DEVELOPMENT AND INVESTIGATION OF LOW COLLAGEN DEGRADABILITY UNHAIRING ENZYME BY GENE MODIFICATION

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Abstract. Unhairing process brought serious pollution, and enzyme application for replacing polluting chemicals in unhairing process attracted much attention in recent years. However, the unhairing enzymes haven't been accepted widely in actual production due to low purity, complex composition and poor stability. To solve these problems, unhairing enzyme is suggested to be improved by genetic modification in this research. The High-keratinase-producing gene (KerT), which was extracted from B. amyloliquefaciens TCCC11319, was introduced into the B.subtilis WB600 by heterologous expression. Because Bacillus subtilis WB600 is deficient in six extracellular proteases, this process successfully reduced the collagenolytic protease content in crude broth as well as improved the keratinase content. Meantime, the recombinant KerT produced by B.subtilis WB600 had the obviously unhairing effect to remove hairs. The results showed that the collagen degradability of recombinant KerT was slightly and it did not cause any adverse effects on the hide quality. This research will contribute to the development of unhairing enzyme, and the novel unhairing enzyme might be applied as the key factor for the advanced cleaning biotechnology in leather production process.

Keywords: KerT gene; B.subtilis WB600; Enzyme unhairing; Low collagen degradability; Heterologous expression.

1 Introduction

In leather production, sulfur pollution mainly is produced in unhairing process. Besides, It's known that every ton of hides processed into hair-free leather will generate 150- 250 kg of unhairing-liming solid waste and 30 m^3 wastewater, which lead to environmental pollution and toxic effect on human health.[1] Thus there is an urgent demand for reducing pollution in unhairing process. It is considered that the enzyme can be used to replace the sodium sulfur for many years. However, traditional unhairing enzyme application needs strict control, otherwise it may lead to the collagen fiber degradation thereby the quality of finished leather will reduce.[2] Until now, complete replacement of sulfide by enzyme unhairing cannot been achieved. It should be attributed to the high content of collagenolytic protease existed in traditional unhairing enzyme.

In unhairing, keratins compose the bulk of the horny layer of the epidermis, the epidermal appendages and hairs, which are typically durable and tough.[3] All of them should be removed in leather production. Though hydrogen bonding and hydrophobic interaction in keratin molecules create a fine filament-matrix structure by tightly bonded polypeptide that withstands degradation by common protease,[4] keratinase can degrade the insoluble structure by hydrolyzing disulfide bond of keratin.[5] The purified keratinase can effectively remove hair without damaging hide collagen. However, taking into account industrial costs, the unhairing enzymes used for production are usually of low purity. In order to obtain an ideal unhairing enzyme, the substrate specificity to keratin of unhairing enzyme should be highly improved, while its collagen degradation ability must be inhibited.

The traditional methods to avoid collagen degradation are usually by addition of some chemicals. But the keratinase activity will be inhibited simultaneously. With the development of genetic engineering, heterologous expression provides a means for characterizing the biosynthetic

pathways in a genetically amenable host, whilst allowing the modification of such pathways for the generation of required products.[6] Therefore, by construction of a producing strain of keratinase and adjusting the fermentation conditions, the keratinase activity in fermentation broth can be improved and the collagenolytic protease activity can be controlled to low level. The fermentation broth can be used as unhairing enzyme, which has a acceptable industrial cost.

In this paper, it investigates the unhairing enzyme characteristics which are produced by transferring keratinase gene KerT into *Bacillus subtilis WB600*. Because *B.subtilis WB600* is deficient in six extracellular proteases,[7] it is suitable for the extracellular production of the recombinant proteins and is helpful to reduce collagenolytic protease productivity. Furthermore, the application conditions and effects are explored and evaluated.

2 Experimental

2.1 KerT Gene Clone and Express

2.1.1 Cloning of Keratinase KerT gene

DNA fragments encoding for keratinase (KerT) from *B. amyloliquefaciens* TCCC11319 were amplified with the reaction primers following Liu's methods.[8] The primer pair kerT-F and kerT-R were designed as Primer kerT-F:'- GCGGATCCATGAGAGGCAAAAAGGTATGGA -3' and Primer kerT-R: 5'- CGGAATTCTTACTGAGCTGCCGCCTGT -3'. The PCR cycling condition comprised an initial step of 5 min at 95 °C, a second step of 30 cycles including 10 s at 95 °C, 30 s at 56 °C and 90 s at 72 °C, and a final extension step of 10 min at 72 °C. The PCR product was purified and cloned into the pMD19-T simple vector using standard procedures,(Hu et al., 2013) and transformed into E.coli JM109. In addition, the plasmids were extracted from E.coli JM109 using Plasmid Mini kit (Omega, USA) according to the manual, and the restriction enzymes were purchased from Takara. The recombinant E.coli JM109/pMD19-T-kerT was confirmed by DNA sequencing.

2.1.2 Construction Plasmids and Expression

The kerT fragments were amplified by PCR using kerT primer, and the original plasmid carrying the kerT gene were digested with relevant restriction enzyme including EcoRI and BamHI,[9] then objective DNA fragments were inserted into pLY plasmid digested with EcoRI and BamHI, resulting in pLY- kerT (Fig.1). The recombinant plasmids were then transformed into B. subtilis WB600. The recombinant cell of Bacillus WB600-kerT was inoculated into 5 mL of LB medium and incubated at 37 °C in a 220 r/min shaker overnight. 5 mL overnight culture was inoculated into SPI medium for about 4 h at 37 °C until the cells grew to OD600nm=1.1-1.3. And then 200 µL of the culture suspension was further inoculated into 2 ml SPII medium and incubated at 37 °C in a 100 r/min shaker for 1.5 h. DNA samples and 10 µL competent cell were mixed and incubated at 37 °C for 30 min. After a centrifugation at 1377 ×g for 5 min, 200 µL of supernatant was plated on the LB medium and further incubated at 37 °C overnight.

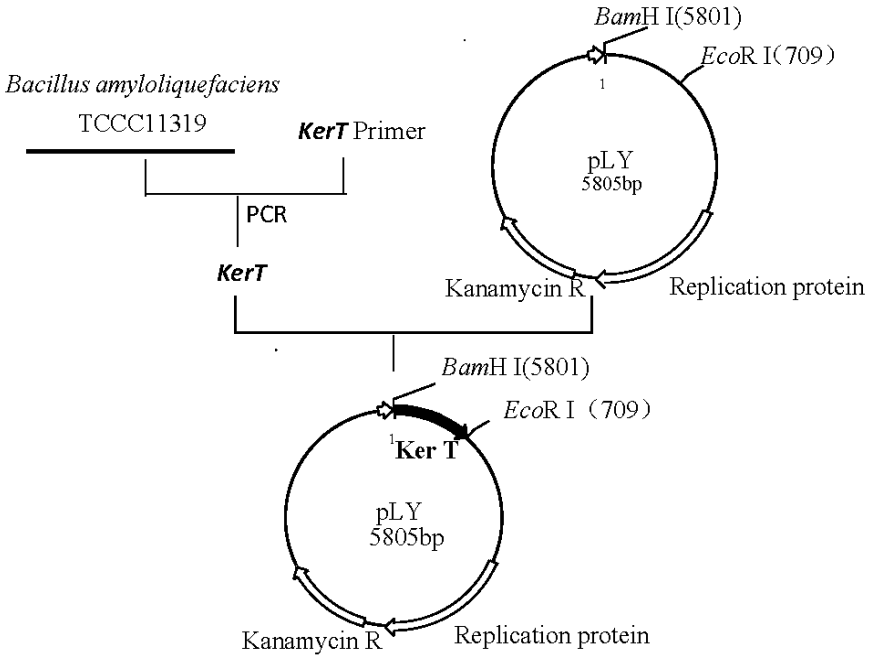

Fig. 1. Construction of the recombinant plasmid pLY-kerT.

2.2 KerT/Wb600 Characterization and Application Evaluation

2.2.1 Keratinolytic Activity and Protein Analysis

After purification,[10] the keratinolytic activity was determined by using keratin as substrates by A280 method.[11] One unit (U) of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.01 under the above conditions. The characteristics of purified *KerT/WB600* were evaluated and the suitable conditions of fermentation broth were optimized.

2.2.2 Enzyme Unhairing Process and Evaluation

In unhairing process, the control group was carried out with 150% (w/w) water, 2.5% (w/w) sodium sulfide and 0.5% (w/w) calcium hydroxide as the normal unhairing process. *The KerT/Wb600* was used under different conditions. The hide samples were collected after deliming and analyzed by Emission Scanning Electron Microscopy (FESEM, Hitachi, S4800, Japan). Meantime, the liquids before and after unhairing process were collected and analyzed using HPLC analysis (AFS-8220, Beijing Titan Instruments, China). Besides, the hydroxyproline measurement was performed with Stoilov's methods.[12]

3 Results and Discussion

3.1 Expression of recombinant KerT in *B.subtilis* WB600 and Purification

As illustrated in Fig.2A, it revealed that the inserted DNA fragment in the *kerT* gene with the expected size. In order to confirm the correct insertion of the *kerT* gene, the recombinant plasmid pLY-*kerT* was extracted and digested by *Eco*RI and *Bam*HI. The molecular mass of double-digested DNA fragment was the same as the PCT product (Fig.2B), which indicating the *kerT* gene was successfully cloned into pLY plasmid. Furthermore, the expressed product was analyzed by SDS-Page, and the molecular weight of the purified enzyme was found to be 28 kDa (Fig.2C).

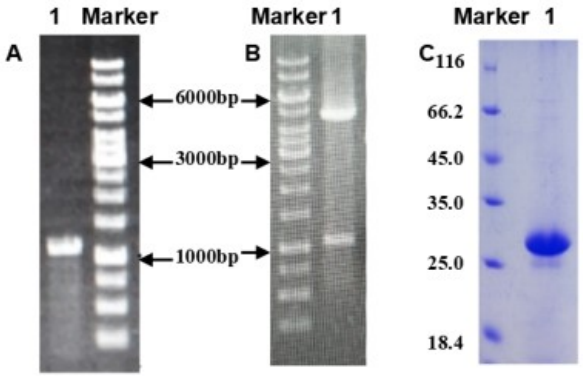

Fig. 2. (A) kerT gene amplified by PCR; (B) Double digestion of recombinant plasmid pLY-kerT. (C) Purified recombinant KerT. Lane M, Markers.

3.2 Characterization of KerT/WB600 and the Novel Unhairing Enzyme

From Fig.3, when temperature was 60 ℃ and pH was approximately 10, the pure KerT displayed it optimum activity. After incubating for 2 h, little loss of activity was observed when temperature was below 50 °C (Fig.2A). Meantime, it showed that more than 60% of the keratinase activity was detectable where pH ranged from 6.0 to 10.0 with the temperature of 70 °C for 2 h. In addition, the unhairing enzyme without purification displayed the optimal keratinase activity when temperature was 40 °C as well as pH was 10.5. Thus this enzyme is suitable for unhairing process. Besides, in order to study the influence of different chemicals, Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, EDTA and PMSF at 5 mmol/L was incubated with pure KerT. From Fig.2E, it was seen that KerT had a well activity with the addition of Na^{+}, K^{+}, Ca^{2+}, EDTA and PMSF. Meanwhile, Cu^{2+} completely inhibited keratinase activity at 5 mmol/L, and Mg^{2+}, Fe^{3+}, $Mn^{2+}$and Co^{2+} showed high inhibition to KerT activity.

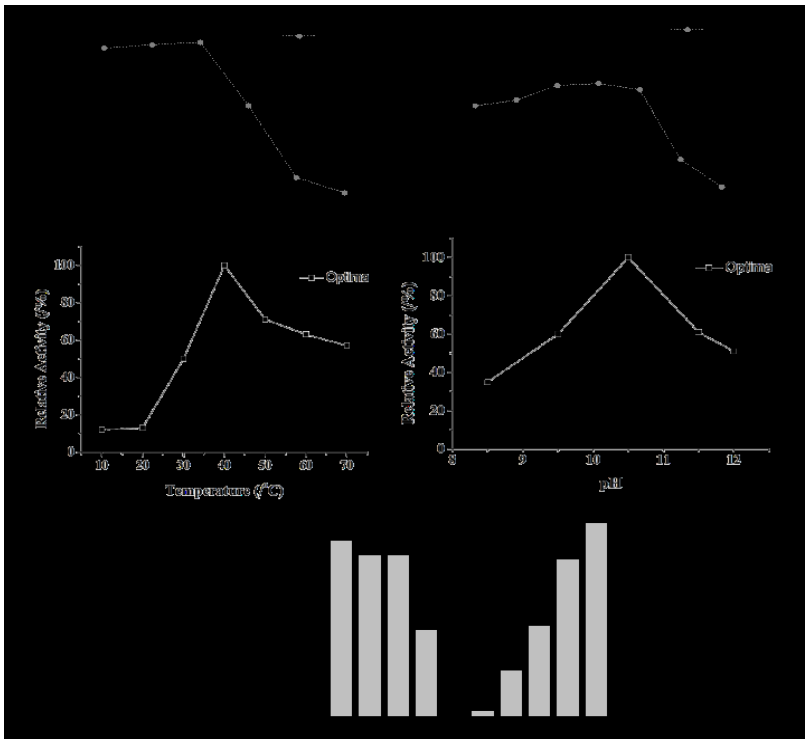

Fig. 3. (A) Relative keratinase activities of pure KerT at different temperatures; (B) Relative keratinase activities of pure KerT at different pH; (C) Optimal temperature of unhairing enzyme. (D) Optimal pH of unhairing enzyme. (E) Relative keratinase activities influenced with different chemicals; *Optima enzyme activities were assayed by incubating for 5 min; The stability enzyme activities were incubated for 120 min while the optima enzyme activities were used as a control.*

3.3 Analysis of hydroxyproline

Hydroxyproline is considered as the signature amino acid for fibrillary collagens. It stabilized the collagen triple helix structure by forming hydrogen bonds with neighboring collagen α-chains.[13] Therefore, Hyp can be measured to evaluate the degradation degree of hide collagen fibers. From Fig.4A, it was seen that the Hyp and Gly contents in *WB600/KerT* unhairing liquid were both lower than that of before gene modification. It represented the collagen degradation activity of the designed unhairing enzyme was successfully reduced by heterologous expression of its producer. In order to observe applicability of *WB600/KerT*, the Hyp concentration changes influenced by time were analysis in Fig.4B. The Hyp concentration in *TCCC11319/*KerT unhairing liquid continued to grow in 24 h. Meantime, the Hyp contents in *WB600/*KerT unhairing liquid increased obviously in the first 8 h, and it only showed a slightly increase in the range of 8 h to 24 h. Thus the *WB600/KerT* would be more safety and easier to apply in unhairing-liming steps. Even overnight, the hide isn't damaged.

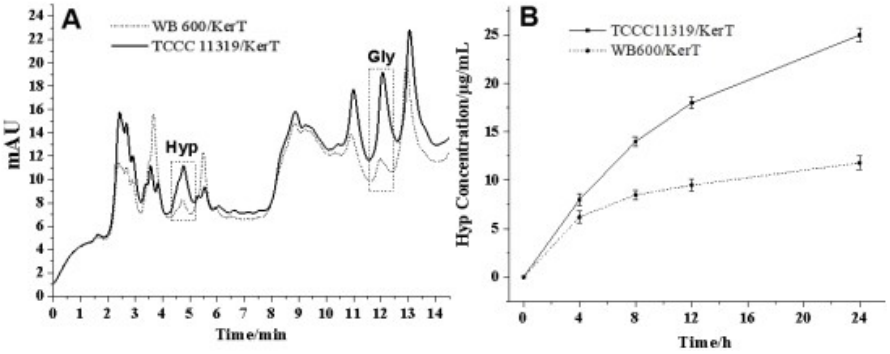

Fig. 4. Changes of hydroxyproline concentration. (A) HPLC analysis; (B) Hyp concentration influenced by time.

3.4 Unhairing Effect Evaluation

The Unhairing process was performed with different conditions, and the results was shown in Table I. The unhairing time was observed continuously and recorded until the hair were remove completely. In fig.1, the sensory properties of hide were evaluated by traditional evaluation method. The hides were evaluated by three experienced tanners and classified into different grades according to their appearance and touch sense. Higher points indicate better properties of the hide. The results showed the optimal temperature was 30 °C and the enzyme activity was 200 U/ml. When the enzyme activity was under 800 U/ml, the hide wouldn't be damaged. As known, the activity of industrial enzyme is usually under 300 U/ml. Therefore, the KerT/WB600 is remarkably safe when it is used in unhairing process.

Table 1. The unhairing effect evaluation for selecting suitable conditions.

Different Ts	20 °C	30 °C	40 °C	50 °C
Unhairing Time /h	12	10	6	4
Sensory Evaluation	A+	A++	A+	A
Different Dosage	200 U/ml	400 U/ml	800 U/ml	1200 U/ml
Unhairing Time /h	15	10	6	4
Sensory Evaluation	A++	A+	A	B

3.5 Observation of Unhairing Effect

From Fig.5, it was seen that the hairs could be fully removed when time was over 9 h. Some fine hairs still existed when the time was 8 h. After unhairing, it was observed that the hide surface

wasn't be damaged, and it possessed an excellent grain surface. By comparing the unhairing effect of KerT/Wb600 unhairing and traditional sodium sulfur unhairing, the appearances of their surfaces were looked similar. Afterwards, their microstructures were analyzed by SEM. The results showed that the hair pores remained intact by KerT/Wb600 Unhairing. It indicated this enzyme didn't degrade collagen fibers in unhairing process.

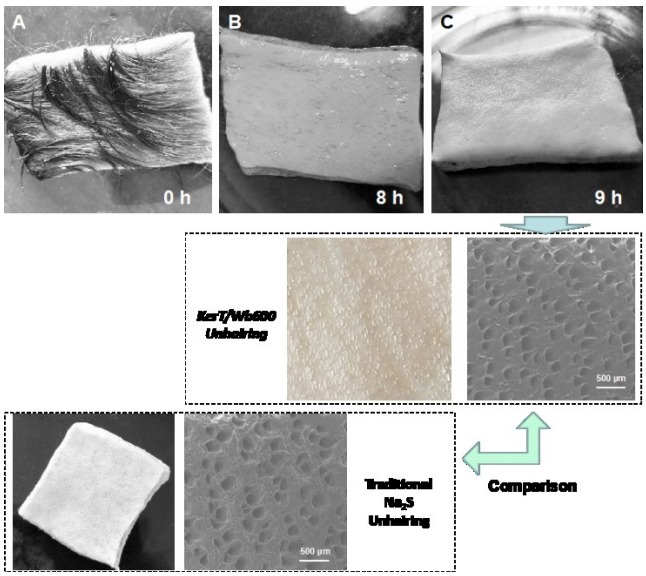

Fig. 5. Observation of unhairing process with KerT/Wb600.

4 Conclusion

In this research, the keratinase gene KerT from *B. amyloliquefaciens TCCC11319* was introduced into the recombinant *B.subtilis WB600* expression system. It is positive to improve the substrate specificity to keratin while reducing collagenolytic protease activity in unhairing enzyme. The fermentation broth is directly used as unhairing enzyme, which can save cost effectively. The hide structure wouldn't be destroyed when unhairing time is 15 h and enzyme activity is 200 U/mL. Therefore, it ensures the economic value of leather. The KerT/Wb600 unhairing effect is similar with traditional sodium sulphide method.

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