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Purification and characterization of keratinase from a new *Bacillus subtilis* strain

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Abstract: The aim of this study was to purify and characterize a keratinase produced by a new isolated *Bacillus subtilis* KD-N2 strain. The keratinase produced by the isolate was purified using ammonium sulphate precipitation, Sephadex G-75 and DEAE (diethylaminoethyl)-Sepharose chromatographic techniques. The purified enzyme was shown to have a molecular mass of 30.5 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The optimum pH at 50 °C was 8.5 and the optimum temperature at pH 8.5 was 55 °C. The keratinase was partially inactivated by some metal ions, organic solvents and serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF). Sodium dodecyl sulfate (SDS) and ethylene diamine tetraacetic acid (EDTA) had positive effect on the keratinase activity. Reducing agents including dithiothreitol (DTT), mercaptoethanol, L-cysteine, sodium sulphite, as well as chemicals of SDS, ammonium sulfamate and dimethylsulfoxide (DMSO) stimulated the enzyme activity upon a feather meal substrate. Besides feather keratin, the enzyme is active upon the soluble proteins ovalbumin, bovine serum albumin (BSA), casein and insoluble ones as sheep wool and human hair. Calf hair, silk and collagen could not be hydrolyzed by the keratinase.

Key words: Ammonium sulfatate, *Bacillus subtilis*, Characterization, Feather, Keratin, Keratinase, Purification, Reducing agents
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INTRODUCTION

Feathers are largely produced as a waste by-product at poultry plants (Williams *et al*., 1991). They are insoluble structural proteins cross-linked by disulfide, hydrogen and hydrophobic bonds but could represent a rich protein resource because they contain over 90% (w/w) keratins. Keratins cannot be degraded by the usual proteolytic enzymes such as pepsin, trypsin and papain. Nevertheless, feathers do not accumulate in nature because keratins could be degraded by keratinases (EC 3.4.21/24/99.11) produced by some microorganisms (Onifade *et al*., 1998). Many keratinases from species of *Bacillus* (Williams *et al*., 1990; Riffel *et al*., 2003; Lucas *et al*., 2003), fungi (El-Naghy *et al*., 1998; Gradišar *et al*., 2000; Friedrich *et al*., 2005) and *Actinomycetes* (Ignatova *et al*., 1999; Gushterova *et al*., 2005) had been reported and some of them were purified and characterized (Lin *et al*., 1992; Böckle *et al*., 1995; Nam *et al*., 2002). Different keratinases vary in their characteristics. Until now, only a few keratinases from *Bacillus* sp. had been purified.

Keratinases from microorganisms have many applications in the feed, fertilizer, detergent, leather and pharmaceutical industries (Gupta and Ramnani, 2006). For example, the feather hydrolysates of *Bacillus licheniformis* PWD-1 and *Vibrio* sp. strain kr2 (Williams *et al*., 1991; Grazziotin *et al*., 2006) can be used as animal food. Indeed, addition of the crude keratinase from *B. licheniformis* PWD-1 improved the poultry growth (Odetallah *et al*., 2003). Also, the keratinase can degrade the infectious form of prion, PrP^{sc}, in the presence of detergents and heat treatment (Langeveld *et al*., 2003), which could be important for the utilization of animal meal as feed. As for

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leather industry, the keratinase from *Bacillus subtilis* S14 exhibits remarkable dehairing capabilities (Macedo *et al*., 2005) without the degradation of collagen and this ecofriendly dehairing approach shows great utilization potential.

We had screened a new feather-degrading *Bacillus subtilis* KD-N2 strain which degraded feathers completely within 30 h in submerged cultivation (Cai *et al*., 2008), and the crude keratinase from the isolate showed capabilities of removing calf hair and sheep wool from the skins. The aim of this study was to purify and characterize the keratinase produced in feathers substrate by this *Bacillus* spp. isolate.

MATERIALS AND METHODS

Bacteria and growth conditions

Strain KD-N2 was screened from a local poultry plant and kept in our laboratory (Cai *et al*., 2008). The medium (pH 7.2) used for keratinase production contained the following constituents (g/L): NaCl 0.5, KH_2PO_4 0.7, K_2HPO_4 1.4, $MgSO_4$ 0.1 and feathers 10. Cultivation was performed using 500 ml Erlenmeyer flasks containing 100 ml medium for 24 h at 28 °C with constant shaking at 200 r/min. As inocula, 5% (v/v) bacteria grew in Luria-Bertani broth [peptone 1% (w/v), yeast extract 0.3% (w/v) and NaCl 0.5% (w/v), pH 7.2] for 20 h. Culture supernatants obtained after centrifugation at 8000×*g* for 20 min were used for further study.

Assay of keratinase activity

Keratin azure (Sigma-Aldrich, USA) was used as the substrate. It was first frozen at −20 °C and then ground into a fine powder. The 5 mg keratin azure powder was suspended in 1 ml 50 mmol/L Tris-HCl buffer (pH 8.0). The reaction mixture contained 1 ml keratin azure suspension and 1 ml appropriately diluted enzyme. The reactions were carried out at 50 °C in a water bath with constant agitation of 200 r/min for 30 min. After incubation, the reactions were stopped by adding 2 ml 0.4 mol/L trichloroacetic acid (TCA) and followed by centrifuging at 3000×*g* for 20 min to remove the substrate. The supernatant was spectrophotometrically measured for release of the azo dye at 595 nm. The 1 ml keratin azure suspension in the same buffer (like that of the sample) was agitated for 30 min at 50 °C, then was added 2 ml 0.4 mol/L TCA and 1 ml enzyme solution as a control. One unit (U) keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under the conditions given.

Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin (BSA) (Sigma, USA) as standard. The specific activity was expressed as the enzymatic activity (U) per mg of protein.

Purification of keratinase

All operations were performed at room temperature. After centrifugation at 8000×*g* for 20 min, solid ammonium sulphate was added to the supernatant to achieve 30% saturation, and then centrifuged to remove the pellet. The enzyme was precipitated from the supernatant by addition of solid ammonium sulphate, with gentle stirring until 80% saturation, and then allowed to stand for 12 h followed by centrifugation at 8000×*g*. The pellet was dissolved in 20 mmol/L Tris-HCl buffer (pH 8.0) and applied to a Sephadex G-75 column (6.0 cm×60.0 cm), which was processed at a flow rate of 10 ml/h with 20 mmol/L Tris-HCl buffer (pH 8.0) and every 3 ml fraction was collected. Active fractions were collected, concentrated by polyethylene glycol 2000 (PEG 2000) and applied to a DEAE (diethylaminoethyl)-Sepharose Fast Flow (FF) column (1.9 cm× 20.0 cm). Samples were eluted at a flow rate of 6 ml/min with different concentrations of sodium chloride solution in 20 mmol/L Tris-HCl buffer (pH 8.0) and every 6 ml fraction was collected. Then the active fractions with PEG 2000 were concentrated and applied to a Sephadex G-75 column (1.0 cm×60.0 cm) at a flow rate of 6 ml/h. The 3 ml fractions were collected. The active fractions were then concentrated by PEG 2000 for further analysis.

Molecular mass determination

Polyacrylamide gel electrophoresis (PAGE) (12%, w/w) in the presence of sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (1970). The electrophoresed protein gels were stained with Coomassie brilliant blue R250 (Sigma, USA).

Characterization of the keratinase

To determine the optimal temperature for keratinolysis, enzyme reactions were carried out at different temperatures for 30 min. In order to investigate thermostability, the enzyme solution was pre-incubated for 20, 40, 60, 80, 100 and 120 min at 55 °C, and then the residual activity was measured. The optimum pH was determined at 55 °C using the following buffers (50 mmol/L): sodium phosphate buffer (pH 6.0~7.5), Tris-HCl buffer (pH 7.5~9.0), and Glycine/NaOH buffer (pH 9.0~10.0).

The effects of metal ions, enzyme inhibitor, detergent and organic solvents on keratinase activity were studied by assaying the enzyme activity as described above after pre-incubation with each chemical for 10 min at room temperature. The concentrations of the chemicals are as follows: 5.0 mmol/L of Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+}, SDS and phenylmethanesulfonyl fluoride (PMSF); 2.5, 5.0 and 10.0 mmol/L ethylene diamine tetraacetic acid (EDTA); 1% (v/v) of methanol, ethanol, dimethyl sulfoxide and isopropyl alcohol.

Substrate specificity of keratinase

To determine the keratinase specificity, the following substrates were used: casein (Sigma, USA), BSA (Sigma, USA), ovalbumin, type I collagen (Worthington, USA), chicken feather, goat hair, calf hair and human hair. The incubation procedures were the same as described for keratinase activity determination except that every incubation contained 20 mg substrate and 50 μl purified keratinase. The extent of hydrolysis was determined spectrophotometrically at 280 nm by measuring TCA-soluble peptides released from the substrates during incubation.

Effects of chemicals on feather meal hydrolysis

To determine the effects of chemicals on feather meal hydrolysis, 1 or 5 mmol/L dithiothreitol (DTT), SDS, sodium sulphite, L-cysteine, ammonium sulfamate and 1% (v/v) or 5% (v/v) mercaptoethanol, dimethylsulfoxide (DMSO) were added to 2 ml of 50 mmol/L Tris-HCl (pH 8.5) buffer containing 20 mg feather meal and 50 μl purified enzyme. After incubation for 1 h, the reaction was stopped by adding 2 ml 0.4 mol/L TCA. An incubation of feather meal and TCA for 1 h before enzyme addition was carried out as a control. The extent of feather meal hydrolysis was determined spectrophotometrically at 280 nm by measuring TCA-soluble peptides released from the substrate during incubation.

RESULTS AND DISCUSSION

Feather degradation and keratinase production

B. subtilis KD-N2 strain produced inducible keratinase in feathers substrate and degraded feathers completely in 30 h (Cai *et al*., 2008). Feathers were the mostly utilized substrate for keratinase production. Accompanied by feather degradation and keratinase production, the pH value of the culture increased to about 8.5. *B. subtilis* strains had been widely utilized for enzyme production, including the keratinases (Lal *et al*., 1999; Suh and Lee, 2001; Kim *et al*., 2001; Macedo *et al*., 2005). Other *Bacillus* species including *B. licheniformis*, *B. pumilis*, *B. cereus*, *B. halodurans* and *B. pseudofirmus* (Williams *et al*., 1990; Takami *et al*., 1999; Rozs *et al*., 2001; Kim *et al*., 2001; Gessesse *et al*., 2003; El-Refai *et al*., 2005) had also been reported for their keratinolytic activity. Among these strains, *B. subtilis* KD-N2 degraded feathers more quickly than any other reported until now, except for *B. licheniformis* RG1, which degraded feathers completely in 24 h (Ramnani and Gupta, 2004). Among the keratinases reported, only two of them had been purified (Suh and Lee, 2001; Macedo *et al*., 2005).

Enzyme purification

The results of the purification procedures are summarized in Table 1. SDS-PAGE analysis of the sample revealed a single band (Fig.1a), indicating that the keratinase was purified. The overall purification factor was about 12.7-fold, and the final yield was 4.6%. The final product had a specific activity of about 63.3 U/mg.

Molecular mass of the keratinase

The molecular mass of the keratinase was estimated by comparing the electrophoretic mobility of the enzyme with the electrophoretic mobilities of marker proteins. The apparent molecular mass was 30.5 kDa (Fig.1b). Molecular masses of keratinases range from 18 to 200 kDa, except for a unique enzyme from a pathogenic fungi (Gupta and Ramnani,

Table 1 Purification of keratinase from *B. subtilis* KD-N2

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification	Yield (%)
Crude enzyme	206.40	1040.0	5.0	1.0-fold	100.0
Sephadex G-75 (6.0 cm×60.0 cm)	10.20	132.6	13.0	2.6-fold	12.8
DEAE-Sepharose FF (1.9 cm×20.0 cm)	1.40	73.0	51.9	10.4-fold	7.0
Sephadex G-75 (1.0 cm×60.0 cm)	0.76	48.0	63.3	12.7-fold	4.6

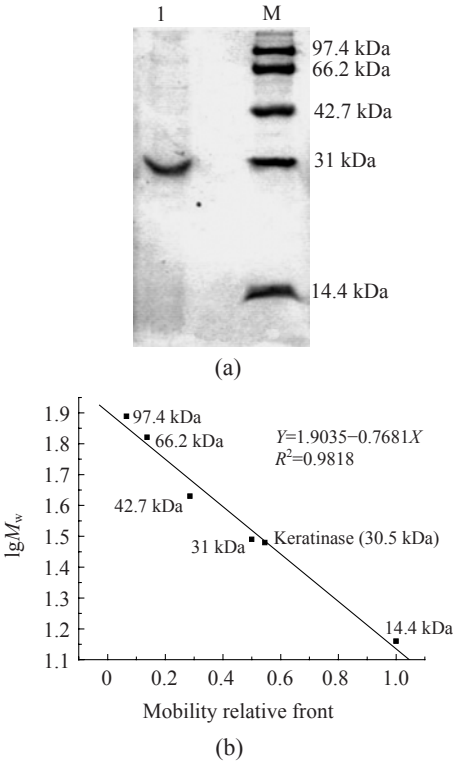

Fig.1 (a) SDS-PAGE of the purified keratinase and marker proteins and (b) molecular weight determination of the purified keratinase

Lane 1: Purified keratinase; Lane M: Molecular mass of marker proteins (Promega, USA): rabbit muscle phosphorylase b 97.4 kDa, bovine serum albumin 66.2 kDa, chicken egg ovalbumin 42.7 kDa, bovine erythrocytes carbonic anhydrase 31 kDa and chicken egg white lysozyme 14.4 kDa

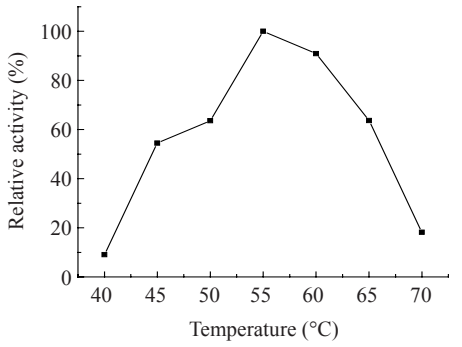

Fig.2 Effect of temperature on the activity of keratinase from *Bacillus subtilis* KD-N2

Assays were performed at different temperatures in 50 mmol/L Tris-HCl buffer (pH 8.0) for 30 min

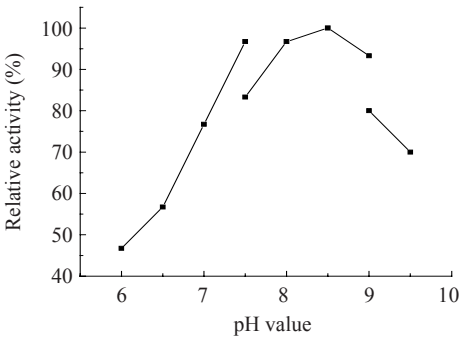

Fig.3 Effect of pH on the activity of keratinase from *Bacillus subtilis* KD-N2

Assays were performed at 55 °C using the following buffers (50 mmol/L): sodium phosphate buffer (pH 6.0~7.5), Tris-HCl buffer (pH 7.5~9.0), and glycine/NaOH buffer (pH 9.0~10.0)

2006). For *Bacillus* species, they are of medium size, such as 33 kDa (*B. licheniformis*) (Lin *et al*., 1992), 25.4 kDa (*B. subtilis*) (Suh and Lee, 2001) and 24 kDa (*B. paeudofirmis*) (Gessesse *et al*., 2003).

Effects of temperature and pH on the keratinase activity

Fig.2 shows that the keratinase was active over a temperature range of 40~70 °C, with an optimum at 55 °C. Fig.3 shows that the keratinase was active at neutral and alkaline conditions, with optimum at pH 8.5, and the most suitable buffer seemed to be Tris-HCl. Most keratinases possess an activity optimum in the range of 30~80 °C, for example, keratinase from *B. pseudofirmus* AL-89 is of 60~70 °C (Gessesse *et al*., 2003), *Nocardiopsis* sp. TOA-1 is of 60 °C (Mitsuiki *et al*., 2004), and a few have exceptionally high temperature optimum of 100 °C (Nam *et al*., 2002). Most keratinases are active in neutral to alkali conditions, from pH 7.0 to pH 9.5. For example, the activity optimum of keratinase from *Microbacterium* kr10 is pH 7.0 (Thys *et al*., 2004), *B. pumilus* FH9 of pH 8.0 (El-Refai *et al*., 2005), *Fervidobacterium islandicum* AW-1 of pH 9.0 (Nam *et al*., 2002), and a

few of extreme alkalophilic optima at pH 12~13 (Takami *et al*., 1999) and pH 12.5 (Mitsuiki *et al*., 2004).

Effects of metal ions, enzyme inhibitor, detergent and organic solvents on the keratinase activity

The keratinase from *B*. *subtilis* KD-N2 was totally inhibited by Cu^{2+}, Mn^{2+} and partially by Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+}, methanol, ethanol, dimethyl sulfoxide, isopropyl alcohol, and was stimulated by SDS and EDTA (Table 2). Generally, heavy metal ions such as Cu^{2+} (Nam *et al*., 2002; Riffel *et al*., 2003; Thys *et al*., 2004), Hg^{2+} (Riffel *et al*., 2003; Thys *et al*., 2004) and Zn^{2+} (Thys *et al*., 2004) have inhibitory effects on keratinolytic activity. Contrarily, Ca^{2+}, Mg^{2+} and Mn^{2+} stimulate some keratinases (Nam *et al*., 2002; Riffel *et al*., 2003). But for the keratinase from *B*. *subtilis* KD-N2 strain, all metal ions have negative effects on its activity. Serine-proteinase inhibitor PMSF partially inhibited the keratinase activity, which is identical to most of the keratinases studied except for a few from *Paecilomyces marquandii*, *Doratomyces microsporus* and *Xanthomonas maltophilia*, which are fully inhibited (de Toni *et al*., 2002; Gradišar *et al*., 2005). EDTA has negative effects on activities of mostly reported keratinases, but we observed in this study that the keratinase from *B*. *subtilis* KD-N2 strain was stimulated by 5 mmol/L EDTA, similar to the keratinase from *Fervidobacterium islandicum* AW-1 (Nam *et al*., 2002). Further results indicate that 2.5 and 10 mmol/L EDTA could also stimulate the keratinase activity (Table 2). Organic solvents inhibited the keratinase activity to some degree, which is different from keratinases from *Chryseobacterium* sp. (Riffel *et al*., 2003) and *Nocardiopsis* sp. TOA-1 (Mitsuiki *et al*., 2004). The detergent SDS has positive effects on keratinase activity, which is similar to the keratinase from *Streptomyces albidoflavus* (Bressollier *et al*., 1999), but differs from the keratinases from *Streptomyces pactum* DSM 40530 (Böckle *et al*., 1995), *Chryseobacterium* sp. (Riffel *et al*., 2003) and *Nocardiopsis* sp. TOA-1 (Mitsuiki *et al*., 2004). Proteases are widely used in the detergent industry, and it is important for the keratinase to be used in the presence of detergents such as SDS. Compared with previously reported keratinolytic enzymes, the keratinase from *B*. *subtilis* KD-N2 showed some novel characterizations and utilization potential.

Table 2 Effect of chemicals on the keratinolytic activity of *Bacillus subtilis* KD-N2

Chemicals	Concentration	Relative activity* (%)
None		100
Ca^{2+}	5 mmol/L	44.8±3.4
Mg^{2+}	5 mmol/L	41.4±3.2
Cu^{2+}	5 mmol/L	0
Mn^{2+}	5 mmol/L	0
Zn^{2+}	5 mmol/L	34.5±3.1
Al^{3+}	5 mmol/L	75.9±6.9
SDS	5 mmol/L	158.6±13.8
EDTA	2.5 mmol/L	131.8±5.6
EDTA	5 mmol/L	136.4±6.2
EDTA	10 mmol/L	144.8±6.6
Methanol	1%, v/v	79.3±7.9
Ethanol	1%, v/v	62.1
Dimethyl sulfoxide	1%, v/v	51.7±3.5
Isopropyl alcohol	1%, v/v	75.9±6.9
PMSF	5 mmol/L	79.3±3.4

*Values are mean of three independent determinations

Substrate specificity of the keratinase

The keratinase from KD-N2 shows activity on the soluble proteins casein, BSA, ovalbumin and the insoluble substrates feather meal, feather keratin, human hair and sheep wool, but no activity was observed upon collagen, calf hair and silk (Table 3). Many keratinases are capable of hydrolyzing a broad range of soluble and insoluble proteins, as the enzymes from *Streptomyces pactum* DSM40530 (Böckle *et al*., 1995), *B*. *subtilis* KS-1 (Suh and Lee, 2001) and *Fervidobacterium islandicum* AW-1 (Nam *et al*., 2002). The keratinase from the isolate had the ability to remove hair from calf and sheep skins (data not shown), but could not hydrolyze collagen, which is the main constituent of the dermis. Indeed, similar result had been reported for keratinase from *B*. *subtilis* S14 (Macedo *et al*., 2005). Type I collagen could be hydrolyzed by keratinases from *Streptomyces albidoflavus* (Bressollier *et al*., 1999), *B*. *subtilis* KS-1 (Suh and Lee, 2001) and *Fervidobacterium islandicum* AW-1 (Nam *et al*., 2002). And keratinases from *Paecilomyces marquandii* and *Doratomyces microsporus* were able to hydrolyze different keratin-containing substrates such as stratum corneum keratin, human nail, porcine nail and bovine keratin (Gradišar *et al*., 2005). Comparing with the substrates listed in Table 3, it seems that insoluble substrates such as human hair and feather meal, which have

more disulfide bonds, were more easily hydrolyzed by the purified keratinase than sheep wool, which has less disulfide bonds. Previous studies showed that ball milling could destroy the keratin structure, which makes the substrates easily to be degraded by proteases (Stahl *et al*., 1949; Noval and Nickerson, 1959). The keratinase from *B*. *subtilis* KD-N2 has a broad range of substrates specificity and could degrade substrates containing both α and β keratins.

Table 3 Hydrolysis of various proteins by keratinase from *Bacillus subtilis* KD-N2

Substrates	Relative activity (%)	Substrates	Relative activity (%)
Ovalbumin	100	Collagen	0
BSA	56±5.2	Human hair	62±4.5
Casein	68±6.7	Wool	12±0.83
Feather keratin	20±2.4	Calf hair	0
Feather meal	80±5.3	Silk	0

The enzyme was incubated with 20 mg of substrate in 2 ml of 50 mmol/L Tris-HCl buffer (pH 8.5) for 30 min at 55 °C. Feather keratin was prepared by the method of Wawrzkiewicz *et al*.(1987) with little modification. After solubilization of native feather keratin in dimethyl sulfoxide and precipitation by cold acetone, the keratin was washed twice with distilled water and dried at 40 °C in a vacuum drier to constant weight. Other insoluble substrates were cut into short fragments

Effects of chemicals on feather meal hydrolysis

Fig.4 shows that sulphur-containing chemicals, especially reducing agents, stimulated the keratinase activity towards feather meal. All tested chemicals had positive effects on the feather meal hydrolysis except 1 mmol/L sodium sulfite. Five mmol/L DTT, 5 mmol/L ammonium sulfamate and 5% (v/v) β-mercaptoethanol enhanced keratinolytic activity maximally. Five mmol/L of DTT, β-mercaptoethanol, ammonium sulfamate and sodium sulfite had more positive effects than 1 mmol/L, but for L-cysteine, SDS and DMSO, lower concentrations had more effects. Reducing agents such as DTT, β-mercaptoethanol, cysteine and sodium sulfite had been largely discussed on their positive effects on keratinases (Böckle *et al*., 1995; Bressollier *et al*., 1999; Ignatova *et al*., 1999; Nam *et al*., 2002; Gradišar *et al*., 2005) and some of the enzymes are thiol-activated (Gupta and Ramnani, 2006). To test the effects of some sulphur-containing chemicals on the keratinase activity, ammonium sulfamate was firstly utilized in the study, and it had positive effects in both low and high concentrations. DMSO is able to solubilize feather keratin (Wawrzkiewicz *et al*., 1987), and has positive effects on feather meal hydrolysis in this study. Generally, purified keratinases cannot degrade keratin in vitro without the presence of reducing agents (Böckle *et al*., 1995; Bressollier *et al*., 1999; Suh and Lee, 2001; Riffel *et al*., 2003). And until now, detailed mechanisms of keratin hydrolysis has not been elucidated. One of the possible mechanisms is the reduction of disulfide bonds or sulfitolysis of the disulfide bonds by secreted sulfite. In this study, the reducing agents, DTT, β-mercaptoethanol, cysteine, sodium sulfite and sulphur-containing chemicals as SDS, ammonium sulfatate and DMSO stimulated the feather meal hydrolysis, which was probably due to the direct breakdown of the disulfide bonds by the reducing agents or due to reactions caused by the sulphur-containing chemicals.

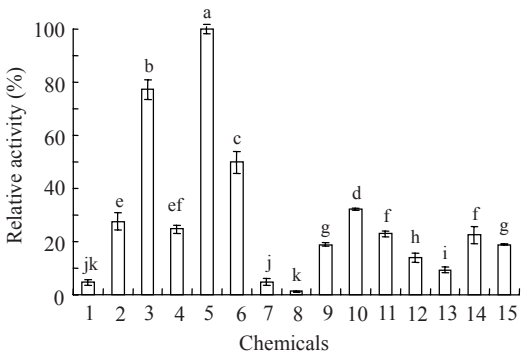

Fig.4 Effects of chemicals on the purified keratinase activity on feather meal

Chemicals were added to the incubation mixtures to reach a final concentration as follows: 1: Control; 2: 1 mmol/L DTT; 3: 5 mmol/L DTT; 4: 1% (v/v) β-mercaptoethanol; 5: 5% (v/v) β-mercaptoethanol; 6: 1 mmol/L SDS; 7: 5 mmol/L SDS; 8: 1 mmol/L sodium sulphite; 9: 5 mmol/L sodium sulphite; 10: 1 mmol/L L-cysteine; 11: 5 mmol/L L-cysteine; 12: 1 mmol/L ammonium sulfamate; 13: 5 mmol/L ammonium sulfamate; 14: 1% (v/v) DMSO; 15: 5% (v/v) DMSO. Bar represents standard error (*SE*) of the mean. Means with the different letters are significantly different according to Duncan's multiple range test at P=0.05 using SAS (SAS Institute, version 6.12, Cary, NC)

CONCLUSION

The *B*. *subtilis* KD-N2 degraded feathers quickly, and the enzyme produced by this isolate seems to be a new keratinase. The enzyme had a molecular mass of 30.5 kDa and was partially inactivated by PMSF. The keratinase is different from another purified *B*. *subtilis* keratinase (Suh and Lee, 2001) in molecular mass and sensitivity to inhibitors, which has a molecular

mass of 25.4 kDa and was completely inactivated by PMSF. The keratinase from *B. subtilis* KD-N2 was inhibited by metal ions and organic solvents to some degree and stimulated by SDS and EDTA. The enzyme showed activity upon feather meal, human hair as well as sheep wool. SDS and some reducing agents had positive effects on the keratinase activity. The keratinase was able to remove hair from the calf and sheep skins. The hydrolysis of collagen and degradation of the dermis should be further characterized, although the enzyme was not able to hydrolyze pure collagen. All the results present potential utilization of the keratinase industrially in keratin hydrolysis, feed nutrient improvement, and hair removing.

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