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Immobilization of Keratinase from *Aspergillus flavus* K-03 for Degradation of Feather Keratin

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Extracellular keratinase isolated from *Aspergillus flavus* K-03 was immobilized on calcium alginate. The properties and reaction activities of free and immobilized keratinase with calcium alginate were characterized. The immobilized keratinase showed proteolytic activity against soluble azo-casein and azo-keratin, and insoluble feather keratin. Heat stability and pH tolerance of keratinase were greatly enhanced by immobilization. It also displayed a higher level of heat stability and an increased tolerance toward alkaline pHs compared with free keratinase. During the durability test at 40°C, 48% of the original enzyme activity of the immobilized keratinase was remained after 7 days of incubation. The immobilized keratinase exhibited better stability, thus increasing its potential for use in industrial application.

KEYWORDS: Durability, Heat stability, Immobilization, Keratinase, pH tolerance

Microbial proteases are among the important hydrolysis enzymes, and have been studied extensively since the advent of enzymology (Gupta *et al.*, 2002). Several techniques have been developed for surface derivatization and covalent immobilization of microbial enzymes on different types of porous silica (Rurgess *et al.*, 1975; Janolino and Swaisgood, 1982; Swaisgood and Catignani, 1987). Immobilized proteases have been used for many applications, such as hydrolysis of protein to amino acids (Church *et al.*, 1984; Swaisgood and Horton, 1989), probing of protein structure (Grima *et al.*, 1986; Swaisgood *et al*, 1994), and evaluation of protein digestibility (Porter *et al.*, 1984; Swaisgood and Catiagnani, 1991). One of the advantages of the protein immobilization is that it minimizes autolysis of enzymes because the proteases exist in solid phase. Since the products, such as amino acids and peptides, are easily separated, it is easy to clean and reuse the immobilized enzyme.

A keratinase was isolated from the feather-degrading fungus, *Aspergillus flavus* K-03. (Kim, 2003a). The purified keratinase was considered as an alkaline serine-type protease, which exhibited strong proteolytic activity toward the feather keratin and other insoluble and soluble protein substrates. In this study, I determined the effects of immobilization of keratinase with calcium alginate on microbial resistance, excellent mechanical strength, and extraordinary chemical durability.

Cell growth and enzyme production. A thermophilic fungus, *A. flavus* K-03 from poultry farming soil (Kim, 2003a) was cultivated on a mineral salt medium (0.5 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g Mg $SO_4 \cdot 7H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$ per liter) supplemented with 0.2% peptone and 1.0% glucose at 40°C, pH 9.0 for 15 days. Keratinase was purified from the culture filtrate by carboxylmethyl cellulose ion exchange and Sephadex G-100 chromatography (2 × 85 cm; Pharmacia, Sweden), and the purity and the protein concentration were determined by SDS-PAGE analysis as previously described (Bradford, 1976).

Immobilization of keratinase. The calcium alginate gel beads were prepared by dropping 2% calcium alginate solution. A suitable amount of enzyme had been diluted into 0.2 M calcium chloride solution under continuous stirring. Keratinolytic and proteolytic activities were measured by three different methods. Hydrolyses of azo-keratin and azo-casein (Sigma) were determined as previously described with some modifications (Lin *et al.*, 1992; Kim, 2003b). The free amino group released by keratinolysis was estimated by the ninhydrin method (Rosen, 1957). No enzyme activity was observed in the final preparation and stored at 4°C. After immobilization, the excess enzyme keratinase was collected for reuse. As shown at Table 1, approximately 100 μg of purified keratinase was immobilized on beads. In free and soluble forms, keratinase was found to have higher proteolytic activity. Immobilization reduced the keratinolytic and caseinolytic activity by 71~ 78%. In comparison with chemically immobilized keratinase (Lin *et al.*, 1996), the specific activity of bio-immobilized keratinase was improved from 17 to 29% when casein was used as the substrate.

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Table 1. Specific activity of free and immobilized keratinase

Enzyme	Specific activity (U/mg)		Feather keratin[a] (μM Leu eq./mg)
	Azo-keratin[b]	Azo-casein[c]	
Keratinase	2,680.00	56,740.00	7.85
Immobilized keratinase	581.00	16,641.00	1.35
Immobilized keratinase/keratinase	0.22	0.29	0.17

[a]Ninhydrin method: Increase of free amino groups as measured by leucine equivalent.
[b]Azo-keratin assay: 1 U, an increase in A_{450} of 0.01/h at 40°C.
[c]Azo-casein assay: 1 U, an increase in A_{450} of 0.01/h at 40°C.

Effect of hydrogen ion concentration. Soluble and immobilized keratinase were pretreated at various pH values. Soluble keratinase and immobilized keratinase (1.5 μg and 20 μg, respectively) were added into 50 μl of 50 mM buffers viz. 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, and incubated at 4°C for 15 min. After pretreatment, 0.8 *ml* of 0.5% azo-casein dissolved in 50 mM Tris-HCl buffer (pH 9.0) was added to measure the remaining activity of free and immobilized keratinase. Comparing the stabilities of soluble and immobilized keratinase at 40°C, 10 μg of keratinase and 0.3 *ml* of immobilized keratinase were added separately into two tubes containing 10 *ml* of 50 mM Tris-HCl buffer, pH 9.0. As shown in Fig. 1, both free and immobilized keratinase showed sensitivity in acidic condition, but were less sensitive in alkaline pH. Comparing with soluble keratinase, the immobilized keratinase was much more stable in extreme pH values. A high pH (pH 12) did not decrease keratinase activity of both forms, while acidic pH did. In both cases, the highest hydrolysis of feather was achieved at pH 8.5. However, when casein was used as the substrate, both forms of keratinase showed the highest activity at pH 9.0. This result is consistent with the previous report (Kim, 2003b). The shift in pH optimum is most likely due to differences in substrate solubility, steric hindrance, etc. Nevertheless, immobilization of keratinase causes little change the enzymatic properties.

Heat stability. As shown in Fig. 2, the immobilized keratinase showed higher heat stability than the free enzyme, indicating that the enzyme stability of the immobilized keratinase was significantly improved. The half-life of immobilized and free keratinase was approximately 50 and 15 h, respectively. After three-day incubation at 40°C, the immobilized keratinase maintained 77% activity, whereas the soluble enzyme was 18% active.

Durability. Casein hydrolysis was used to measure keratinase activity over a 7-day period. Free keratinase (10 μg) and immobilized keratinase (0.3 *ml*) were added separately into the tube of azo-casein and incubated at 40°C with rotation. A 5.0 *ml* aliquot from each reaction mixture was taken every 2 h, and casein hydrolysis was measured. During a seven-day reaction, immobilized keratinase hydrolyzed casein continuously, and it retained 48% of activity by day 7. However, free keratinase lost its

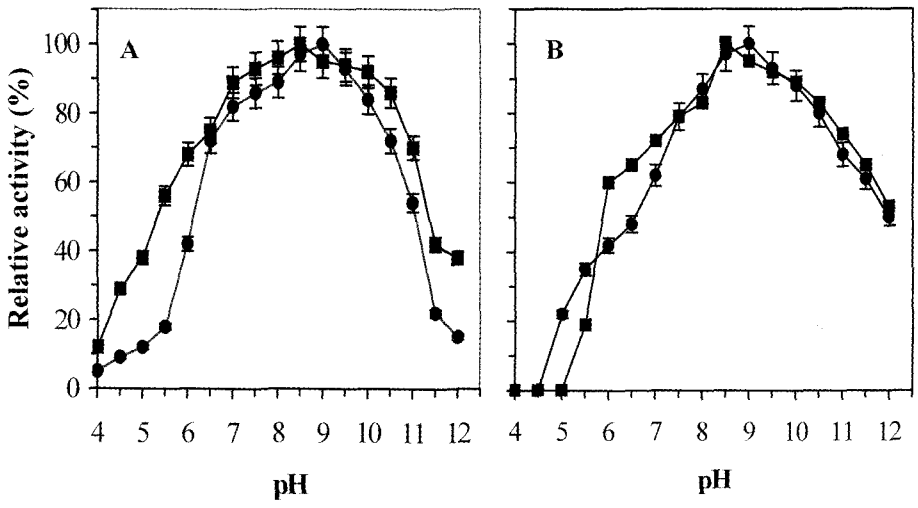

Fig. 1. Activity profiles of free (A) and immobilized (B) keratinase against the feather-keratin (●) and azo-casein (■) as substrate at different pH.

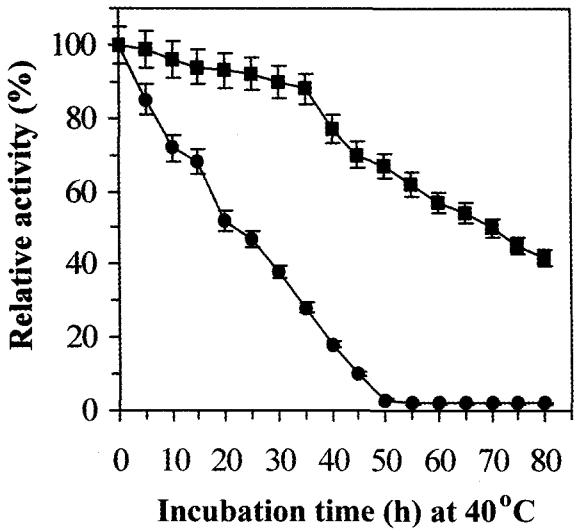

Fig. 2. Heat stability of free (●) and immobilized (■) keratinase at 40°C. The enzyme activity was performed by azo-casein hydrolysis.

activity after two days, probably because of autolysis and denaturation (Lin *et al.*, 1992). This suggests that immobilized keratinase has a higher durability than free enzyme, allowing an extended reaction period (Fig. 3).

In conclusion, immobilized keratinase is able to hydrolyze soluble casein and keratin and the insoluble feather keratin. With increased heat stability and pH tolerance, immobilized keratinase could be utilized in some industrial applications. For example, bioreactors with immobilized keratinase can convert ground feathers to peptides and amino acids. The results of the present study demonstrated that the level of keratinase attachment is low. Therefore, further studies on improving binding ability of keratinase to various carriers are currently in progress.

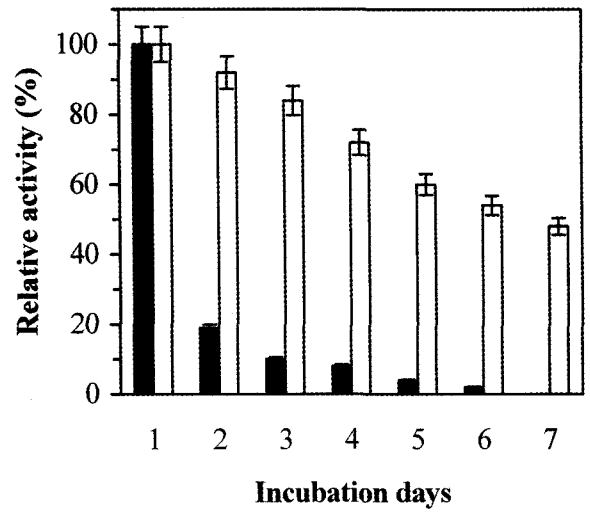

Fig. 3. Long-term hydrolysis of azo-casein by free (■) and immobilized (□) keratinase. Values for the casein hydrolysis on day one were changes in A_{450} of 6.69 and 1.26 units for free and immobilized keratinase, respectively.

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