymes to the chemicals reducing the disulfide bonds with reducing agents can occur directly (whose mechanism has not been elucidated until now) or by excretion of sulfite, which causes the sulfitolysis of the disulfide bonds.

Hydrolysis of Various Proteins

The purified keratinolytic protease was examined for its ability to hydrolyze some proteins (Fig. 3). Casein was effectively hydrolyzed by the enzyme; however, the activity was significantly lower toward BSA, collagen, and elastin. By contrast, keratinolytic protease had a higher activity on feather rather than keratin. Keratinolytic protease from *Bacillus* sp. SCB-3 hydrolyzed casein and feather, but the enzyme hydrolyzed casein more effectively than feather. Lin et al. *(21)* found that keratinolytic protease from *B. licheniformis* was capable of hydrolyzing all the protein substrates tested, including BSA, collagen, elastin, and feather keratin. The soluble substrates BSA and casein were readily degradable, whereas the insoluble substrates collagen, elastin, and keratin were less so. Consistent with these findings, Bockle et al. *(22)* observed the liberation of peptides from different soluble substrates (casein and gelatin) and insoluble substrates (native and autoclaved) of chicken feathers. By contrast, Dozie et al. *(8)* reported a thermophilic keratinolytic protease from *Crysosporium keratinophyllum* that hydrolyzed only keratin but showed no activity on casein, BSA, or keratin powder.

The K_m values obtained were 0.17 and 0.67 mg/mL for casein and feather, respectively. The corresponding V_{max} values were 1047.2 and 111.4 U. The corresponding k_{cat} values were 7.47 and 0.79 s^{-1}. Varying microbial keratinolytic activities and enzymatic substrate specificities may be owing to methodologic and species differences.

For the evaluation of a biotechnologic application of keratinolytic protease from *Bacillus* sp. SCB-3, more detailed understanding of the keratinolytic metalloprotease would be helpful. Therefore, further research needs to be conducted on the specific molecular characteristics of this interesting enzyme.

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