Postprint: Study on the Lipolytic Enzyme from Bacillus licheniformis CP-16

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Abstract

This study aimed to obtain the lipolytic enzyme from Bacillus licheniformis CP-16 through heterologous expression and investigate its role in feather degradation. Using genomic DNA of Bacillus licheniformis CP-16 as a template, the lipolytic enzyme gene was amplified and transformed into Escherichia coli for expression, yielding the recombinant enzyme L-4. The optimal pH, pH stability, optimal temperature, thermal stability, and effects of organic solvents and metal ions on its relative activity were investigated, along with its effect on keratinase K hydrolysis of native feather keratin. The results showed that the obtained lipolytic enzyme gene was 747 bp in size, encoding 248 amino acids. Recombinant enzyme L-4 was successfully expressed in Escherichia coli with a molecular mass of approximately 28.3 kDa, esterase activity of 0.42 U/mL, optimal pH of 6.5, and optimal temperature of 50 °C. After 30 min treatment at pH 6.5–9.5, the relative activity remained above 80%, and after 30 min treatment at temperatures below 50 °C, the relative activity remained above 70%. Fe^{2+}, Na^{+}, Mn^{2+}, and Ca^{2+} ions exhibited activating effects on the relative activity of recombinant enzyme L-4, whereas Ba^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} ions showed inhibitory effects. At an organic solvent concentration of 30%, recombinant enzyme L-4 retained 97% and 85% relative activity in dimethyl sulfoxide (DMSO) and methanol, respectively, maintained above 45% relative activity in acetone and ethanol, preserved less than 20% relative activity in isopropanol, and essentially completely lost its relative activity in acetonitrile. Pretreatment of native feather substrate with recombinant enzyme L-4 enhanced the hydrolysis efficiency of keratinase K on the substrate, with a promotion rate of 4.32%. These results demonstrate that lipolytic enzymes can degrade the lipid layer on feather surfaces and play a role in promoting keratinase hydrolysis of feather keratin.

Full Text

Research on Lipolytic Enzymes from *Bacillus licheniformis* CP-16

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Abstract: This study aimed to obtain a lipolytic enzyme from *Bacillus licheniformis* CP-16 through heterologous expression and investigate its role in feather degradation. Using the genomic DNA of *Bacillus licheniformis* CP-16 as a template, the lipolytic enzyme gene was amplified and transformed into *Escherichia coli* for expression to obtain recombinant enzyme L-4. The optimal pH, pH stability, optimal temperature, temperature stability, and effects of organic solvents and metal ions on its relative activity were investigated, while its effect on keratinase K hydrolysis of natural feather keratin was also explored. The results showed that the obtained lipolytic enzyme gene was 747 bp in length, encoding 248 amino acids. Recombinant enzyme L-4 was successfully expressed in *E. coli* with a molecular weight of approximately 28.3 ku and esterase activity of 0.42 U/mL. The optimal pH was 6.5 and the optimal temperature was 50 °C. After treatment for 30 min at pH 6.5–9.5, the relative activity remained above 80%, and after treatment for 30 min at temperatures below 50 °C, the relative activity remained above 70%. Divalent iron (Fe^{2+}), sodium (Na^{+}), manganese (Mn^{2+}), and calcium (Ca^{2+}) ions enhanced the relative activity of recombinant enzyme L-4, whereas barium (Ba^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), and nickel (Ni^{2+}) ions inhibited its relative activity. When the organic solvent concentration was 30%, recombinant enzyme L-4 retained 97% and 85% relative activity in dimethyl sulfoxide (DMSO) and methanol, respectively, over 45% relative activity in acetone and ethanol, less than 20% relative activity in isopropanol, and essentially complete loss of relative activity in acetonitrile. Pretreatment of natural feather substrates with recombinant enzyme L-4 improved the hydrolysis efficiency of keratinase K on the substrate, with a promotion rate of 4.32%. These results demonstrate that lipolytic enzymes can degrade feather surface lipids and may play a role in promoting keratinase hydrolysis of feather keratin.

Keywords: *Bacillus licheniformis*; lipolytic enzymes; keratin; feather lipid; cloning and expression

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Introduction

China faces a chronic shortage of protein feed resources with long-term dependence on imports, a situation unlikely to change in the short term. With over 10 billion poultry slaughtered annually, China generates 700,000–800,000 tons of feathers. Feathers represent an excellent protein resource due to their high crude protein content (85%), low cost, and abundant sulfur-containing amino acids. Developing this resource could alleviate protein feed shortages while reducing environmental pollution. Current feather processing methods include physical, chemical, and biological approaches. Physical and chemical methods suffer from high energy consumption, severe amino acid destruction, and environmental pollution. Biological methods primarily utilize keratin-degrading microorganisms or their enzymes. While microbial degradation produces less utilizable bacterial protein, enzymatic hydrolysis directly converts keratin into highly utilizable small peptides and amino acids. Consequently, keratinase treatment of feather keratin has attracted increasing attention and shows great application potential.

Feather surfaces are coated with lipids [1-4], mostly mixed lipids secreted by the uropygial gland [5] with minor amounts from cell membrane layers. This surface lipid forms a natural barrier that prevents contact with external substances. The interior consists of structurally stable keratin with numerous disulfide bonds, hydrogen bonds, and hydrophobic groups that resist degradation. Destroying the surface lipid layer is critical for improving keratinase hydrolysis efficiency. Investigating the role of lipolytic enzymes in degrading feather surface lipids will complete the feather enzymatic hydrolysis system and provide guidance for efficient enzymatic processing.

Physical and chemical degreasing treatments alter feather properties. Treatment with sodium hydroxide (NaOH) solution decreases contact angles and changes spatial structure [6]. Wang et al. [7] found that petroleum ether degreasing increased protease hydrolysis rates of feathers by up to 1-fold. Numerous studies have detected lipolytic enzyme production in various keratin-degrading bacteria. Vasileva-Tonkova et al. [8] observed lipase production by 20 actinomycete strains in feather medium. Zhang et al. [9] detected lipase activity in *Streptomyces fradiae* fermentation broth. Wang et al. [7] measured lipase activities of 6 U/mL and 1 U/mL in the fermentation broth and intracellular fluid of *Bacillus licheniformis* CP-16, respectively. These findings suggest that lipolytic enzymes from keratin-degrading bacteria may degrade feather surface lipids and promote keratinase efficiency, though limited research has addressed this hypothesis. Therefore, this study obtained the lipolytic enzyme from the feather-degrading strain *Bacillus licheniformis* CP-16 to investigate its role in keratinase K hydrolysis of

feather keratin, thereby improving the feather enzymatic hydrolysis system and providing theoretical guidance for efficient feather keratin degradation.

Materials and Methods

1.1.1 Strain Sources

Bacillus licheniformis CP-16, a feather-degrading strain previously screened and preserved in our laboratory, was used as the source strain. *Escherichia coli* DH5α and BL21(DE3) were purchased from Beijing Biomed Technology Development Co., Ltd.

1.1.2 Vectors and Enzymes

The pMD19-T vector, LA Taq DNA polymerase, and DNA Marker were purchased from TaKaRa (Dalian) Co., Ltd. T4 DNA ligase was obtained from Beijing Biomed Technology Development Co., Ltd. Restriction enzymes BamHI and XhoI were from NEB. The pET-22b vector was preserved in our laboratory. Keratinase K was purchased from Jinan Nuoneng Bioengineering Co., Ltd.

1.1.3 Reagents

Isopropyl-β-D-thiogalactoside (IPTG), ampicillin (Amp), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were purchased from Promega. Bacterial genome extraction kit, plasmid extraction kit, and agarose gel recovery kit were from Beijing Biomed Technology Development Co., Ltd. Nickel-agarose gel column was from Beijing CoWin Biotech Co., Ltd. α-Naphthyl acetate and Fast Blue B salt were from Sigma-Aldrich (USA).

1.1.4 Culture Media

Luria-Bertani (LB) broth: 0.5 g yeast extract, 1.0 g tryptone, 1.0 g NaCl, 100 mL deionized water, natural pH, sterilized at 121 °C for 30 min.

LB solid medium (with Amp): 0.5 g yeast extract, 1.0 g tryptone, 1.0 g NaCl, 1.5–2.0 g agar powder, 100 mL deionized water, natural pH, sterilized at 121 °C for 30 min. Amp was added to a final concentration of 100 μg/mL when cooled to 55–60 °C before pouring plates, which were stored at 4 °C.

1.2 Experimental Methods

1.2.1 Target Gene Cloning Genomic DNA of *Bacillus licheniformis* CP-16 was extracted using a bacterial genome extraction kit according to the manufacturer' s instructions. The full-length lipolytic enzyme gene sequence was obtained by searching *Bacillus licheniformis* protein sequences in UniProt

(http://www.uniprot.org/). Primers were designed to amplify the full-length gene, with BamHI and XhoI restriction sites introduced at the forward and reverse primers, respectively (underlined sequences indicate restriction sites):

Forward primer: 5′-CGGGATCCGAAAATTGTCAAACC-*3′*
Reverse primer: 5′-CCGCTCGAGTGTCTGCCAATC-3′

PCR was performed according to the DNA polymerase manufacturer' s instructions. Amplification products were detected by 1% agarose gel electrophoresis. The purified PCR product was ligated with pMD19-T vector at 16 °C for 12 h and transformed into *E. coli* DH5α. Positive clones were selected through blue-white screening and colony PCR, then sent to Beijing Tsingke Biotech Co., Ltd. for sequencing. Sequencing results were analyzed using DNAMAN 6.0 software and subjected to BLAST analysis in NCBI to identify suitable clones for subsequent experiments.

1.2.2 Expression Plasmid Construction and Recombinant Enzyme Expression Plasmid pMD19-T-L-4 and vector pET-22b were digested with BamHI and XhoI. The digested L-4 gene and pET-22b vector were ligated with T4 DNA ligase at 25 °C for 2 h. The resulting expression plasmid pET-22b-L-4 was transformed into *E. coli* DH5α competent cells. Potential positive clones were selected based on ampicillin resistance, then confirmed by colony PCR, restriction digestion, and sequencing. The correctly sequenced recombinant plasmid was transformed into *E. coli* BL21(DE3) competent cells to construct the expression strain.

A single positive colony was inoculated into LB medium (containing 100 μg/mL Amp) and cultured overnight at 37 °C with shaking at 180 r/min to obtain seed culture. The seed culture was inoculated at 2% (v/v) into 50 mL LB medium (containing 100 μg/mL Amp) and grown at 37 °C to an OD value of 0.5–0.6. Protein expression was induced with IPTG (final concentration 0.2 mmol/L) at 30 °C for 4 h. Cells were harvested by centrifugation at 4 °C, 10,000 r/min for 10 min, then subjected to ultrasonic disruption. The supernatant and precipitate were collected separately after centrifugation.

1.2.3 SDS-PAGE Analysis and Inclusion Body Protein Purification SDS-PAGE was performed on the supernatant and precipitate after ultrasonic disruption to detect protein expression patterns and yield. Inclusion body proteins were purified using a nickel-agarose gel column and dissolved in elution buffer containing 8 mol/L urea. The purified inclusion body protein solution was loaded into a prepared dialysis bag at an appropriate concentration. The dialysis bag was placed in renaturation buffer containing sequentially decreasing urea concentrations (6, 4, 2, and 0 mol/L) and dialyzed at 4 °C. Each concentration gradient lasted 24 h to ensure complete urea removal. After completion, the dialysis bag was transferred to phosphate-buffered saline (PBS) for an additional 24 h. During dialysis, a magnetic stirrer was used to continuously mix

the solution, allowing urea to diffuse out and denatured proteins to gradually refold.

1.2.4 Esterase Activity Assay Esterase activity was determined according to methods described by Liu et al. [10] and Liang et al. [11]. The assay procedure was as follows: 50 µL of enzyme solution was mixed with 800 µL phosphate buffer, followed by addition of 50 µL α-naphthyl acetate substrate solution. The mixture was incubated in a 50 °C water bath for 10 min, then the reaction was terminated by adding 3% sodium dodecyl sulfate (SDS) solution. After mixing, 50 µL of 0.02% Fast Blue B salt solution was added for color development. After 30 s, 50 µL of hydrochloric acid solution was added to stabilize the color. A control reaction was prepared by adding SDS before incubation. The absorbance at 535 nm was measured using a microplate reader.

1.2.5 Enzymatic Properties of Recombinant Enzyme L-4 1.2.5.1 Optimal pH and pH Stability

Optimal pH: Enzyme activity was measured at 50 °C in 15 different buffer systems with pH ranging from 2.5 to 9.5 (at 0.5 pH intervals): glycine-HCl (pH 2.5–3.0), citrate-sodium citrate (pH 3.5–5.0), MES buffer (pH 5.5–6.5), sodium phosphate (pH 7.0–8.0), and Tris-HCl (pH 8.5–9.5). Each treatment was performed in triplicate to determine the optimal pH for enzymatic reaction.

pH Stability: Recombinant enzyme was diluted 1:9 (v/v) with different pH buffers and incubated in a 50 °C water bath for 30 min, followed by cooling in an ice-water mixture for 30 min. Each treatment was performed in triplicate. Residual enzyme activity was measured at 50 °C. Untreated enzyme solution served as the control, and the percentage of residual activity relative to the control was calculated for each pH condition.

1.2.5.2 Optimal Temperature and Temperature Stability

Optimal Temperature: Enzyme activity was measured at temperatures ranging from 30 to 80 °C (at 5 °C intervals) under optimal pH conditions. Each treatment was performed in triplicate to determine the optimal reaction temperature.

Temperature Stability: Recombinant enzyme L-4 was treated at various temperatures (0–80 °C, at 10 °C intervals) for 30 min, then cooled in an ice-water bath for 30 min. Residual enzyme activity was measured under optimal pH conditions. Untreated enzyme solution served as the control, and the percentage of residual activity relative to the control was calculated for each temperature condition.

1.2.5.3 Effects of Organic Solvents on Recombinant Enzyme L-4 Activity

Six organic solvents (methanol, ethanol, isopropanol, acetone, DMSO, and acetonitrile) were tested at final concentrations of 30% and 80%. Each solvent was incubated with recombinant enzyme L-4 at 30 °C for 60 min, followed by rapid

cooling in an ice-water bath for 30 min. Residual enzyme activity was then measured, with each treatment performed in triplicate. The activity of enzyme without organic solvent was defined as 100%.

1.2.5.4 Effects of Metal Ions on Recombinant Enzyme L-4 Activity

Metal ions [Na^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, K^+, Ni^{2+}, Fe^{3+}] dissolved in optimal pH buffer at 10 mmol/L were mixed with equal volumes of recombinant enzyme L-4 solution and incubated at 30 °C for 60 min, followed by cooling in an ice-water bath for 30 min. Residual enzyme activity was measured, with each treatment performed in triplicate. The activity of enzyme without metal ions was defined as 100%.

1.2.6 Effects of Recombinant Enzyme L-4 on Keratinase Feather Hydrolysis 1.2.6.1 Combined Degradation by Recombinant Enzyme L-4 and Keratinase K

The keratinase activity of recombinant enzyme L-4, keratinase K, and their combination was measured. Considering the enzymatic properties of both enzymes, keratinase activity was determined at 50 °C in Tris-HCl buffer (pH 8.0). The keratinase activity assay followed the method of Wang [12]: 1.0 mL enzyme solution was mixed with 2.0 mL feather powder substrate (5 mg/mL) in 0.05 mol/L Tris-HCl buffer (pH 9.5) and incubated at optimal temperature with shaking at 180 r/min for 1 h. To prevent substrate sedimentation, the reaction mixture was removed and mixed every 10 min. The reaction was terminated by adding 2.0 mL of 10% trichloroacetic acid. After standing for 5 min, the mixture was centrifuged at 10,000 r/min for 10 min (with a second centrifugation if necessary). The supernatant absorbance at 280 nm was measured, with the blank control being samples where terminator was added before incubation. Enzyme combination ratios are shown in Table 1 .

1.2.6.2 Pretreatment of Natural Feather Substrate by Recombinant Enzyme L-4

Recombinant enzyme L-4 was mixed with natural feather substrate (5 mg/mL) at equal volume and incubated in a 50 °C water bath for 1 h. Keratinase K activity was then measured at 55 °C, with each treatment performed in triplicate.

1.3 Statistical Analysis Experimental data were processed and analyzed using SAS 9.3 software, and graphs were generated using GraphPad Prism 5 to create line charts and bar graphs.

Results

2.1 Target Gene Cloning and Sequencing

The full-length gene was amplified using *Bacillus licheniformis* CP-16 genomic DNA as template. Agarose gel electrophoresis (1%) confirmed the amplified product size matched the expected result (Figure 1 [Figure 1: see original paper]). The amplified gene sequence was 747 bp with a complete open reading frame encoding 248 amino acids. The recombinant enzyme L-4 gene sequence and predicted amino acid sequence are as follows:

[Gene sequence would appear here]

2.2 Expression Plasmid Construction

After digesting the target gene and plasmid with BamHI and XhoI restriction enzymes, they were ligated with T4 DNA ligase and transformed into *E. coli* DH5α. Positive clones were initially identified by colony PCR, then plasmids were extracted for double digestion verification (Figure 2 [Figure 2: see original paper]), confirming successful construction of the expression plasmid.

2.3 Recombinant Enzyme Expression and Inclusion Body Purification

E. coli BL21(DE3) containing the verified plasmid was induced with IPTG. SDS-PAGE analysis (Figure 3 [Figure 3: see original paper]) showed successful expression of recombinant enzyme L-4, with the expressed protein size matching the predicted 28.3 ku. Further analysis of ultrasonic supernatant and precipitate (Figure 4 [Figure 4: see original paper]) revealed that recombinant enzyme L-4 was primarily expressed as inclusion bodies in the precipitate. Inclusion body proteins were collected, purified by nickel-agarose gel chromatography, and refolded through gradient urea dialysis to obtain active enzyme protein with esterase activity of 0.42 U/mL.

2.4 Enzymatic Properties of Recombinant Enzyme L-4

2.4.1 Optimal pH and pH Stability As shown in Figure 5 [Figure 5: see original paper], the optimal pH for recombinant enzyme L-4 was 6.5, with relative activity maintained above 60% within pH 5.5–7.0, but decreasing sharply outside this range. After 30 min treatment at pH 6.5–9.5, the relative activity remained above 80%, demonstrating good stability and classifying it as a neutral lipolytic enzyme.

2.4.2 Optimal Temperature and Temperature Stability Figure 6 [Figure 6: see original paper] shows that the optimal reaction temperature for recombinant enzyme L-4 was 50 °C, with relative activity above 65% at 30–50 °C, but decreasing sharply above 50 °C and essentially lost above 65 °C. After 30 min treatment at temperatures below 55 °C, the relative activity remained

stable above 90%. Based on both optimal temperature and stability, lipolytic enzyme L-4 belongs to the mesophilic enzyme category.

2.4.3 Effects of Organic Solvents on Recombinant Enzyme L-4 Activity As shown in Figure 7 [Figure 7: see original paper], at a final organic solvent concentration of 30%, recombinant enzyme L-4 retained 97% relative activity in DMSO, 85% in methanol, over 45% in acetone and ethanol, less than 20% in isopropanol, and essentially complete loss of activity in acetonitrile. At 80% organic solvent concentration, recombinant enzyme L-4 retained only low relative activity in methanol and isopropanol, with activity essentially lost in other solvents.

2.4.4 Effects of Metal Ions on Recombinant Enzyme L-4 Activity Figure 8 [Figure 8: see original paper] demonstrates that at 10 mmol/L metal ion concentration, Fe^{2+}, Na^{+}, Mn^{2+}, and Ca^{2+} activated recombinant enzyme L-4, while Ba^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} inhibited its activity. Fe^{2+} showed the most significant activation effect, doubling the relative activity, whereas Cu^{2+} showed the strongest inhibition, retaining only 7.53% relative activity.

2.5 Effects of Recombinant Enzyme L-4 on Keratinase Hydrolysis

2.5.1 Combined Degradation by Recombinant Enzyme L-4 and Keratinase K Table 2 shows that recombinant enzyme L-4 exhibited no keratinase activity, and the mixture of recombinant enzyme L-4 and keratinase K negatively affected keratinase K activity. Recombinant enzyme L-4 had an adverse effect on keratinase K feather hydrolysis, with mechanisms requiring further investigation.

2.5.2 Pretreatment of Natural Feather Substrate by Recombinant Enzyme L-4 Table 3 demonstrates that pretreatment of natural feather substrate with recombinant enzyme L-4 for 1 h improved keratinase K activity, with a promotion rate of 4.32%.

Discussion

E. coli is the most widely used and well-studied prokaryotic expression system in modern research, offering advantages including rapid proliferation, low production cost, high heterologous gene expression levels, and clear genetic maps and regulatory mechanisms [13]. However, when using *E. coli* as expression host, rapid expression leads to minimal modification and inefficient secretion of expressed proteins [14]. In this study, recombinant enzyme L-4 was expressed as inclusion bodies at high levels but with mostly no biological activity. For industrial production, *E. coli* expression of lipolytic enzymes shows no advantages, necessitating exploration of alternative expression systems.

Enzymatic properties determine the application potential and fields of enzyme proteins. Recombinant enzyme L-4 inclusion bodies were purified by nickel-agarose gel chromatography and refolded by gradient urea dialysis to obtain active enzyme for characterization. Lipases generally exhibit optimal pH in the weakly acidic to alkaline range of 6.0–8.0, with pH stability ranges of 4.0–11.0 [15-17]. The recombinant enzyme L-4 obtained in this study had an optimal pH of 6.5 and good stability at pH 6.5–9.5, retaining over 80% relative activity after 30 min treatment. This may be related to *Bacillus licheniformis* CP-16 using feather keratin as its sole carbon and nitrogen source. During fermentation, *B. licheniformis* CP-16 performs deamination to generate carbon skeletons, causing the fermentation broth to become alkaline over time [18]. The protease produced by *B. licheniformis* CP-16 is alkaline [12], which accelerates keratin degradation and provides more nutrients for bacterial survival.

The optimal temperature for recombinant enzyme L-4 was 50 °C, with stable relative activity above 90% after 30 min treatment below 55 °C, classifying it as a mesophilic lipolytic enzyme. Lipases typically have optimal reaction temperatures of 30–50 °C [19-20]. Temperature stability varies among lipases from different sources, with some researchers identifying thermostable lipases with broader applicability [21]. Industrial applications such as pulp degreasing/deinking and production of human milk fat substitute structured lipids (1,3-dioleoyl-2-palmitoylglycerol, OPO) require thermostable lipases [22-23].

Metal ions can serve as enzyme activators or inhibitors by forming active centers, stabilizing enzyme conformation, or bridging enzyme and substrate. In this study, Fe^{2+}, Na^{+}, Mn^{2+}, and Ca^{2+} at 10 mmol/L activated recombinant enzyme L-4, while Ba^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} inhibited activity, with Cu^{2+} showing the most pronounced inhibition. Yan [24] reported that Ba^{2+} significantly promoted lipase activity, contrary to our results, possibly due to different enzyme gene types. Many enzymatic reactions occur in non-aqueous organic solvents, such as fatty acid ester and biodiesel synthesis [25-26], making organic solvent effects worth investigating. At 30% organic solvent concentration, recombinant enzyme L-4 retained high relative activity in DMSO and methanol, over 45% in acetone and ethanol, but essentially lost activity in isopropanol and acetonitrile. At 80% concentration, organic solvents severely affected recombinant enzyme L-4 activity, causing substantial loss.

Feather surface lipids are primarily mixed lipids secreted by the uropygial gland, with various esterification patterns including aliphatic monoesters and diesters [27]. Traditional degreasing methods include alkaline saponification, surfactant emulsification, and organic solvent extraction [28]. These conventional methods not only pollute the environment but also leave chemical residues that limit feed applications. Lipolytic enzymes, including esterases and lipases, catalyze ester bond hydrolysis and formation, enabling efficient and environmentally friendly degreasing. Microbial enzymatic degreasing is widely used in wool processing to remove surface hydrophobic lipids [4,29], with cutinases hydrolyzing fatty acid esters in fibers [30]. Wang [31] modified wool through combined action

of lipase, cutinase, and keratinase. Although lipase activity has been detected in fermentation broths of various keratin-degrading bacteria [7-9], no studies have reported the effect of lipolytic enzymes from keratin-degrading bacteria on natural feather degradation. In investigating lipolytic enzyme effects on keratinase feather hydrolysis, we found that mixing recombinant enzyme L-4 with keratinase K for 1 h reduced keratinase activity compared to keratinase alone, suggesting lipolytic enzymes may affect keratinase activity. The specific mechanisms require further study. In actual feather degradation by microorganisms, these two enzymes may act sequentially. Pretreatment of natural feather substrate with recombinant enzyme L-4 for 1 h promoted keratinase K hydrolysis with a 4.32% promotion rate, indicating that recombinant enzyme L-4 degrades feather surface lipids, increases feather hydrophilicity, and facilitates direct keratinase action on feather keratin. However, the promotion efficiency was modest, warranting further investigation of other lipolytic enzymes from *Bacillus licheniformis* CP-16 with faster feather lipid degradation capabilities.

Conclusions

1. The lipolytic enzyme gene was amplified from *Bacillus licheniformis* CP-16 genome and successfully expressed in *E. coli* to obtain recombinant enzyme L-4.
2. Recombinant enzyme L-4 had an optimal temperature of 50 °C and optimal pH of 6.5, with relatively stable activity under alkaline conditions. Fe^{2+}, Na^{+}, Mn^{2+}, and Ca^{2+} enhanced its relative activity. The enzyme retained 97% relative activity in DMSO, 85% in methanol, and over 45% in acetone and ethanol, while other organic solvents essentially abolished activity.
3. Pretreatment of natural feather substrate with recombinant enzyme L-4 for 1 h showed a promoting trend on keratinase K hydrolysis, though with limited efficiency.
4. Lipolytic enzymes hydrolyze feather surface lipids, increase feather hydrophilicity, and promote direct keratinase action on feather keratin.

References

[1] JI Cheng. Animal Nutrition [M]. Beijing: Higher Education Press, 2008: 75.
[2] ZHAO Quanfang. Analysis of hydrophobic and hydrophilic properties of bird feathers [J]. Journal of Management, 2005, 18(2): 121–122.
[3] SARAVANAN K, DHURAI B. Exploration on amino acid content and morphological structure in chicken feather fiber [J]. Journal of Textile and Apparel: Technology and Management, 2012, 7(3): 1–6.

[4] MALL J K, SIMS P, CARR C M. Surface chemical analysis of lipase enzyme treatments on wool and mohair [J]. The Journal of the Textile Institute, 2002, 93(1): 43-51.
[5] FENG Yulong, XIE Ming, WEN Zhiguo, et al. Research progress on avian uropygial gland and its biological functions [J]. China Feed, 2013(9): 9-12.
[6] REN Luquan, SHANG Guangrui, YANG Xiaodong. Effects of feather structure and surface lipids on wettability [J]. Journal of Jilin University: Engineering and Technology Edition, 2006, 36(2): 213-218.
[7] WANG Deshan, ZHANG Tieying, LIANG Zhengwen, et al. Effects of lipids in feathers on feather keratin degradation [J]. Feed Industry, 2014, 35(19): 26-29.
[8] VASILEVA-TONKOVA E, GOUSTEROVA A, NESHEV G. Ecologically safe method for improved feather wastes biodegradation [J]. International Biodeterioration & Biodegradation, 2009, 63(8): 1008-1012.
[9] ZHANG Y H, MENG K, WANG Y R, et al. A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae*, var. k11 [J]. Enzyme and Microbial Technology, 2008, 42(4): 346-352.
[10] LIU Chunhong, FENG Zhibiao. Optimization of conditions for plant esterase activity determination using α-naphthyl acetate as substrate [J]. Science and Technology of Food Industry, 2008(6): 145-147, 151.
[11] LIANG Zhengwen, ZHANG Tieying, LI Shuang, et al. Identification, fermentation optimization and enzymatic properties of a plant cuticle-degrading bacterium [J]. Chinese Journal of Animal Nutrition, 2016, 28(11): 3567-3575.
[12] WANG Deshan. Isolation, expression and relationship of feather keratin-degrading related proteases from *Bacillus licheniformis* CP-16 [D]. Master' s thesis. Beijing: Chinese Academy of Agricultural Sciences, 2014.
[13] NUC P, NUC K. Recombinant protein production in *Escherichia coli* [J]. Postepy Biochemii, 2006, 52(4): 448-456.
[14] JHAMB K, SAHOO D K. Production of soluble recombinant proteins in *Escherichia coli*: effects of process conditions and chaperone co-expression on cell growth and production of xylanase [J]. Bioresource Technology, 2012, 123: 135-143.
[15] LIU Z Q, CHI Z M, WANG C L, et al. Production, purification and characterization of an extracellular lipase from *Aureobasidium pullulans* HN2.3 with potential application for the hydrolysis of edible oils [J]. Biochemical Engineering Journal, 2008, 40(3): 445-451.
[16] ÜLKER S, KARAOĞLU S A. Purification and characterization of an extracellular lipase from *Mucor hiemalis* f. *corticola* isolated from soil [J]. Journal of Bioscience and Bioengineering, 2012, 114(4): 385-390.
[17] DAOUD L, KAMOUN J, ALI M B, et al. Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase [J]. International Journal of Biological Macromolecules, 2013, 57: 232-237.
[18] QI Zhiguo. Study on keratin-degrading bacteria with high disulfide bond reduction capacity [D]. Master' s thesis. Beijing: Chinese Academy of Agricultural Sciences, 2012.
[19] GOLDBECK R, FILHO F M. Screening, characterization, and biocatalytic

capacity of lipases producing yeasts Brazil biomes [J]. Food Science and Biotechnology, 2013, 22(S1): 79–87.
[20] MHETRAS N C, BASTAWDE K B, GOKHALE D V. Purification and characterization of acidic lipase *Aspergillus niger* 1207 [J]. Bioresource Technology, 2009, 100(3): 1486–1490.
[21] MUÑOZ P A, CORREA-LLANTÉN D N, BLAMEY J M. Production, purification and partial characterization of lipases from a thermophile isolated from Deception Island [J]. Lipids, 2013, 48(5): 527–533.
[22] LEVISSON M, VAN DER OOST J, KENGEN S W M. Carboxylic ester hydrolases from hyperthermophiles [J]. Extremophiles, 2009, 13: 567.
[23] ESTEBAN L, JIMÉNEZ M J, HITA E, et al. Production of structured triacylglycerols rich in palmitic acid at sn-2 position and oleic acid at sn-1,3 positions as human milk fat substitutes by enzymatic acidolysis [J]. Biochemical Engineering Journal, 2011, 54(1): 62–69.
[24] YAN Jinyong. Cloning and expression of lipase gene from *Geotrichum candidum* Y162 [D]. Master' s thesis. Wuhan: Huazhong University of Science and Technology, 2007.
[25] JOSEPH B, RAMTEKE P W. Extracellular solvent stable cold-active lipase from psychrotrophic *Bacillus sphaericus* MTCC 7526: partial purification and characterization [J]. Annals of Microbiology, 2013, 63(1): 363–370.
[26] UGUR A, SARAC N, BORAN R, et al. New lipase for biodiesel production: partial purification and characterization of LipSB 25-4 [J]. ISRN Biochemistry, 2014, 2014: 289749.
[27] SALIBIAN A, MONTALTI D. Physiological and biochemical aspects of the avian uropygial gland [J]. Brazilian Journal of Biology, 2009, 69(2): 437–446.
[28] CHEN Zhenlin. Selection of degreasing methods for goatskin [J]. China Leather, 1989(9): 12–13.
[29] FRANKA G, MEYER H H, DEUTZ H, et al. Enzyme-catalyzed hydrolysis of 18-methyl eicosanoic acid-cysteine thioester [J]. European Journal of Lipid Science and Technology, 2003, 105(10): 627–632.
[30] DEGANI O, GEPSTEIN S, DOSORETZ C G. Potential use of cutinase in enzymatic scouring of cotton fiber cuticle [J]. Applied Biochemistry and Biotechnology, 2002, 102(1–6): 277–289.
[31] WANG Ping. Study on biological enzyme modification of wool surface and its mechanism [D]. Doctoral dissertation. Wuxi: Jiangnan University, 2010.

Note: Figure translations are in progress. See original paper for figures.