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Preliminary Characterization of Keratinolytic Enzyme of *Aspergillus flavus* K-03 and Its Potential in Biodegradation of Keratin Wastes

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***Aspergillus flavus* K-03 isolated from poultry forming soil in Korea was studied for its ability to produce extracellular proteases on basal medium containing 2% (w/v) chicken feathers. The fungus was observed to be a potent producer of such enzymes. Keratinolytic enzyme secretion was the best at 15 days of incubation period at pH 9 and temperature 40°C. No relationship existed between the enzyme yield and increase of biomass. Enzyme production was suppressed by exogenous sugars in descending order arabinose>maltose>mannose>fructose. But glucose did not influence the enzyme activity. The keratinolytic enzyme released by the fungus demonstrated the ability to decompose keratin substrates as chicken feather when exogenous glucose was present. The keratinolytic activity was inhibited by $HgCl_2$ and serine-protease inhibitors such as phenymethylsulfonyl fluoride (100%), chymostain (88%), crystalline soybean trypsin inhibtor (80%), antipain (45%) and aprotinin (40%), and was not by cystein-protease and aspartyl-protease inhibitors. The enzyme activity is only partially inhibited by metallo-protease inhibitor. Thus, the enzyme secreted by *A. flavus* K-03 belongs to the alkaline serine-type protease.**

KEYWORDS: Alkaline serine-type keratinolytic protease, *Aspergillus flavus*, Extracellular enzyme, Keratinolytic enzyme

Keratin occurs in nature mainly in the form of hair, feathers, wool, horn and nails and is the structural component of vertebrates' skin and then it arises as a waste product in a variety of ways. As a result of shedding and molting of animal appendages, death of animals or as a by-product of poultry and leather industry, keratin ultimately is added to the saprotrophic compartment of our environment. In particular, chicken feather constitute a troublesome waste product that is produced in large quantities in commercial poultry processing plants and their utilization is of economic value as well as ecological significance to reduce pollution problems (Onifade *et al.*, 1998). Currently, a great part of feather waste is utilized on a limited basis as a dietary protein supplement as animal feedstuffs (Bhargave and O'Neil, 1975). However, feather meal has two important nutritional limitations such as an amino acid imbalance and poor digestibility (Baker *et al.*, 1981). Therefore, the utilizing of poultry feathers as a fermentation substrate by keratin-degrading microorganism or enzymatic biodegradation may be a better alternative to improve their nutritional value and to diminish environmental wastes. Several extracellular keratinases have been isolated and characterized from *Chrysosporium keratinophilium* (Dozie *et al.*, 1994), *C. georgiae* (El-Naghy *et al.*, 1998) and *Aspergillus fumigatus* (Santos *et al.*, 1996). Also *Microsporium gallinae* (Wawrzkiewicz *et al.*, 1991) grow on native keratin from chicken feathers. Kim (2003) isolated the five feather degrading fungi from poultry farming soil in Korea and adapted it grow on feathers as its source of carbon, sulfur and energy. The phytopathogenic nature of *Aspergillus flavus*, which was reported its capability to use feather as fermentation substrate in submerged batch culture, makes feasible the use of this fungus in biotechnological processes.

Hence, the present work was to study the secretion conditions and preliminary characterization of keratinolytic enzyme involved in feather degradation of *A. flavus* K-03.

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Materials and Methods

Chemicals. Ninhydrin, keratin-azure, casein and protease inhibitors were purchased from Sigma (St. Louis, MO, USA). White chicken feather were obtained from a poultry processing plant. They washed extensively with tap water, dried and sterilized by autoclaving at 121°C for 15 min and stored at room temperature in sealed bags until used.

Microorganism and culture conditions. The fungus used this study was *A. flavus* K-03 isolated from poultry farming soil in Korea (Kim, 2003). The fungus was maintained by serial passages in a potato-dextrose broth and agar (Difco) medium. The strain K-03 was cultivated in 500 *ml* Erlenmeyer flasks, containing feathers and basal medium as described by Kim (2003). This medium contained glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.025 g; $CaCl_2$, 0.025 g; $FeSO_4 \cdot 7H_2O$, 0.015 g; $ZnSO_4 \cdot 7H_2O$, 0.005 g; and distilled water 1,000 *ml*. The pH was adjusted to 8. Erlenmeyer flasks containing 50 *ml* of sterilized basal medium supplemented with 1 g of pre-sterilized feather as a keratin

source were inoculated with the strain K-03 and were incubated at 28±2°C and 120 rpm for 21 days. Erlenmeyer flasks containing the basal medium with a disc of agar without the fungus served as control. For inoculation, inoculum discs (8 mm diameter) were obtained from the periphery of activity growing 7 day-old subcultures of *A. flavus* K-03. When the effect of different carbon and nitrogen sources was investigated, 1% (w/v) of fructose, mannose, maltose, and were substituted to glucose in the basal medium. The fungal mass was harvested after 15 days incubation. The purity of the culture was determined on potato dextrose agar (Difco) plates with 500 *μg/l* streptomycin.

Proteolytic activity assays. Proteolytic activity was estimated by a modified method after Kunitz (1947). To 500 *μl* of 0.25% (w/v) casein in 50 mM Tris-HCl buffer with pH 8, a volume of centrifuged culture broth containing 5 *μ*g of protein sample was added. The reaction was carried out at 28±2°C for 45 min and proteins were precipitated with 500 *μl* 0.5% (w/v) trichloroacetic acid (TCA). After 15 min in an ice bath, the supernatant was separated by centrifugation at 15,000 rpm for 10 min and absorbance determined at 280 nm (ModeHP8453B, Hewlett Packard, Waldbronn, Germany). The blank was prepared adding 2.5 *ml* of TCA solution before adding the sample. One unit of proteolytic activity was defined as μmol tryptophan released per hour.

Keratinolytic activity assays. Keratinolytic activity was determined with keratin-azure as substrate by a modified method of Letourneau *et al.* (1998). To one milliliter of reaction mixture contained 30 mg keratin-azure in 50 mM Tris-HCl buffer with pH 8, a volume of centrifuged culture broth containing 0.05 *μ*g of protein sample was added. The reaction was carried out at 28±2°C for 1 h with constant agitation at 120 rpm and stopped by placing the tubes in an ice bath and recovering the supernatant after centrifugation of the reaction mixture at 15,000 rpm for 10 min. An assay without sample was carried out under the same conditions described above and used as blank. All assays were done as the amount of enzyme required to cause an increase of 0.01 at $A_{595\,nm}$ in 1 h.

Determination of protein. The protein content was determined by the Bradford method (1976) and bovine serum albumin as the standard.

Effects of temperature and pH on enzyme activity. The optimal temperature was tested by assaying the keratinolytic activity at different temperatures from 20 to 80°C in Tris-HCl buffer pH 8. To determine the optimal pH, keratinolytic activity was investigated in the pH range of 4~12, using the following 50 mM buffers such as citric acid/Na_2HPO_4 for pH 4~6, $NaHPO_4$/Na_2HPO_4 for pH 6~8, Tris-HCl for pH 7~9, glycine/NaOH for pH 9~11 and $NaHCO_3$/NaOH for pH 11~12.

Effects of protease inhibitors and modulators on keratinolytic protease activity. Aliquots of the culture broth of *A. flavus* K-03 were pre-incubated with the various proteases inhibitors viz. phenymethanesulphonyl fluoride (PMSF), ethylenediaminetetraacetate (EDTA), aprotinin, crystalline soybean trypsin inhibitor (ITS), trans-epoxysuccinyl-L-leucylamido (4-gguanidino) butane (E-64), leupeptin, pepstain A, antipain and chymostatin. All of them were in 50 mM Tris-HCl buffer with pH 9 for 1 h at 40°C. Keratinolytic activity was then determined and residual activity was expressed as percentage activity of the uninhibited enzyme.

Results and Discussion

Influence of culture temperature on proteolytic activity. Proteolytic activity as revealed by the clearing of particulate casein was exhibited by the fungus *A. flavus* K-03. The time course for the production of extracellular proteolytic activity in the basal medium containing 2% (w/v) of feathers is shown in Fig. 1. The maximum growth and the biomass of *A. flavus* K-03 on milled chicken feathers was observed at 40°C. The results are suggesting that free amino group levels and maximum feather degradation were achieved at 40°C in chicken feather batch cultures of *A. flavus* K-03 and that the secretion of keratinolytic enzymes allow the strain K-03 to survive when the only available sources of carbon are feathers. The proteolytic activity and the biomass production of the fungus K-03 was gradual increased in the basal medium including chicken feathers from 8th incubation day onward until a peak was reached on 15th day. After

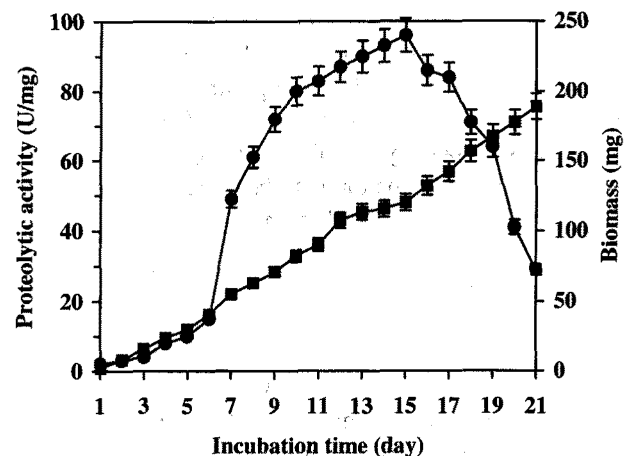

Fig. 1. Proteolytic activity (●) and biomass (■) of *A. flavus* K-03 cultured in basal medium containing 2% (w/v) feather for 21 days at 28±2°C, pH 8 and 120 rpm.

Table 1. Effect of various carbon sources on biomass and protease secretion of *A. flavus* K-03, which cultivated in Erlenmeyer flasks on basal medium containing 2% (w/v) of feathers for 15 days at 28±2°C, pH 8 and 120 rpm

Supplement (10 g/*l*)	Caseinolytic (U/mg)	Keratinolytic (U/mg)	Biomass (Dry weight mg/*l*)
Glucose	96.4 (±2.0)	26.2 (±0.5)	120.1 (±0.9)
Arabinose	58.9 (±1.9)	9.8 (±0.9)	102.9 (±0.8)
Fructose	18.2 (±1.7)	6.1 (±0.0)	98.8 (±0.1)
Maltose	30.2 (±1.0)	8.9 (±0.3)	92.3 (±0.2)
Mannose	22.7 (±1.8)	10.5 (±0.4)	95.5 (±0.5)
Control[a]	97.8 (±1.9)	25.7 (±0.9)	94.8 (±0.5)

[a] *A. flavus* K-03 was cultivated in the basal medium without glucose.

that there was a dramatically decrease in proteolytic activity, but the biomass was steadily bigger with the incubation time. Therefore, no relation was observed between enzyme production and biomass in the basal medium. The similar production of protease has been reported for fungi such as *Endothia parastica* (McGarrol and Thore, 1985), *Hendersonula toruloidea, Scytalidum hyalinum. S. japonicum* (Oyeka and Gugnani, 1995), *Chrysosporium tropicum* (Singh and Singh, 1995), *C. keratinophilum* (Dozie *et al.*, 1994), *Trichiphyton simii* (Singh, 1997) and *Malbranchea gypsea* (Singh, 1999).

Effects of various carbon sources on protease production. The effect on cell growth and enzyme production of the addition of carbon supplements to the basal medium containing 2% chicken feathers is given in Table 1. Of the five carbon sources tested, glucose promoted the highest values of caseinolytic and keratinolytic activities as well as biomass accumulation. The declining order of enzyme yield in other carbohydrates was as follows: arabinose>maltose>mannose or fructose. On other hand, keratinolytic activities in *Trichophyton mentagrophytes* (Yu *et al.*, 1969) and *T. rubrum* (Meevootisom and Niederpruem, 1979) were partly suppressed by 0.1% (w/v) concentration, and completely inhibited by 2% (w/v) of glucose contained in a keratin salts broth, while the enzyme release of *A. flavus* K-03 was not inhibited by 2%. Thus, the result is suggesting that the present study obviously demonstrated sugars except glucose suppressed enzyme production of the fungus. The specificity of a particular carbon source on enzyme production was repressive when various sugars were added to the medium. Sugars suppression of protease appears to be common among fungi (Chandra and Banerjee, 1972; Meevootisom and Niederpruem, 1979; Somkuti and Babel, 1967; Yu *et al.*, 1969).

Effect of temperature on keratinolytic protease activity. The influence of temperature on the keratinolytic activity of *A. flavus* K-03 is demonstrated in Fig. 2. The

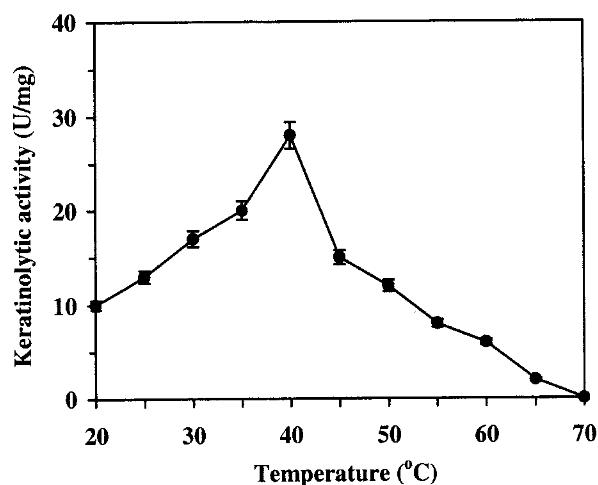

Fig. 2. Effect of temperature on keratinolytic activity of centrifuged culture broth of *A. flavus* K-03 cultivated for 15 days at pH 8 and 120 rpm in Erlenmeyer flasks. Enzyme activity was calculated at pH 8 with keratin-azure as the standard.

keratinolytic activity of the culture broth was detectable between 20 and 60°C, showing a maximum activity at 40°C. A quick drop in keratinolytic activity was observed between 40~60°C. Fungal activity up to 60°C with a peak at 40°C for keratinase release is considered worthy of attention as this corresponds to the thermotolerant nature of the fungus. Some researchers reported similar results for keratinase of *Scopulariopsis breviculis* (Malviya *et al.*, 1992), *Streptomyces pactum* (Böckle *et al.*, 1995) and *Chrysosporium keratinophilum* (Dozie *et al.*, 1994). The above-mentioned microorganisms listed optimal temperature near 40°C. Investigating thermal stability, the keratinolytic activity present in the culture broth remained fully active at 5°C for several weeks and 24 h when stored at room temperature. The enzyme activity retained up to 40°C, but was gradually inactivated at higher temperature (data not shown).

Effect of pH on keratinolytic protease activity. The keratinolytic activity of the culture broth of *A. flavus* K-03 was detectible over a wide range of pH values, with an optimum at pH 9 (Fig. 3), dependent on the buffer used. Tris-HCl buffer appeared to be the most suitable. The activity declined rapidly over 10 and was negligible above 11. Other keratinolytic enzymes have been reported to be active at alkaline pH (Gradišar *et al.*, 2000; Letourneau *et al.*, 1998; Mitsiki *et al.*, 2002). The accumulation of ammonium ions as the products of deamination stimulated the pH increasing for fermentation with increasing keratinolytic activity (Gradišar *et al.*, 2000). The enzyme activity was quite stable in the pH 8~11 and maintained more than 80% activity after 24 h at room temperature (data not shown).

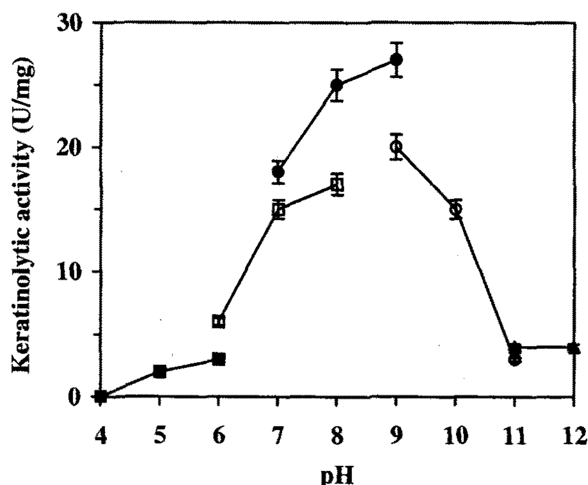

Fig. 3. Effect of pH on keratinolytic activity of centrifuged culture broth of *A. flavus* K-03 incubated for 15 day at 40°C and 120 rpm in Erlenmeyer flasks. Enzyme activity was calculated at 40°C with keratin-azure as the standard, using 50 mM buffers. (■), citrate/Na_2HPO_4 buffer (pH 4~6); (□), $NaHPO_4$/Na_2HPO_4 buffer (pH 6~8); (●), Tris-HCl buffer (pH 7~9); (○), glycine/NaOH buffer (pH 9~11); (▲), $NaHCO_3$/NaOH buffer (pH 11~12).

Effect of protease inhibitors on keratinolytic protease activity. The keratinolytic activity in the culture broth of *A. flavus* K-03 influenced by various protease inhibitors was shown in Table 2. Interestingly, only serine protease inhibitors suppressed the activity with the strongest effect exerted by PMSF (100%), chymostain (88%), ITS (80%), antipain (45%) and aprotinin (40%). In contrast, cysteine protease inhibitor (E-64) and aspartyl-protease inhibitor (pepstatin A) did not decrease the keratinolytic activity in significant. The enzyme activity is only partially inhibited by metallo-protease inhibitor (EDTA). The similar inhibitory patterns were reported for other keratinase (Böckle *et al.*, 1995; Bressolier *et al.*, 1999; Friedrich and Antranikian, 1996; Galas and Kaluzewska, 1989; Grzywnowicz *et al.*, 1989; Ignatova *et al.*, 1999). Partial inhibitory by EDTA indicates that cations may act as cofactors or as stabilizing agents, as already found for several other alkaline serine endopeptidases (Beynon and Bond, 1990; Böckle *et al.*, 1995; Galas and Kaluzewska, 1989; Gradišar *et al.*, 2000; Grzywnowicz *et al.*, 1989). Alkaline peptidases require a divalent cation such as Ca^{2+}, Mg^{2+}, or Mn^{2+}. In addition, $HgCl_2$ inhibited the enzyme activity. Inhibition by $HgCl_2$ was observed in the case of proteinase K, another keratin-degrading serine endopeptidase (Müller and Saenger, 1993). Thus, it was proposed the main proteases secreted by *A. flavus* K-03 are alkaline serine-type peptidases. *A. flavus* K-03 cultured on simple medium supplemented with chicken feather, produced a protease mixture with high keratinolytic activity at high pH, and was very effective in feather degradation, suggesting its potential use in biotechnological processes involving keratin hydrolysis. Moreover, the serine-type keatainase produced by the strain K-03 was active over a wide range of pH values and temperature. These are interesting properties for regarding industrial use of the enzyme for the ultimate degradation of keratin wastes.

Table 2. Effects of protease inhibitors on the keratinolytic activity of centrifuged culture broth of *A. flavus* K-03 incubated for 15 days at 28±2°C, pH 9, and 120 rpm

Inhibitor[a]	Concentration (mM)	Residual activity (%)
PMSF[b]	1.0	0
EDTA[c]	10.0	96
Aprotinin	0.1	60
ITS[d]	2.5	20
E-64[e]	0.01	100
Leupeptin	0.1	76
Pepstatin A	1.0	100
Antipain	0.1	55
Chymostatin	0.1	12
$HgCl_2$	1.0	0

[a]There were 3 replications per treatment.
[b]Phenymethylsulfonyl fluoride.
[c]Ethylenediaminetetraacetate.
[d]Crystalline soybean trypsin inhibtor.
[e]Trans-epoxysuccinyl-L-leucylamido (4-gguanidino) butane.

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