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Full Length Research Paper

Isolation and purification of alkaline keratinase from *Bacillus* sp. 50-3

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A new alkaline keratinase extracted from *Bacillus* sp. 50-3 was isolated and purified in this study. Solid ammonium sulfate was selected to precipitate the enzyme. Its proper adding mass was also determined. Through solid ammonium sulfate precipitation and liquid chromatography via the DEAE-Sephadex-A50 column using with azokeratin as a substrate, 17.7-fold purification with a yield of 46.5% was obtained. The purification effect was determined through SDS-PAGE, and the molecular weight of the enzyme was found at 27 423 Da by the MALDI-TOF-MS. Its simple purification step and high yield using a cheap medium attest to the great biotechnological potential of keratinase, especially in environment protection and in recycling valuable materials from wastes.

Key words: *Bacillus*, chicken feathers, environment protection, keratinase, purification.

INTRODUCTION

Microorganisms and their enzymes play an important role in improving industrial processes. Given that the enzyme-catalyzed reactions are highly efficient, can easily be selected and produce less pollution, they usually require mild conditions and less energy, resulting in lower costs (Cherry and Fidantset, 2003). The world market has a big need for industrial enzymes, estimated to be about US$ 1.6 billion; this market is split among food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%) (Outtrup and Jorgensen, 2002). Thus, conducting a study on isolating new enzymes and improving the purification technology used in industrial conversions is significant.

Keratin is the most abundant structural protein present in animal skin, horn, hair, wool, and feathers. It is rich in α-helix or β-sheet linked with cystine bridges (Bockle et al., 1995). Feather contains over 90% of crude protein in the form of keratin. Feather waste represents a potential protein alternative to more expensive dietary ingredients for animal feed (Onifade et al., 1998; El-Refai et al., 2005). However, the commonly known proteases could not degrade the keratin to a large degree; however it can be broken down by some keratinase-secreting microorganisms that turn native keratin into smaller molecular entities that, in turn, can subsequently be absorbed by cells (Bernal et al., 2006). In recent years, there have been many reports on the purification of keratinase from different microorganisms (Suntornsuk et al., 2005; Kojima et al., 2006; Riffel et al., 2007 Tatineni et al., 2008). However, there is still a need to find new enzymes and improve the purification technique employed in the biotechnological processes involving keratin-containing wastes from poultry and leather industries; this is especially true in the development of nonpolluting processes (Shih, 1993; Onifade et al., 1998; Wang et al., 2007).

Bacillus sp. 50-3 with high keratinase activity has been previously isolated from *Calotes versicolor* feces (Zhang et al., 2008). In this study, we report an efficient method for the isolation and purification of keratinase from the strain culture.

MATERIALS AND METHODS

Chemicals

Chemicals used in the experiment were from Oxoid Ltd. (Basingstoke, UK), Merck AG (Darmstadt, Germany), and Sigma (USA).

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Azokeratin was synthesized based on the method described in a previous study (Riffel et al., 2003). Chicken feathers were from the Beijing Huadu Chicken Factory (Beijing, China).

Microorganism

The *Bacillus* sp. 50-3 was restored from the Luria-Bertani (LB) agar medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, 15 g agar per liter, pH 7.2-7.6) and subsequently pre-inoculated in a 100 ml Erlenmeyer flask containing 20 ml of autoclaved LB (without agar) starter culture. This was followed by 12 h of incubation at 37°C and 150 rpm to obtain 10^6 colony-forming units (CFU)/ml.

Keratinase production

The bacilli from the 10^6 CFU/ml starter culture were inoculated by 2% (v/v) into 20 ml of chicken feather medium (10 g native chicken feather, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, per liter; pH 7.0-7.2) placed in 100 ml Erlenmeyer flasks. These samples were subsequently cultivated for 36 h to reach maximum keratinase activity at 37°C and 150 rpm. The samples were then centrifuged at 10 000 g for 10 min, after which the supernatant fluid was used for the crude enzyme preparation.

Enzyme assays

Keratinase activity was assayed with azokeratin as a substrate, in accordance with the modified method described by Riffel et al. (2003). The reaction mixture contained 200 µl of enzyme preparation and 1.6 ml of 10 g/l azokeratin in 50 mM glycine/NaOH buffer, with a pH of 10.0. The mixture was incubated for 15 min at 60°C, after which the reaction was stopped by adding the trichloroacetic acid to a final concentration of 100 g/l. After conducting centrifugation at 10 000 g for 10 min, the absorbance of the supernatant fluid was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm for 15 min at 60°C.

Protein assay

The protein content of the enzyme preparation was determined with bovine serum albumin as the standard protein according to the method previously described by Bradford et al. (1976). After performing column chromatography, a protein concentration estimation was undertaken by measuring the absorbance at 280 nm.

Determination of enzyme precipitation reagent

Three organic solvents and one salt were selected and added to the enzymatic preparation to precipitate the keratinase. Allowing it to stand overnight, the precipitates were collected by centrifugation at 10 000 g for 10 min at 4°C. Afterwards, these were respectively dissolved and dialyzed against a Tris HCl buffer (50 mM, pH 8.0). The keratinase activity and the protein content were assayed to select the proper precipitation reagent. The precipitating reagents were as follows: methanol (200%) (v/v), acetone (200%) (v/v), ethanol (200%) (v/v), and solid ammonium sulfate powder (90% saturation).

Determination of solid ammonium sulfate powder saturation

Four saturation stages were selected to determine the properly added quantity. Solid ammonium sulfate powder (0–30% saturation) was initially added to precipitate the protein in the supernatant. Next, the mixture was centrifuged at 10 000 g for 10 min at 4°C, after which the first precipitate was collected. Solid ammonium sulfate powder (30–40% saturation) was then continuously added to the resulting supernatant. After conducting centrifugation under the same conditions, the second precipitate was collected. The solid ammonium sulfate powder (40–80% saturation) was continuously added to the resulting supernatant, and the third precipitate was thereby collected. Finally, the 80–90% saturation of solid ammonium sulfate powder was added, also by centrifugation at 10 000 g for 10 min at 4°C, after which the fourth precipitate was collected. Afterwards, the four collected precipitates were respectively dissolved and dialyzed against the Tris HCl buffer (50 mM, pH 8.0). The keratinase activity and the protein content were then assayed.

DEAE-Sephadex-A50 chromatography

The proper dialyzed solution was applied to a column of DEAE-Sephadex-A50 (1.0 × 20 cm) (Pharmacia, Sweden). It was then equilibrated with 50 mM Tris HCl buffer, with a pH of 8.0. Subsequently, the column was eluted with a gradient of 0-1.0 M NaCl in 50 mM Tris HCl buffer with a pH of 8.0, after which 3 ml fractions were collected at a flow rate of 0.5 ml/min. Fractions with enzyme activity were pooled, dialyzed against distilled water, freeze-dried, and stored at -20°C until use. Chromatographic procedures were performed at 4°C.

Electrophoretic analysis

As described by Laemmli (1970), the protein purity and molecular mass of the enzyme were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 1 mm-thick slab gels containing 14.5% (w/v) polyacrylamide resolving gels and 4% (w/v) stacking gels. The lyophilized enzyme samples were solubilized in 65 mM Tris buffer, pH 6.8 and were then boiled for 5 min at 100°C upon the addition of 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. After staining the gels with Coomassie brilliant blue R-250, the electrophoretic migration of the protein was compared with that of low-molecular-mass protein markers (Pharmacia, Sweden). Zymography was determined according to the method described in the study of Riffel et al. (2007).

MALDI-TOF-MS analysis

As was mentioned, the purified keratinase was prepared according to the method of Riffel et al. (2007), which was conducted here prior to performing an analysis on a matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) spectrometer (Bruker-Reflex™□, Germany). Analyses were performed exclusively for positively charged ions in linear mode and were undertaken at the National Center of Biomedical Analysis in Beijing, China.

RESULTS

Keratinase production

Strain 50-3 was isolated from *Calotes versicolor* (an agamid lizard) feces showing high keratinase activity. After 36 h of culturing on native chicken feather as the sole carbon, nitrogen, and energy source, we found that it could completely degrade the chicken feather (Figure 1).

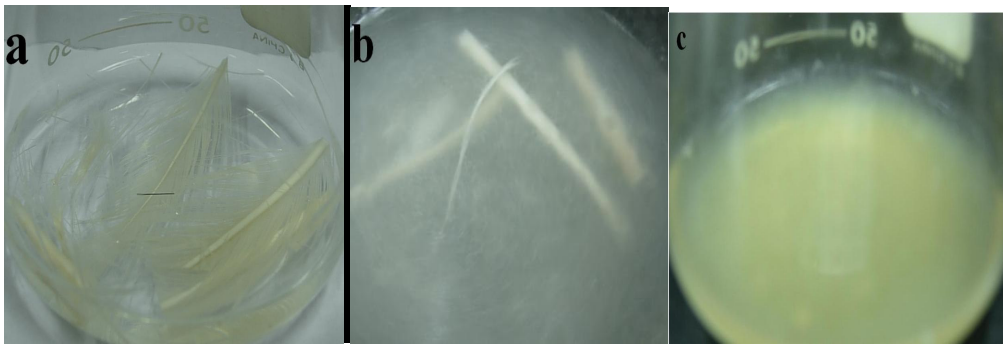

Figure 1. Strain 50-3 degraded the native chicken after 0 h (a), 24 h (b) and 36 h (c) at 37℃.

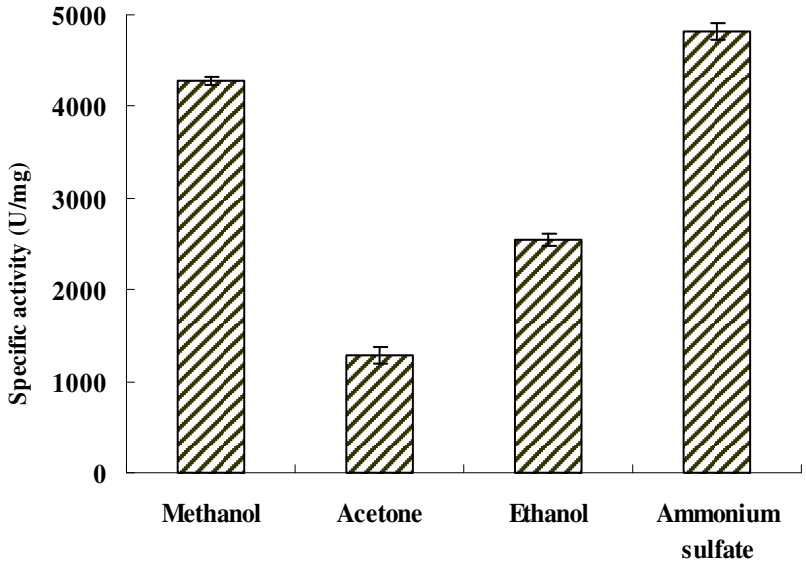

Figure 2. The respective activities after conducting the precipitation process using methanol, acetone, ethanol, and ammonium sulfate powder. The bars show the means and standard errors of the three replicate experiments.

At the same time, we also found that the keratinase reached 680 ± 25 U/ml at 37℃ with an initial pH level of 7.0.

Determination of the precipitation reagent

Three organic solvents and one salt were used to determine the proper precipitation reagent for keratinase by analyzing the specific activities. Among these, the solid ammonium sulfate powder (90% saturation) had the best precipitation effect (Figure 2). Protein also had the highest keratinase activity after precipitating by ammonium sulfate (data not shown). The protein precipitated by methanol, acetone, and ethanol demonstrated the same keratinase activity, but the protein precipitated by acetone was more than that produced by the other two; thus its specific activity was relatively low.

Determination of the ammonium sulfate saturation

To remove unwanted proteins from the crude enzyme solution, the proper amount of ammonium sulfate to be added was determined in the experiment. From Table 1, the specific activity of precipitated keratinase indicated that 40–-80% saturation of $(NH_4)_2SO_4$ had the best effect on enzyme purification. Although the 0–30% saturation of precipitate had enzyme activity, it contained much unwanted proteins. In contrast, at 80–90% saturation stage, no proteins were detected.

Purification through DEAE-Sephadex-A50 chromatography

The keratinase was purified through the ion exchange column. Measurement by keratinase assay enabled 17.7-

Table 1. Effect of different $(NH_4)_2SO_4$ saturation levels on keratinase purification.

Saturation of $(NH_4)_2SO_4$	Keratinase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
0%–30%	260	0.344	755.8
30%–40%	55	0.105	523.8
40%–80%	679	0.141	4815.6
80%–90%	0	0	0

Table 2. Purification of keratinase from *Bacillus* sp. 50-3.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Supernatant	78667	80.2	980.9	100	1
40%–80% $(NH_4)_2SO_4$ precipitation	67899	14.1	4815.6	86.3	4.9
DEAE-Sephadex-A50	36560	2.1	17409.5	46.5	17.7

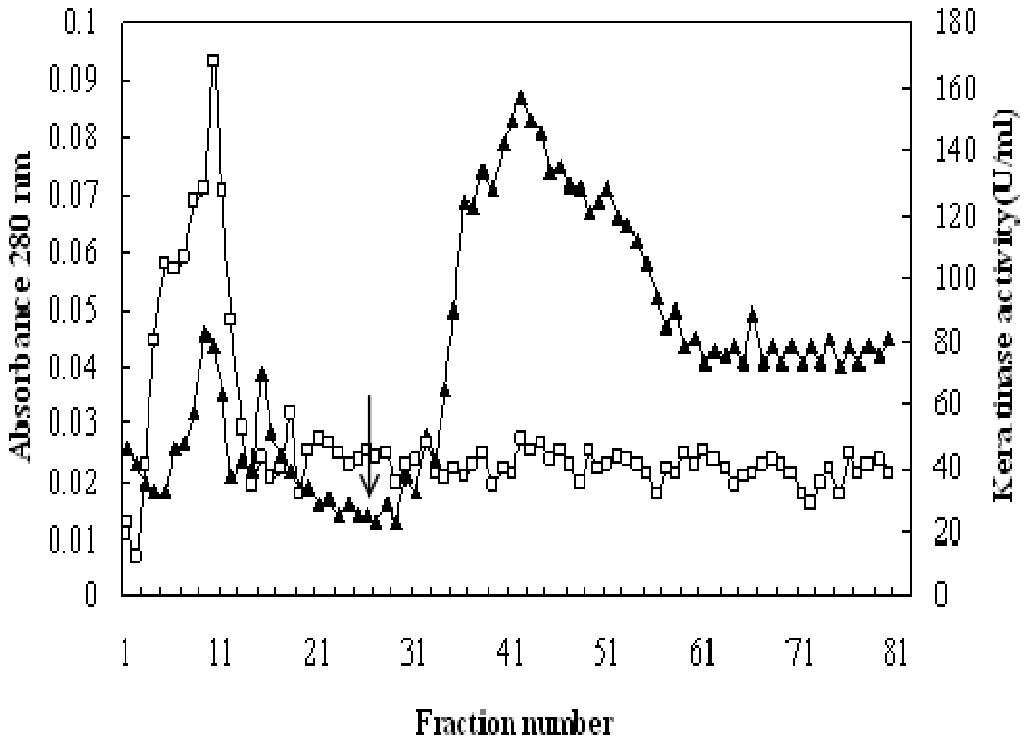

Figure 3. Keratinase purification via the DEAE-Sephadex-A50 ion exchange column (open square) keratinase activity; (closed triangle) protein concentration. Arrows indicate the start of the linear gradient from 0 to 1.0 M NaCl.

fold purification of keratinase, with a yield of 46.5%. The maximum specific enzyme activity was determined at 17 409.5 U/mg. After adding 40-80% ammonium sulfate, the precipitate was then dissolved and dialyzed in 50 mM Tris HCl buffer at pH 8.0, and submitted to ion exchange chromatography on a DEAE-Sephadex-A50 column (Table 2). The enzyme activity eluted as a single peak which coincides with a small absorbance at 280 nm (Figure 3).

Electrophoretic and MALDI-TOF-MS analysis

Electrophoretic and MALDI-TOF-MS analyses were carried out in order to determine the purified keratinase.

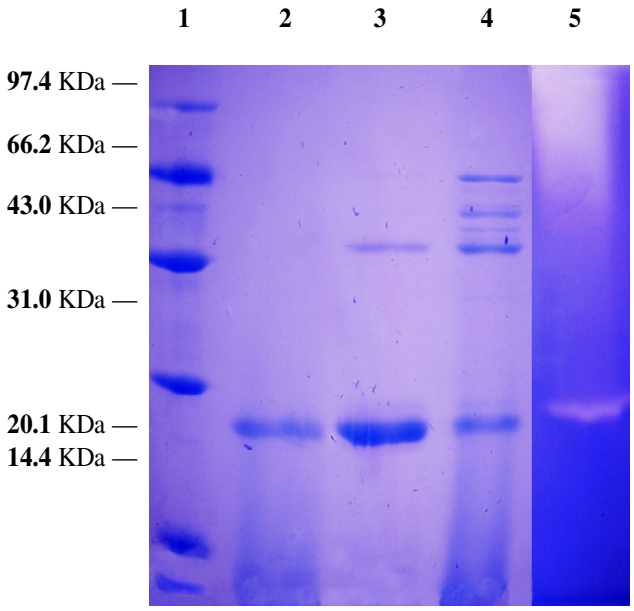

Figure 4. SDS-PAGE of keratinase obtained from *Bacillus* sp. 50-3. Lane 1: molecular weight standards (Rabbit Phosphorylase b, 97.4 KDa; Bovine Serum Albumin, 66.2 KDa; Rabbit Actin, 43 KDa; Bovine Carbonic Anhydrase, 31KDa; Trypsin Inhibitor, 20.1 KDa; Hen Egg White Lysozyme, 14.1 KDa); Lane 2: purified keratinase; Lane 3: crude keratinase after 40%–80% of $(NH_4)_2SO_4$ precipitation; Lane 4: crude keratinase. Lane 5: zymography of purified keratinase.

Specifically, the purified enzyme was analyzed by polyacrylamide gel electrophoresis, and a unique protein band was visualized after ion exchange chromatography in comparison with the two previous purified steps (Figure 4). Compared with molecular weight standards, the enzyme in the SDS-PAGE showed a molecular weight of approximately 30 KDa. Zymogram activity staining also revealed a clear band of proteolytic activity (Figure 4). Mass spectrometry analysis was later carried out to determine the accurate molecular weight of the protein, which also revealed a single peak with a molecular weight of 27 423 Da (data not shown).

DISCUSSION

The newly isolated *Bacillus* sp. 50-3 is a novel *Bacillus* strain which can degrade feather keratin. Compared to most other keratin-degrading strains, it can degrade the native feather in much shorter time (36 h) and has a relatively high keratinase activity (680 ± 25 U/ml) under the same conditions (Lin et al., 1999; Nam et al., 2002; Riffel et al., 2003; El-Refai et al., 2005). This suggests its potential use in biotechnological processes involving environment protection. The strain isolated from *C. versicolor* feces showed the highest keratinase activity, which could be considered a microorganism of environmental origin. As it is known, *C. versicolor* is an agamid lizard found in Asia. Its main food source includes insects such as crickets, beetles, and spiders. Therefore, there could be microorganisms that colonize its gastrointestinal tract and degrade the insects' keratin-filled scute in order to facilitate efficient digestion (Bevilacqua et al., 2003; Angelis et al., 2006).

The keratinase precipitated by the ammonium sulfate powder had been reported in many previous studies (Thys and Brandelli, 2006; Tatineni et al., 2008; Wang et al., 2007). However, the selection of proper precipitating reagents and proper ammonium sulfate saturation to precipitate the enzyme had not been reported so far. It is important to add the proper mass of ammonium sulfate to isolate the keratinase from crude culture solutions, because it can remove many unwanted proteins in the culture, simplifying the following processes and improving efficiency.

The purification process includes just two steps. In contrast, some purification protocols described for *Bacillus* and other microorganisms' keratinases often involve more than two steps that normally contain two kinds of chromatography procedures (Suntornsuk et al., 2005; Kojima et al., 2006; Thys and Brandelli, 2006; Riffel et al., 2007). In this study, the purified enzyme comes from a poor medium containing just keratin protein; thus, keratinase could be purified through a relatively simple procedure (Thys and Brandelli, 2006). On the other hand, the 0–40% ammonium sulfate precipitation may have removed many unwanted proteins. Hence, this approach to the purification of keratinase from *Bacillus* sp. 50-3 expended less time and energy, further increasing its biotechnological potential.

The detection of a unique band through the SDS-PAGE confirmed the purification of the keratinase from *Bacillus* sp. 50-3. The proteolytic activity of the keratinase after the final purification (17409.5 U/mg) increased approxi-mately 18-fold compared to that of the crude extract (980.9 U/mg). The level of purification is higher than those reported in other similar papers (Kojima et al., 2006; Moallaei et al., 2006; Riffel et al., 2007; Tatineni et al., 2008), and the purify-cation procedure also had a higher recovery rate (46.5%). This indicates its potential use in biotechnological processes that hasten the period of recycling the feather keratin from industrial wastes. The study also identified the molecular weight (MW) of the *Bacillus* sp. 50-3 keratinase as 27 423 Da. It belongs to the molecular weight range of major keratinases, vary-ing from 20 to 50 KDa (Bockle et al., 1995). Likewise, it is similar to the keratinase MW of *Bacillus pseudofirmus* FA30-01 at 27 KDa (Kojima et al., 2007) and *Bacillus licheniformis* PWD-1 at 30 KDa (Lin et al., 1992).

The production of keratinase from *Bacillus* sp. 50-3 is straightforward and easy to scale up; the organism grows on simple media with feathers as its sole carbon, nitrogen, and energy source. Hence, it is possible to culture an organism with great commercial potential using an inexpensive substrate, resulting in low production cost. At the same time, it transforms a kind of industrial waste

(chicken feather) into the required nutritional feed additives, thereby protecting the environment by minimizing wastage. Further studies should therefore be carried out in order to evaluate the biotechnological potential of this keratinase in processes involving keratin hydrolysis.

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