Genetics and Molecular Research

Improving production of extracellular proteases by random mutagenesis and biochemical characterization of a serine protease in *Bacillus subtilis* S1-4

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Genet. Mol. Res. 15 (2): gmr.15027831
Received October 15, 2015
Accepted December 23, 2015
Published June 17, 2016
DOI http://dx.doi.org/10.4238/gmr.15027831

ABSTRACT. The feather is a valuable by-product with a huge annual yield produced by the poultry industry. Degradation of feathers by microorganisms is a prerequisite to utilize this insoluble protein resource. To improve the degrading efficiency of feathers, mutagenesis of the bacterium *Bacillus subtilis* S1-4 was performed. By combining ultraviolet irradiation and N-methyl-N'-nitro-N-nitrosoguanidine treatment for mutagenesis, a high protease-producing mutant (UMU4) of *B. subtilis* S1-4 was selected, which exhibited 2.5-fold higher extracellular caseinolytic activity than did the wild-type strain. UMU4 degraded chicken feathers more efficiently, particularly for the release of soluble proteins from the feathers, compared to the wild-type strain. Furthermore, an extracellular protease with a molecular weight of 45 kDa, as determined by SDS-PAGE, was purified from UMU4. Biochemical characterization indicated that the caseinolytic activity of the protease was largely inhibited by phenylmethanesulfonyl fluoride, suggesting that the purified enzyme is

a serine protease. This protease was highly active over a wide range of pHs (6.0 to 12.0) and temperatures (50° to 75°C) with an optimal pH and temperature of 8.0 and 65°C, respectively. The purified enzyme exhibited good thermostability with a 72.2 min half-life of thermal denaturation at 60°C. In addition, this protease was not sensitive to heavy metal ions, surfactants, or oxidative reagents. In conclusion, strain improvement for protease production can serve as an alternative strategy to promote feather degradation. The UMU4 mutant of *B. subtilis* and its serine protease could be potentially used in various industries.

Key words: *Bacillus subtilis*; Mutagenesis; Strain improvement; Purification; Serine protease

INTRODUCTION

Proteases are widely found in many species of animals, plants and microorganisms, and have been intensively studied by various methods, including protein engineering, molecular structure, and catalytic kinetics and mechanisms (Rao et al., 1998; Bryan, 2000; Polgár, 2005). Over the past several years, there has been an increase in the application of proteases in various industries, such as detergent additives, food and feed processing, pharmaceutical, and leather and silk processing (Gupta et al., 2002). Microorganisms are an important source of proteases due to their fast growth, simple fermentation conditions, special metabolic processes, and wide distribution. A large proportion of commercial proteases are produced by microorganisms, particularly *Bacillus* strains (Kumar and Takagi, 1999; Gupta et al., 2002).

Typically, strains of microorganisms isolated from natural niches cannot be directly used in the industrial-scale production of proteases due to their lower yield. To meet the ever-growing demand of proteases, many high protease-producing strains have been selected for industrial production. For example, a hyper-producing mutant of *Bacillus clausii* KSM-K16 was developed and used for production of M-protease at an industrial scale (Saeki et al., 2007). Various technologies have been exploited for this purpose (Rowlands, 1984; Vinci and Byng, 1999; Parekh et al., 2000; Li et al., 2011). Classic mutation or selection techniques like ultraviolet (UV) irradiation and chemical treatment are still used today. For example, protease activity was increased by about 4-fold in *Bacillus stearothermophilus* by chemical mutagenesis (Zamost et al., 1990).

Previously, a strain of *Bacillus subtilis* S1-4 with the capacity to degrade feathers efficiently was isolated from chicken feathers, as it was able to secrete several proteases with hydrolytic activity to various substrates like casein, gelatin, and keratin (Yong et al., 2013). In order to obtain a high protease-producing strain, multiple rounds of mutagenesis by UV and chemical treatment were applied to *B. subtilis* S1-4, and a serine protease was purified from the mutated isolate (UMU4) and its catalytic properties were characterized.

MATERIAL AND METHODS

Microorganisms and growing media

B. subtilis S1-4 was isolated from the waste chicken feathers from a local poultry farm

in China (Yong et al., 2013) and maintained on Luria-Bertani (LB; 1% peptone, 0.5% yeast, 0.5% NaCl) agar plates. Minimal media (MM; 0.15% K_2HPO_4, 0.0025% $CaCl_2$, 0.0015% $FeSO_4 \cdot 7H_2O$, 0.0005% $ZnSO_4 \cdot 7H_2O$, 0.0025% $MgSO_4 \cdot 7H_2O$) was used to formulate the milk-containing medium (MM plus 1% milk), feather medium (MM plus 5% chopped chicken feathers), and the fermentation medium (MM plus 3% soluble starch, 0.2% yeast extract, and 0.5% gelatin) for various purposes.

Mutagenesis and selection of mutants

For UV mutagenesis, *B. subtilis* S1-4 was first incubated in LB broth at 37°C for 16-18 h. After collection by centrifugation at 4,600 *g*, the cells were washed with sterilized water and re-suspended in PBS buffer, pH 7.2 (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4), at a density of about 10^6 cells/mL. Then, 5 mL bacterial suspension was dispensed into a 9 cm-diameter Petri dish and subjected to irradiation by UV under the indicated energy in the UV Crosslinker (Amersham Biosciences, Madison, USA) in the dark. The treated cells were diluted appropriately and spread on milk-containing MM agar plates. The plates were incubated at 37°C in the dark for 48 h. The hydrolytic halos around the mutated colonies were measured and compared against the halo of the wild-type strain. Simultaneously, the colonies with larger transparent hydrolytic halos in diameter were selected. The selected colonies were sub-cultured by streaking on the same agar plates several times until the hydrolytic halo formed stably.

The mutant isolate (UV3), which was obtained from the first UV mutagenesis, was subjected to chemical mutagenesis again. UV3 was grown in LB broth at 37°C for 16-18 h and the cells were collected by centrifugation. The resultant cell suspension (about 10^6 per mL) was treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) solution (1.0 mg/mL final concentration) for 0.5 h, and then washed with sterilized water twice by centrifugation at 4,600 *g*. The cells were diluted appropriately and spread on milk-containing MM plates. After incubation at 37°C for 24-48 h, the colonies were selected based on size of the transparent hydrolytic halos and streaked on the same agar plates. A mutant isolate (MG5) was selected after chemical mutagenesis and subjected to a second UV mutagenesis as described above. Finally, the mutant isolate UMU4 was obtained.

Next, the selected mutant isolates were inoculated into 20 mL fermentation medium and cultured at 37°C for 48 h with shaking at 180 rpm. The supernatant from each culture was used to determine the extracellular protease activity following a protocol described previously (Wan et al., 2009). The mutant isolates were applied to 100 mL feather medium to assess degradation capability. The cultures were incubated at 37°C for 72 h with shaking at 180 rpm. At the end of fermentation, the cultures were filtered through four layers of gauze cloth to remove debris, which was dried and used to calculate feather degradation. The supernatant was further clarified by centrifugation at 15,500 *g* and then used to determine the content of soluble proteins, oligopeptides, and free amino acids as described previously (Yong et al., 2013). All experiments were performed in triplicate.

Purification of the serine protease

For purification of the extracellular protease, the mutated isolate (UMU4) of *B. subtilis* S1-4 was inoculated in 1.0 L fermentation medium in a 3-L Erlenmeyer flask and incubated at 37°C with shaking at 180 rpm for 48 h. The culture was clarified by centrifugation at 4,600 *g*

at 4°C. The resulting supernatant was precipitated by the addition of solid ammonium sulfate at 60% saturation. The pellet was collected by centrifugation at 15,500 *g* rpm for 10 min at 4°C and then solubilized in 5 mL 20 mM Tris-HCl (pH 8.0) plus 1 M $(NH_4)_2SO_4$. After clarification by centrifugation and filtration through a 0.45 mm-diameter filter, the resulting sample was applied to a hydrophobic column (0.8 x 15 cm) pre-equilibrated with buffer A (20 mM Tris-HCl, 1 M $(NH_4)_2SO_4$, pH 8.0). The proteins were eluted with a linear gradient [1 to 0 M $(NH_4)_2SO_4$] at a flow rate of 1 mL/min on the ÄKTA Purifier System (GE Healthcare, Wisconsin, USA). The 2-mL fractions with protease activity were pooled and dialyzed in phosphate buffer (25 mM, pH 8.0) overnight at 4°C.

The dialyzed sample (about 3 mL) was loaded on the Sephacryl S-200 column (0.4 x 60 cm, GE Healthcare, Wisconsin, USA) pre-equilibrated with 25 mM phosphate buffer (pH 8.0) and then eluted with the same buffer at a flow rate of 0.5 mL/min. The 1-mL fractions with protease activity were pooled. The protease sample was stored in storage buffer, pH 8.0 (25 mM phosphate, 50% glycerol, 1 mM $CaCl_2$). Finally, the purified protease was analyzed by 12.5% SDS-PAGE and subjected to zymogram analysis with casein as the substrate (Cedrola et al., 2012).

Characterization of the purified protease

Hydrolytic activity of the protease towards the substrate casein was assayed as described previously (Wan et al., 2009). A standard assay for the caseinolytic reaction was set up in 400 μL borate-NaOH buffer, pH 9.6, containing 5 mg enzyme and 4 mg casein as substrate, and then incubated in a 60°C water bath for exactly 10 min.

To determine the effects of pH and temperature on the hydrolytic activity, the activity assay was performed at different temperatures (40°, 50°, 60°, 65°, 70°, 75°, or 80°C) and different pHs (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13). In addition, thermostability of the protease was also determined by measuring the residual hydrolytic activity after pre-incubating the enzyme solution at 50°, 60°, 65°, or 70°C for the indicated time in the presence of 2 mM $CaCl_2$. Finally, the effect of various chemicals on the catalytic reaction was analyzed. We examined the effect of NaCl concentrations (0, 50, 100, 200, or 500 mM), 2 mM various divalent metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}), 5 mM surfactants and organic solvents, and 5 mM protease inhibitors (Tween-20, Triton X-100, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), Na_2SO_3, β-mercaptoethanol (β-2ME), ethylene bis(oxyethylenenitrilo)-tetra-acetic acid (EGTA), or phenylmethanesulfonyl fluoride (PMSF)). All activity assays were performed in triplicate.

RESULTS

Mutagenesis and selection of mutant isolate

With *B. subtilis* S1-4 as the starting (parent/wild-type) strain, UV irradiation was first applied to the bacterial cells at an energy level of 100 E (E = 100 x 9^2 μJ/cm^2), which resulted in a 99% mortality rate. In total, more than 60,000 surviving colonies were screened after the first mutagenesis. On the milk-containing agar plates, the treated cells formed colonies with various sizes of hydrolytic halos (Figure 1A). Finally, several isolates with stable and larger halos were selected after successively streaking them on the agar plates several times (Figure 1B). As a result, an isolate that we termed UV3 was obtained.

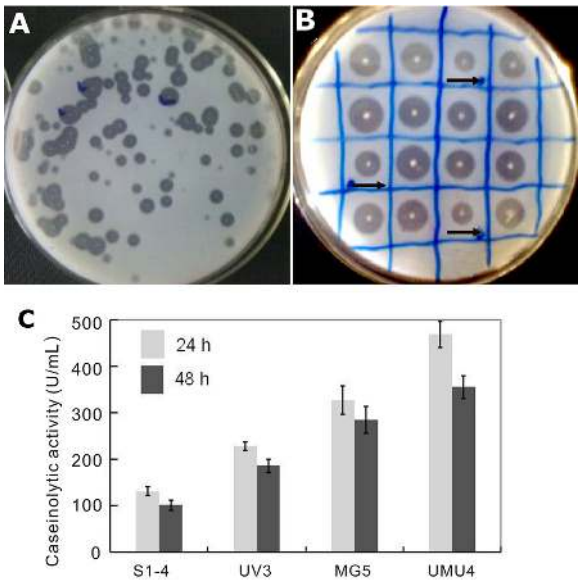

Figure 1. Mutagenesis and screening of mutant isolates of *Bacillus subtilis* S1-4 with improved protease activity. **A.** A representative milk-containing agar plate with bacterial cells after UV irradiation. **B.** Comparison of hydrolytic halos on the milk-containing agar plate of the selected mutant isolates and the wild-type strain (indicated by an arrow) of *B. subtilis* S1-4. **C.** Comparison of protease production in fermentation broth with the selected mutant isolates and the wild-type strain of *B. subtilis* S1-4.

At the second mutagenesis, 1 mg/mL MNNG was applied to the UV3 cells, which resulted in a 98.5% mortality rate. After spreading on the milk-containing agar plates, one isolate (MG5) was selected out of the 57,500 surviving colonies. Finally, the third mutagenesis was performed with UV irradiation with MG5 as the starting strain, and one isolate (UMU4) was selected from about 50,000 surviving colonies.

To confirm the selected isolates had improved caseinolytic activity, three isolates (UV3, MG5 and UMU4) were inoculated in 20 mL fermentation medium and cultured at 37°C. The extracellular protease activity was determined for these cultures at 24 and 48 h, respectively. The results are presented in Figure 1C. The isolate UMU4 achieved a hydrolytic activity up to 469 U/mL at 24 h, an increase of about 2.5-fold compared to the wild-type strain (131 U/mL). The other isolates (UV3, MG5) also showed higher protease activity than the wild-type strain S1-4.

The wild-type strain S1-4 and two isolates (MG5 and UMU4) were chosen to further evaluate their ability to degrade feather. All three strains were inoculated into 100 mL feather medium and incubated at 37°C for 72 h. After fermentation, the extracellular protease activity, content of soluble proteins, oligopeptides and free amino acids, and weight loss of feather in the fermentation broth were determined (Table 1). Specifically, the caseinolytic activity of isolate UMU4 approached 302.40 U/mL, which was much higher than that of the wild-type strain (50.40 U/mL). In comparison with the parent strain S1-4, soluble protein content and weight loss of the feather for the mutant UMU4 were obviously increased. Additionally, the contents of oligopeptides and free amino acids were enhanced. These results indicate that extracellular protease activity results in the degradation of chicken feather.

Table 1. Degradation of chicken feather by the mutant isolates and wild-type strain of *B. subtilis* S1-4*.

Isolates	S1-4	MG5	UMU4
Protease activity (U/mL)	50.40 ± 1.9	152.69 ± 37.2	302.40 ± 31.4
Soluble proteins (mg/mL)	13.96 ± 3.8	15.71 ± 1.9	24.03 ± 1.3
Oligopeptides (mg/mL)	7.46 ± 0.7	5.55 ± 0.8	8.91 ± 1.0
Amino acids (mg/mL)	4.47 ± 0.3	4.23 ± 0.3	4.73 ± 0.4
Degraded feather (g)	2.34 ± 0.2	3.93 ± 0.3	4.43 ± 0.3

*Data are reported as means ± SD derived from experiments performed in triplicate.

Purification and characterization of the protease from mutant UMU4

The protease from the fermentation broth of UMU4 was purified through a multiple-step purification protocol (Table 2). With this purification strategy, the protease was enriched 23.28-fold, but resulted in a lower yield (0.45%). However, the purified protease was nearly homogeneous, as can be seen by a single band on SDS-PAGE (Figure 2A). This was also confirmed by zymogram analysis (Figure 2B). As judged by SDS-PAGE, the molecular weight of the purified protease was estimated at 45.0 kDa.

Table 2. Puriðcation proðle of the protease from the mutant UMU4 of *B. subtilis* S1-4.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Fermentation broth	1,664,000	19,170.51	86.80	1.00	100
Precipitation by $(NH_4)_2SO_4$	46,000	231.17	198.99	2.30	2.76
Hydrophobic column	17,136	42.28	405.30	4.67	1.02
Gel filtration	9,516	4.71	2,020.38	23.28	0.57

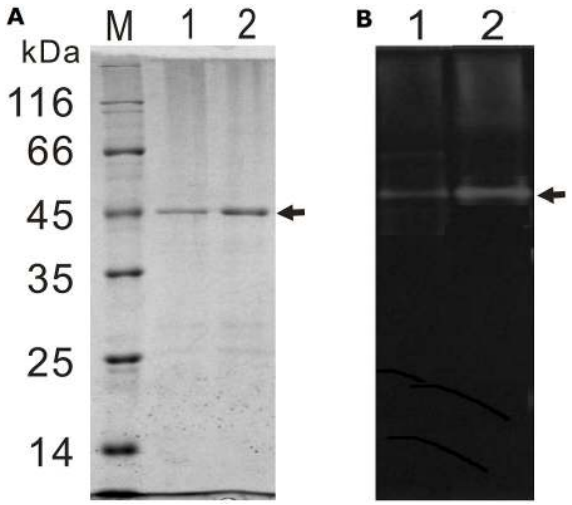

Figure 2. Analysis of SDS-PAGE (**A**) and zymogram (**B**) of the purified protease from the mutant UMU4 of *Bacillus subtilis* S1-4. The arrows indicate the target protein. Two and four micrograms of protease were loaded in lanes 1 and 2, respectively.

The protease purified from UMU4 showed higher hydrolytic activity towards casein over a wide range of temperatures (50°-75°C) and pHs (6-12) (Figure 3A and B), with the optimal temperature and pH being 65°C and 8.0, respectively. The thermostability of the purified protease was determined by pre-incubating the enzyme at various temperatures for

different times. As shown in Figure 4, the caseinolytic activity did not change remarkably at 50°C, maintaining >90% activity for at least 2 h. However, an obvious loss of activity was observed with increasing the time of pre-incubation at 60°C or above. The half-life of thermal denaturation at 60°, 65°, and 70°C was 72.2, 21.3, and 3.2 min, respectively.

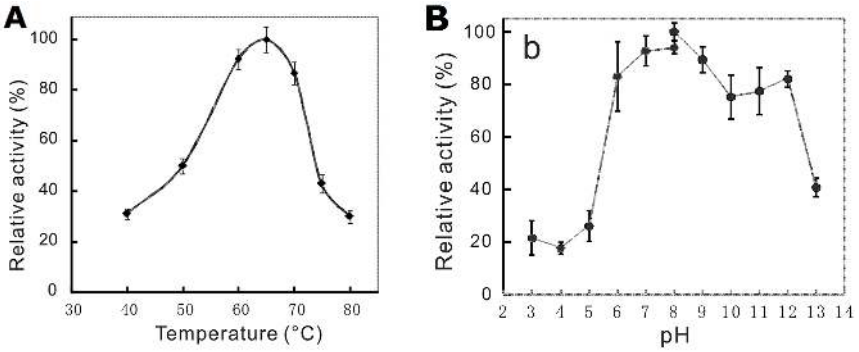

Figure 3. Effects of temperature (**A**) and pH (**B**) on the caseinolytic activity of the purified protease from the mutant UMU4 of *Bacillus subtilis* S1-4. The activity assay was performed at the indicated pH and temperatures using casein as a substrate. The data represent the average value with the standard deviation. The experiment was repeated in triplicate.

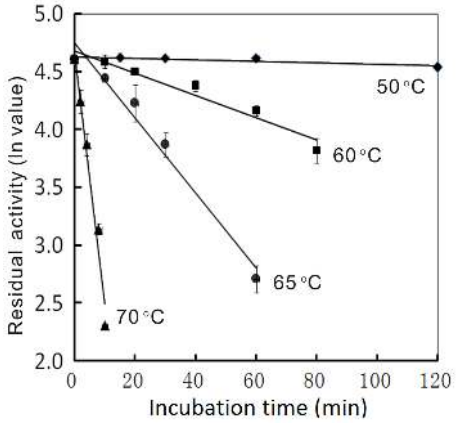

Figure 4. Thermal denaturation of the purified protease from the mutant UMU4 of *Bacillus subtilis* S1-4 at various temperatures in the presence of calcium ions. The thermostability analysis was performed by pre-incubating the protease at the indicated temperatures for various times in 25 mM Tris-HCl buffer (pH 8.0) with 2 mM Ca^{2+}. The residual activity was then assayed at 60°C with casein as substrate. The data represent the average value with the standard deviation. The experiment was repeated in triplicate.

Figure 5A shows the effects of various divalent metal ions on the hydrolytic activity of the protease. In comparison with the control (without addition of any ion), the presence of 2.0 mM metal ions did not result in any obvious change in the hydrolytic activity of this protease, indicating that it is not sensitive to these metal ions. Interestingly, the protease activity could be enhanced by addition of some surfactants and reducing reagents to various extents (Figure 5B). Na_2SO_3 increased hydrolytic activity by about 73%. However, 5 mM EGTA or PMSF

greatly inhibited the hydrolytic activity, indicating that this protease is a serine protease. In addition, the effect of NaCl on hydrolytic activity was also determined. The hydrolytic activity was enhanced by less than 10% at lower concentrations of NaCl (<100 mM) and only slightly inhibited at higher concentrations of NaCl (>100 mM). When 500 mM NaCl was present in the reaction mixture, the hydrolytic activity was decreased to 86.32% in comparison with the control without NaCl.

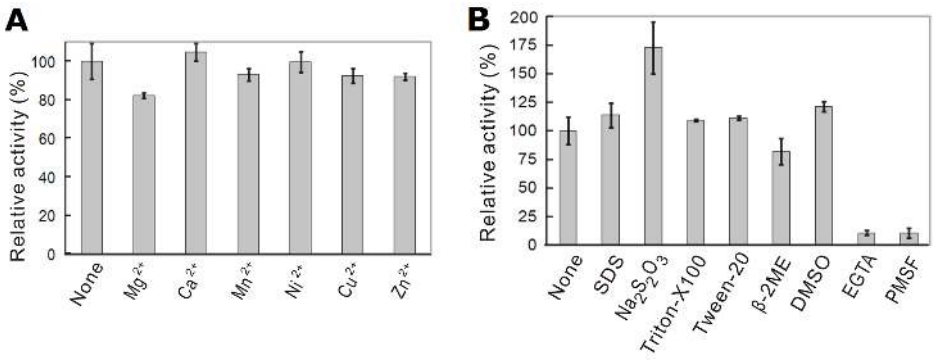

Figure 5. Effects of divalent metal ions (**A**) and surfactants and other chemicals (**B**) on the caseinolytic activity of the purified protease from the mutant UMU4 of *Bacillus subtilis* S1-4. The activity of the protease was assayed at 60°C with casein as substrate in borate-NaOH buffer (pH 9.6). The data represent the average value with the standard deviation. The experiment was repeated in triplicate.

DISCUSSION

Since proteases are important industrial enzymes, strain improvement is necessary for protease production, especially at the industrial scale. *B. subtilis* S1-4 strain was previously isolated from waste chicken feathers, as it was able to degrade the chicken feather efficiently (Yong et al., 2013). Keratinase or protease activity has been recognized as the key player in degradation of feather or keratin (Brandelli et al., 2010). Classic mutagenesis technology was applied to this strain in order to improve protease production. By three successive rounds of mutagenesis (UV, MNNG, and UV), a mutant UMU4 was selected with hydrolytic activity up to 464 U/mL. This is a 2.5-fold increase over the activity of the parent strain S1-4 (131 U/mL) under the same fermentation conditions. These results indicate that UV and MNNG are efficient as the mutagenesis reagents for *B. subtilis* S1-4. UV irradiation does not always work efficiently for improving alkaline β-keratinase activity, as was seen with *Brevibacillus* sp strain AS-S10-II (Mukherjee et al., 2011). Mutagenesis with only UV irradiation seemed less efficient in strain improvement for protease production (Nadeem et al., 2010). The mutant strain selected after UV irradiation only showed a 44% increase in protease activity with an alkalophilic bacterial strain of *B. pantotheneticus* (Shikha et al., 2007). However, the protease activity in *Pseudomonas* sp RAJR044 was enhanced 2.5-fold by UV mutagenesis (Dutta and Banerjee, 2006). This deviation may be due to the various bacterial strains used. In general, chemical mutagenesis is more efficient for improving protease production. The keratinolytic activity was enhanced by about 2.5-fold for the mutant KD-N2 of *B. subtilis* by MNNG mutagenesis (Cai et al., 2008). However, combination of multiple mutagenic treatments may give better results (Xia et al., 2012). The caseinolytic activity was enhanced 5-fold in the

mutant SCU11 of *B. pumilus* by a combination of UV, γ-rays, and MNNG (Wang et al., 2007). In conclusion, the classic mutagenesis strategy is still efficient to increase specific protease production.

As expected, the isolate UMU4 degraded the chicken feathers more efficiently than the parent strain (Table 1), and its extracellular protease activity was about 5-fold higher than the parent strain in the feather degrading cultures. These data indicate that the increase in protease activity is a key factor in feather degradation. Further, the content of soluble proteins was increased in UMU4, although the content of free amino acids and oligopeptides were only increased by a limited amount. Accordingly, the strain improvement for the extracellular protease could serve as an alternative strategy in exploiting feather degradation since many studies have been focused on the isolation of new bacterial strains or characterization and optimal production of the keratinase or protease (Brandelli et al., 2010; Korniłłowicz-Kowalska and Bohacz, 2011).

An extracellular protease from the mutant UMU4 strain was purified and characterized, although both the wild-type and UMU4 strains were shown to produce several extracellular proteases by zymography analysis (data not shown). The size of the purified protease was estimated to be 45 kDa by SDS-PAGE, which is similar to the alkaline protease KP-43 (43 kDa) from *Bacillus* sp strain KSM-KP43 (Saeki et al., 2007). In terms of the molecular weight, this protease is different from subtilisin E (30 kDa). Furthermore, the optimal temperature for this protease to catalyze hydrolysis was higher than that of the other proteases from various *Bacillus* species, such as *B. subtilis* CFR3001 (40°C) (Bhaskar et al., 2007), *B. pumilus* BA06 (50°C) (Wan et al., 2009), *B. proteolyticus* AP MSU6 (40°C) (Maruthiah et al., 2013), and *B. subtilis* GA CAS8 (50°C) (Sathishkumar et al., 2015). Consequently, this protease exhibited better thermostability in comparison with the other bacterial serine proteases (Haddar et al., 2009; Maruthiah et al., 2013), as the half-life of thermal denaturation at 60°C approaches 72.2 min (Figure 4). Regarding pH, the puriðed protease was active over a wide range (6.0 to 12.0) and the optimal pH was 8.0, which was similar with the other proteases from various *Bacillus* strains (Wan et al., 2009; Sathishkumar et al., 2015). However, this protease was less sensitive to the metal ions tested in this study. Usually, heavy metal ions, like Cu^{2+} and Hg^{2+}, lead to moderate inhibition of the hydrolytic activity of some bacterial serine proteases. For example, the protease from *B. subtilis* GA CAS8 was inhibited by Cu^{2+} and Zn^{2+} (Sathishkumar et al., 2015). In contrast, the hydrolytic activity of the protease from *B. subtilis* AP-MSU6 was stimulated by Cu^{2+} and Hg^{2+} (Maruthiah et al., 2013).

As shown in Figure 5, the hydrolytic activity was increased when surfactants like Triton X-100 and Tween-20 were added to the hydrolytic reaction, which was similar to other proteases from the different *Bacillus* species (Had-Alij et al., 2007; Haddar et al., 2009; Shah et al., 2010). Noticeably, Na_2SO_3 resulted in a 73% increase in the hydrolytic activity for this protease in comparison with the control (without addition of the additive). Na_2SO_3 has been previously reported to enhance feather degradation (Yong et al., 2013). In terms of chemical properties, Na_2SO_3 may provide reducing power that would help the substrate unfold to be accessible to the enzyme.

In conclusion, a high protease-producing mutant UMU4 was selected from *B. subtilis* S1-4 by three successive rounds of mutagenesis using UV irradiation and MNNG treatment. Feather degradation was enhanced in this mutant, as can be seen by the increase in soluble protein content, which suggests that strain improvement for the production of protease could be an alternative strategy in feather degradation. The main extracellular protease produced

by UMU4 was purified and characterized, indicating this protease is a typical serine protease with higher than usual optimal temperature and good thermostability in caseinolytic activity. These data suggest that the mutant UMU4 and its serine protease could be of use in various industries.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#31171204) to H. Feng.

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