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Biosynthesis and Control of Keratinase in Recalcitrant Feather-Degrading *Bacillus megaterium* F7-1

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Abstract

This study was performed to investigate the nutritional conditions controlling keratinase activity in *Bacillus megaterium* F7-1. *B. megaterium* F7-1 produced keratinase using chicken feather as a sole source of carbon, nitrogen and sulfur. Addition of the feather medium with glucose enhanced keratinase production (68.9 U/ml), compared to control without glucose (63.2 U/ml). The synthesis of keratinase was repressed by addition of NH_4Cl in *B. megaterium* F7-1. The highest keratinase production (70.9 U/ml) was obtained with the feather medium containing glucose and $MgSO_4 \cdot 7H_2O$. Keratinase was produced in the absence of feather (4.9 U/ml), indicating its constitutive synthesis. Feather degradation resulted in free SH group formation. *B. megaterium* F7-1 effectively degraded chicken feather meal (86%), whereas duck feather, human nail, human hair and sheep wool displayed relatively low degradation rates (8-34%).

Key Words : *Bacillus megaterium*, Feather, Keratinase, Biosynthesis control

1. Introduction

Feathers, which are almost pure keratin protein, are produced in large amounts as waste byproduct at poultry-processing plants, reaching millions of tons per year worldwide (Onifade et al., 1998). The tight packing of the keratin chain in the α-helix or β-sheet into a supercoiled polypeptide chain results in mechanical stability and resistance to proteolysis (Joshi et al., 2007). In addition, cross-linking of protein chains by cysteine bridges also confers high mechanical stability and resistance to proteolytic degradation of keratins. The structural rigidity of this protein makes feathers to accumulate in large quantities as wastes of commercial poultry processing leading to serious environmental problems.

The feathers, which are hydrolyzed by mechanical or chemical treatment, can be converted to feedstuffs, fertilizers, glues, and foils or used for the production of amino acids and peptides (Haddar et al., 2009). However, these treatments are expensive and also destroy certain amino acids, yielding a product with poor digestibility and variable nutrient quality (Brandelli et al., 2010). The chemical processing also causes environmental pollution. Therefore, the use of microbial keratinase in the production of amino acids and peptides is becoming attractive for biotechnological applications.

Control of enzyme biosynthesis is involved in successful production of enzyme by fermentation. For a large-scale production of keratinase, therefore,

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its biosynthesis must be carefully controlled. Although the literature shows that there are some researches on nutritional control of keratinase biosynthesis in fungi, to the best of our knowledge there is no report on the control of the production of this enzyme in bacterial strains, except *Bacillus pumilis* (Son et al., 2008).

We have previously reported on the isolation and characterization of *Bacillus megaterium* F7-1, which is able to produce chicken feather-degrading keratinase (Park and Son, 2009; Son et al., 2004). The objective of this investigation was to study the nutritional conditions controlling keratinase biosynthesis in *B. megaterium* F7-1. Degradation of various keratin substrates was also investigated.

2. Materials and Methods

2.1. Microorganism and culture conditions

B. megaterium F7-1 used in this study was isolated from a poultry waste in Korea (Son et al., 2004). The bacterial strain F7-1 was cultivated in a basal salts medium containing 0.1% chicken feather unless otherwise indicated. The strain was precultured in nutrient broth at 30℃ for 24 h. The cells were washed three times with 0.1 M phosphate-buffered saline, and 2% (v/v) of inoculum (2×10^7 cells/ml) was then inoculated to 250-ml flasks containing 50 ml of the medium. Cultivations were conducted at 30℃ and 200 rpm for 5 days in a rotary shaker. The basal salts medium used contained the following: 2.8 mM KH_2PO_4, 3.4 mM K_2HPO_4, 10.3 mM NaCl, 1.5 mM $MgCl_2 \cdot 6H_2O$ and 136 μM $CaCl_2 \cdot 2H_2O$ (Son et al., 2008). The pH was adjusted to 7.5 prior autoclaving. Chicken feathers were obtained from a poultry-processing plant. They were washed extensively with tap water and dried at 60℃ for 72 h, and then kept at 4℃ until used.

To study nutritional control of the keratinase biosynthesis in *B. megaterium* F7-1, cells were grown on basal salt medium supplemented with nine different combinations as described by Son et al. (2008): 0.1% chicken feather; 0.2% glucose; 9.3 mM NH_4Cl; and 406 μM $MgSO_4 \cdot 7H_2O$. In one set 0.1% chicken feather was the sole source of carbon, nitrogen and sulfur; the other seven sets had all possible combinations of single, double and triple supplementation with additional carbon, nitrogen or sulfur. The remaining one set which lacked chicken feather, contained all three major elements (as glucose, NH_4Cl and $MgSO_4 \cdot 7H_2O$).

The keratin-degrading ability of *B. megaterium* F7-1 was also investigated using chicken feather meal, duck feather, human hair, human nail and sheep wool as substrate. All keratin substrates except feather meal were cut into fragments (0.3 cm long) and washed with distilled water. Various keratin sources were added separately to the medium at 0.1%.

2.2. Feather keratin preparation

Feather keratin was prepared from chicken feathers by the method of Wawrzkiewicz et al. (1987). Chicken feathers (10 g) suspended in 500 ml of dimethylsulfoxide were solubilized by heat treatment in a reflux condenser at 100℃ for 1 h. Feather keratin was then precipitated by addition of cold acetone for 2 h followed by centrifugation at 17,418 *g* for 10 min. The precipitate was washed twice with distilled water, and then dried at 60℃. The dried feather keratin was crushed in mortal and pestle. The keratin powder obtained was used as a substrate for keratinase determination.

2.3. Keratinase assay

The bacterial cultures were centrifuged at 17,418 *g* for 15 min, and the supernatant was used as a crude enzyme preparation. Keratinase was assayed with feather keratin as a substrate, according to the method of Park and Son (2009). An appropriately enzyme solution (2 ml) was mixed with 3 ml of 0.06% feather

keratin in 0.1 M phosphate buffer (pH 7.5) and incubated for 3 h at 30℃. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid and centrifuged at 17,418 *g* for 10 min at 4℃. The absorbance of the supernatant was measured at 280 nm. One unit (U) of keratinase was defined as the amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.01 per h under the above conditions. The protein concentration was determined by the Bradford (1976) method with bovine serum albumin as the standard.

2.4. Analytical methods

Cell growth was determined by total plate count on nutrient agar plate. Keratins in culture were collected by filtration with Whatman no. 3 filter paper, washed twice with distilled water and dried at 105℃ to constant weight. The keratin degradation rate was calculated from the differences in residual keratin dry weight between a control (keratin without bacterial inoculation) and treated sample. The concentration of SH groups in the culture medium of *B. megaterium* F7-1 was determined according to the method of Ellman (1959). All experiments were repeated at least three times. The data presented in the tables correspond to mean values.

3. Results and Discussion

Table 1 shows the production of keratinase by *B. megaterium* F7-1 in the presence of feather and different combinations of nutrients. Among 9 experimental cultures tested, addition of glucose to the feather medium enhanced cell growth (9.97×10^9 CFU/ml) as well as keratinase activity (68.9 U/ml) compared to the feather medium. In the absence of feather, *B. megaterium* F7-1 also produced a low level of keratinase (4.9 U/ml) when incubated in medium containing glucose. Production of proteases by bacteria is generally regulated by repression and induction (Rao et al., 1998). Carbohydrates including glucose are known as a common catabolic repressor for a number of protease in *Bacillus* strains (Fisher and Sonenshein, 1991). That is, the supplementation of additional carbon source has a negative effect on the production of protease (Gioppo et al., 2009). Cheng et al. (1995) reported complete inhibition of the keratinase production from *Bacillus licheniformis* PWD-1 in presence of glucose. However, these effects are not common for all microorganisms. For example, the addition of glucose to the feather medium has a positive effect on the production of keratinase by *B. pumilis* F3-4 (Son et al., 2008). Our

Table 1. Biosynthesis of keratinase by *Bacillus megaterium* F7-1 in the presence of feather and different combinations of nutrients

Treatment	Cell growth (CFU/ml)	Protein (μg/ml)	Keratinase activity (U/ml)	Feather degradation (%)	SH group (μM)
Feather	6.12×10^9	410	63.2	74	1.87
Feather+glucose	9.97×10^9	453	68.9	78	2.01
Feather+NH_4Cl	6.95×10^9	406	49.6	34	0.89
Feather+$MgSO_4 \cdot 7H_2O$	6.45×10^9	501	62.7	79	1.99
Feather+glucose+NH_4Cl	8.24×10^9	433	51.5	41	0.81
Feather+glucose+$MgSO_4 \cdot 7H_2O$	1.12×10^{10}	467	70.9	83	2.11
Feather+NH_4Cl+$MgSO_4 \cdot 7H_2O$	7.30×10^9	454	52.5	48	1.15
Feather+glucose+NH_4Cl+$MgSO_4 \cdot 7H_2O$	1.09×10^{10}	495	61.1	69	1.57
Glucose+NH_4Cl+$MgSO_4 \cdot 7H_2O$	9.02×10^9	532	4.9	-	-

results are in accordance with the data of *B. pumilis* F3-4. Therefore, our results indicate that the synthesis of keratinase by *B. megaterium* F7-1 is constitutive and is increased by additional carbon sources. This new phenomenon suggests a changed general enzyme-regulatory circuit in the background (Rozs et al., 2001).

Addition of NH_4Cl to the feather medium enhanced cell growth (6.95×10^9 CFU/ml), but repressed keratinase production (49.6 U/ml); suggesting this enzyme is regulated by nitrogen catabolite repression. The addition of NH_4Cl to the feather medium together with glucose, although it enhanced cell growth, also repressed keratinase production. This result is in accordance with the previous findings of Son et al. (2008) and Malviya et al. (1992) for *B. pumilis* F3-4 and *Chrysosporium queenslandicum*. It is also reported that the addition of nitrate repressed the keratinase production of *Thermoactinomyces candidus* and *Aspergillus fumigatus* (Noronha et al., 2002).

In case of *B. pumilis* F3-4, the addition of the feather medium with $MgSO_4 \cdot 7H_2O$ enhanced keratinase production (Son et al., 2008). However, the addition of $MgSO_4 \cdot 7H_2O$ to the feather medium did not influence on keratinase production of *B. megaterium* F7-1 (Table 1). In the presence of glucose, the addition of $MgSO_4 \cdot 7H_2O$ enhanced keratinase production (70.9 U/ml). This result indicates that glucose and $MgSO_4 \cdot 7H_2O$ may play a synergetic role in the production of keratinase by *B. megaterium* F7-1. On the other hand, the addition of $MgSO_4 \cdot 7H_2O$ to the feather medium containing NH_4Cl enhanced cell growth, but repressed keratinase production. Addition of glucose to this medium also enhanced cell growth, but did not enhance keratinase production.

Reduction of disulfide bonds has a significant influence on feather degradation (Kunert and Stransky, 1988). Thus, we measured the free SH group concentration in culture broth. As shown in Table 1, feather degradation resulted in free SH group formation; higher feather degradation resulted in high free SH group formation. The maximum free SH group was observed in the medium containing feather, glucose and $MgSO_4 \cdot 7H_2O$. Since keratin has a very rigid structure through the formation of cystine disulfide bridges, feather degradation is likely to require reduction of the disulfide bonds. In other words, free SH group formation indicates the presence of disulfide reductase activity (Kumar et al., 2008; Sangali and Brandelli, 2000). Thus, our results suggest that *B. megaterium* F7-1 possesses disulfide reductase activity along with keratinase activity. Reduction of disulfide bridges was observed for *Chryseobacterium* sp. kr6 (Riffel et al., 2003) and *Streptomyces pactum* (Böckle and Müller, 1997) grown on feathers.

Keratin-degrading ability of *B. megaterium* F7-1 was studied using duck feather, chicken feather meal,

Table 2. Biosynthesis of keratinase by *Bacillus megaterium* F7-1 grown on various keratin substrates

Substrates	Cell growth (CFU/ml)	Protein (μg/ml)	Keratinase activity (U/ml)	Keratin degradation (%)
Duck feather	5.11×10^9	496	59.6	34
Chicken feather meal	1.45×10^{10}	624	74.8	86
Human nail	3.01×10^9	211	12.5	28
Human hair	1.08×10^9	107	22.1	8
Sheep wool	1.19×10^9	115	4.3	19

human nail, human hair and sheep wool as substrate. As shown in Table 2, the keratin substrates displayed different degradation rates by *B. megaterium* F7-1. The chicken feather meal was the most strongly degraded (86%) followed by duck feather (34%). Human nail (28%), sheep wool (19%) and human hair (8%) showed relatively low degradation rate. The degradation rate of feather meal by *B. megaterium* F7-1 was higher than that by *Streptomyces albidoflavus* (67%) (Bressollier et al., 1999). *B. megaterium* F7-1 is also comparable to *Thermoactinomyces candidus* which could degrade 11% of sheep wool (Ignatova et al., 1999). It is reported that *Bacillus* sp. PA-001A degraded 90, 60 and 50 % of the sheep skin, feather and horn, and hair was supported poor growth (Atalo and Gashe, 1993). On the other hand, keratin degradation rate was not proportional to the keratinase activity (Table 2). The lower level of degradation of keratin at the expense of higher production of keratinase was suggested to be due to the repression of some accessory proteins capable of cleavaging the disulfide bonds present in the keratin during the degradation process, an essential step in the enzymatic degradation of keratin (Singh, 1997). However, *B. megaterium* F7-1 possessed disulfide reductase activity. Therefore, it seems more likely that these differences in the degradation rate are conditioned by a different chemical composition and the molecular structure of keratin.

As shown in Table 2, chicken feather meal greatly enhanced the production of the keratinase (74.8 U/ml) followed by duck feather (59.6 U/ml). The other keratin substrates supported very low keratinase activity (4.3-22.1 U/ml). Cell growth and protein production had the same tendency as the production of keratinase; the highest concentration of protein was observed in feather meal medium followed by duck feather medium. Thus, the keratin degradation was related to the release of a large amount of protein into the medium.

4. Conclusion

From the present investigation, followings could be concluded. *B. megaterium* F7-1 could utilize chicken feather as a sole source of carbon, nitrogen and sulfur. Keratinase was produced in the absence of chicken feather. Supplementation of the feather medium with glucose increased keratinase production. Supplementation of nitrogen sources was not effective in producing keratinase. These results suggest that synthesis of keratinase by *B. megaterium* F7-1 is constitutive and is enhanced by additional glucose, and is regulated by nitrogen catabolite repression. Since the keratinase assay methods and growth media are different, it is difficult to compare the enzyme productivity of our result with those in the literature directly from the activity level in culture broth. However, *B. megaterium* F7-1 was suitable for the biodegradation of chicken feather and feather meal, providing potential use for biotechnological processes of keratin hydrolysis.

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