Degradation of Native Feathers by a Novel Keratinase-Producing, Thermophilic Isolate, *Brevibacillus thermoruber* T1E

Zoltán Bihari[a,*], Diána Vidéki[a], Erzsébet Mihalik[b], Attila Szvetnik[a], Zsolt Szabó[a], Margit Balázs[a], Péter Kesserű[a], and István Kiss[a]

[a] Department of Applied Microbiology, Institute for Biotechnology, Bay Zoltán Foundation for Applied Research, Derkovits fasor 2., H-6726 Szeged, Hungary. Fax: +36-62-432250. E-mail: bihari@bay.u-szeged.hu

[b] Department of Plant Biology, University of Szeged, Szeged, Hungary

* Author for correspondence and reprint requests

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Strain T1E, isolated and identified as *Brevibacillus thermoruber*, and evolutionally distant from the known keratinolytic isolates, proved to have feather-degrading ability. During the 7-day fermentation period, T1E consumed 10 g/l native goose feathers as the sole source of carbon and energy at 50 ºC under aerobic conditions. The isolate secreted a thermostable, keratinolytic protease, which exhibited activity optimally at pH 6.5, whilst it was inhibited at alkaline pH. The keratin cleavage and catabolism resulted in the accumulation of free aspartic acid and soluble peptides with maximum values of 31.6 and 720 mg/l, respectively. The majority of the fermentation end-products were found to be small oligopeptides with an average molecular mass of 2275 Da.

Key words: Feather, *Brevibacillus thermoruber*, Keratinase

Introduction

Keratinases are currently gaining importance in biotechnology. The growing number of their applications stem from their ability to act on highly rigid and cross-linked polypeptides, even in insoluble form. The application of keratinolytic proteases in pre-tanning and dehairing steps furnishes a more effective and more environmentally friendly technology for the leather industry. Such enzymes are also used for the production of detergents, biodegradable films, coatings and glues. Furthermore, through the utilization of keratinases, the decomposition of casein, gelatin, collagen, wool, silk and also feathers can be achieved (Gupta and Rammani, 2006).

The poultry feathers generated as by-product in millions of tons per year worldwide are generally composted, incinerated or converted to feather meal during energy-consuming treatment (Cao *et al*., 2009). This latter method has several drawbacks and the biotechnological recycling of keratinous wastes is of great theoretical potential for the production of nutritionally rich animal feedstuffs (Onifade *et al*., 1998). Since native feathers are quite resistant at normal temperatures, thermoactive keratinases are preferred for efficient degradation. Such enzymes have been isolated from various mesophilic bacteria and fungi, as surveyed recently by Gupta and Rammani (2006), but the strains producing strong keratinases are mostly pathogenic and unsuitable for industrial applications (Suzuki *et al*., 2006). Consequently, direct fermentative applications of thermophilic keratinolytic microbes would be more advantageous.

Although, *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996), *F. islandicum* AW-1 (Nam *et al*., 2002), and *Thermoanaerobacter keratinophilus* 2KXI (Riessen and Antranikian, 2001) exhibit a high feather decomposition rate, they require anaerobic and hyperthermophilic (70 °C) culture conditions, which hinder their practical utilization. Among the moderately thermophilic, aerobic bacteria, *Bacillus licheniformis* PWD-1 (Williams *et al*., 1990), *Streptomyces thermonitrificans* MG104 (Mohamedin, 1999), and *Meiothermus ruber* H328 (Matsui *et al*., 2009) isolates with temperature optima of 50–55 °C have proved to possess keratinolytic activity.

In spite of their relevance, only limited numbers of thermophilic bacterial strains are able to degrade feathers, as indicated above. In consequence of environmental, industrial and sani-

tary demands, the isolation and description of novel non-pathogenic, thermophilic, keratinolytic strains is highly desirable. In this paper, we describe a novel *Brevibacillus* isolate with special feather-degrading features.

Material and Methods

Isolation and identification of isolate T1E

In order to isolate aerobic, thermophilic, feather-degrading bacteria, 0.5 ml of the waste stream of a Hungarian goose-processing plant was injected into 50 ml of KTM minimal medium [0.2 g/l KNO_3, 0.2 g/l $CaCl_2 \cdot 2\ H_2O$, 0.02 g/l K_2HPO_4, 0.02 g/l $MgSO_4$, 5 ml/l trace element solution SL-4 (http://www.dsmz.de/microorganisms/media_list.php, see Media 14 and 27), pH 7.0] supplemented with 10 g/l raw goose feathers as the sole carbon source and incubated aerobically at 50 °C. After 168 h, fractions of the cultures were serially diluted and spread on LB (Sambrook *et al.*, 1989) plates. One isolate, designated T1E, was identified and used for further studies.

16S rDNA analysis was performed on a genomic DNA template, prepared as described previously (Wilson, 1994). PCR amplification was carried out using the EubB(27F)-EubA(1522R) primer pair and the PCR method described by Suzuki and Giovannoni (1996). The resulting 1449-bp partial nucleotide sequence of the 16S rDNA gene has been submitted to the GenBank database and is reported under accession number DQ011668. The biochemical characterization of isolate T1E was achieved according to Shida *et al.* (1996) and standard public health protocols. The isolated strain T1E has been deposited in the Hungarian National Collection of Agricultural and Industrial Microorganisms as NCAIM B02377.

Cultivation conditions

Cells were routinely cultivated in 100-ml Erlenmeyer flasks containing 50 ml of KTM minimal medium supplemented with 10 g/l UV-sterilized raw goose feathers. Media were inoculated with 0.5 ml of the 24-h midlog phase culture grown on KTM minimal medium amended with 5 g/l yeast extract. Flasks were incubated at 50 °C and 160 rpm for 0–168 h.

Microbial growth was followed via the increases in optical density (OD_{600}) and in total cell numbers, which were counted microscopically. In order to determine the dry weight of the residual solid matter, triplicate cultures were centrifuged (16,000 × *g*, 20 min) every 24 h. After pH measurements, the supernatants were stored at -20 °C until use. The pelleted solid material was washed and filtered through a PVDF membrane (pore size 0.2 μm), air-dried at 105 °C, weighed and documented by a CCD camera (MegaView III, Soft Imaging System).

Analytical procedures

The total soluble protein concentrations of the cell-free supernatants were determined by the methods of Bradford (1976) and Lowry *et al.* (1951) using a Unicam Helios α spectrophotometer, applying bovine serum albumin as a standard. Gas chromatographic free amino acid analysis was performed according to Husek (1991).

For investigation of the molecular weight distributions of the proteinaceous products in the fermentation broths, SDS-PAGE (Laemmli, 1970) and gel filtration HPLC separations were carried out. 20 μl of the cell-free supernatants were injected onto a TSK G3000 PW_{XL} column (Tosoh, Tokyo, Japan) and eluted with 50 mM potassium phosphate buffer, pH 7.0, at a flow rate of 1.0 ml/min for 15 min at 40 °C. The Dionex chromatographic system was calibrated with polyethylene glycol standards; peaks were detected via the UV absorbance at 205 nm and via the refractive index.

The keratinase activity was determined according to Suntornsuk and Suntornsuk (2003) using keratin azure (Sigma) powder as substrate. 0.5 ml of enzyme-containing supernatants was added to 0.5 ml of keratin azure suspension (4 g/l) dissolved in 10 mM Tris-HCl, pH 7.5 or pH 6–9, when the effect of pH on enzyme activity was investigated. The reaction mixtures were incubated at 50 °C and 160 rpm for 1 h. After subsequent boiling for 5 min, the tubes were centrifuged (16,000 × *g*, 10 min), and the absorbance of the supernatants was measured at 595 nm. The values were corrected by the absorbance of the controls containing previously boiled (20 min) supernatant samples. One unit of keratinase was defined as the amount of enzyme causing 0.1 absorbance increase at 595 nm under the given conditions.

Protease enzyme assays were performed with the use of Hammarsten casein (Merck) as substrate according to Nam *et al.* (2002).

Scanning electron microscopy (SEM)

Feather samples were taken from inoculated flasks daily. After slicing, the samples were dehydrated in graded ethanol and acetone series, followed by critical point drying. The specimens were then sputter-coated with gold under vacuum. The preparations were observed under a Hitachi S-2400 scanning electron microscope at an accelerating voltage of 10–20 kV.

Results

Identification of strain T1E

The feather-degrading isolate T1E was classified by 16S rDNA analysis and phenotypic characterization. The PCR-amplified and sequenced 1449-bp 16S rRNA gene displayed the highest similarity (99.7%, 99.2%, and 98.8% identity) to that of the Lon protease-producing *Brevibacillus thermoruber* sp. WR-249 (AY196006; Lee *et al.*, 2004), the undersea isolate *Br. thermoruber* JAM-FM2501 (AB362290; Kobayashi *et al.*, 2008), and *Br. thermoruber* DSM 7064^T (Z26921; Rainey *et al.*, 1994), respectively. Comparison with the available 16S rDNA sequences revealed that the isolate T1E was evolutionally distant from the known thermophilic feather-degrading bacteria, but very similar to the *Br. thermoruber* type strain.

Further biochemical characterization was performed to confirm that this rod-shaped, endospore-forming, reddish pigment-producing Gram-positive isolate is a member of this genus. The strain was proved to be oxidase-positive, and negative for the reduction of nitrate and nitrite and also for starch hydrolysis. Growth was demonstrated in the interval 50–65 °C, but not at 37 °C. Acid was not produced from glucose, xylose, maltose, lactose, sucrose, arabinose, salicine or mannitol. In view of these biochemical properties and the similarity of the 16S rDNA sequences, we concluded that strain T1E is indeed a member of the *Br. thermoruber* species.

Degradation of native feathers

During a 7-d incubation period, strain T1E aerobically utilized 10 g/l raw goose feathers as the sole carbon and energy source at 50 °C, while the optical density of the culture steadily increased, reaching a plateau of OD_{600} = 2.2. This was equivalent to an increase in total cell number from initial 10^6 cells/ml to final $9{\cdot}10^8$ cells/ml. In line with this, the pH value of the medium increased from 7.0 to 8.65, and the dry weight content of the residual solid matter decreased from 10 g/l to 6 g/l. Apart from some larger barbs and quills, the decomposition of the feathers was complete. SEM micrographs demonstrated that the microbial attack began with colonization and strong adherence of the cells to the keratin surfaces (figure not shown). The barbs were depleted after 72 h of incubation, whilst the quills appeared to be intact. Intensive fragmentation in the quills could be observed on day 5 and it led to the break-down of the keratin structures.

Production of soluble catabolic products

Catabolic products were monitored daily in the native feather fermentation broths. Gas chromatographic amino acid analysis identified only free aspartic acid in the supernatants from day 2. Its concentration reached a maximum of (31.6 ± 0.6) mg/l on day 4 (Fig. 1), and then decreased to around 11 mg/l, suggesting that it is not only formed, but also consumed by the microbe. The Folin phenol measurement revealed that the concentration of T1E-produced soluble proteins increased up to day 6, reaching (720 ± 1) mg/l. Nevertheless, in comparison with the Folin phenol technique, the Bradford method detected significantly lower protein concentrations in the cell-free superna-

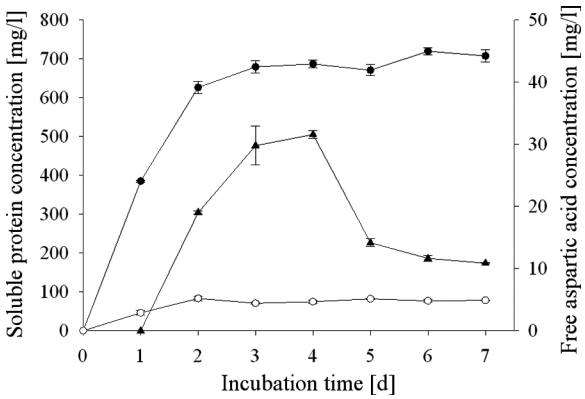

Fig. 1. Production of soluble proteins and aspartic acid by *Br. thermoruber* T1E during the fermentation of 10 g/l goose feathers under aerobic conditions in 50 ml of KTM minimal medium at 50 °C. Protein levels were determined in cell-free supernatants by the Folin phenol (full circles) or the Bradford method (open circles), whilst free aspartic acid concentrations (triangles) were measured by GC.

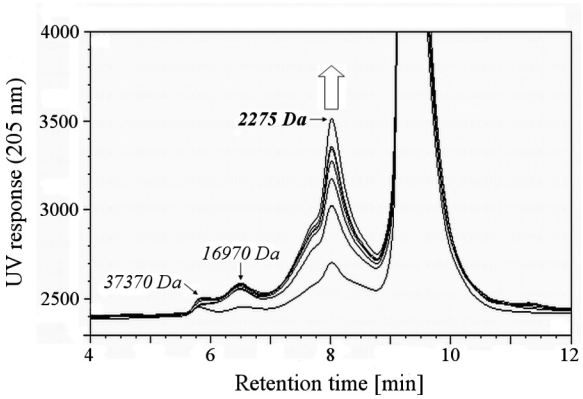

Fig. 2. Gel filtration HPLC chromatograms of soluble proteinaceous T1E products sampled every 24 h of fermentation. The open arrow indicates the accumulation of small oligopeptides with an average molecular mass of 2275 Da. Aspartic acid and minerals of the medium coeluted at a retention time of 9.5 min.

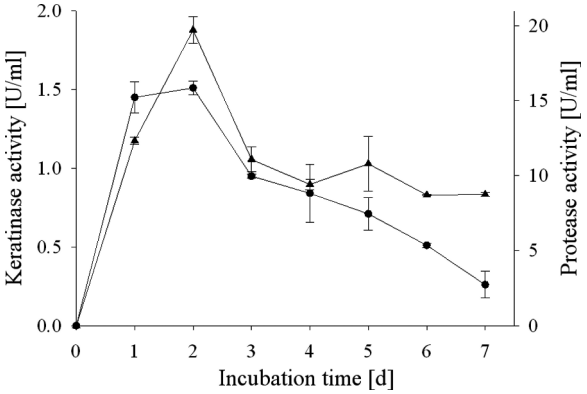

Fig. 3. Keratinase (circles) and protease (triangles) activities detected in cell-free supernatants during the biodegradation of 10 g/l goose feathers by *Br. thermoruber* T1E under aerobic conditions at 50 °C.

tants. The maximum value [(83.1 ± 1.4) mg/l] was reached on day 2 and then remained within the range 70–80 mg/l (Fig. 1). These results indicated that the catabolic products are mainly short peptides and not proteins.

This theory was verified by gel filtration HPLC-based molecular weight distribution analysis of the proteinaceous compounds, which demonstrated that the majority of these fermentation end-products were indeed small oligopeptides with an average molecular mass of 2275 Da. As illustrated in Fig. 2, the quantity of this oligopeptide pool steadily increased in the cell-free supernatants, suggesting that the keratinolytic proteases creating short peptides and free aspartic acid are involved in feather digestion.

Analysis of the keratinolytic protease

Keratinase and protease activities were successfully detected in the cell-free supernatants. In accordance with the kinetics of the changes in the protein levels, their activity increased up to day 2, reaching maxima of (1.51 ± 0.04) U/ml and (19.71 ± 1.51) U/ml, respectively (Fig. 3). The increases were followed by a drop in both activities in the fermentation broths, which is probably due to the unfavourable impact of the increasing pH value on the enzyme production. In order to evaluate the effect of pH on the keratinase activity, an enzyme assay was performed on a 48-h supernatant sample. The optimum pH value for keratinase was found to be 6.5, while an alkaline pH value significantly impaired the enzyme activity (Fig. 4). These findings suggest that an alkaline pH value has a dual adverse effect on keratinolysis, inhibiting either the production or the activity of the enzyme.

Since denaturing SDS-PAGE detected only one band with a molecular mass of 75 kDa in the cell-free supernatants, but not in the abiotic controls (Fig. 5), this protein was suspected of being the secreted keratinolytic protease of strain T1E. The specific keratinase activity of the enzyme was calculated to be 18.17 U/mg.

Discussion

A novel aerobic, thermophilic, keratinolytic strain was isolated from a Hungarian goose-processing plant, and classified as *Br. thermoruber*. Isolate T1E is a non-pathogenic, Gram-positive rod exhibiting optimal growth on native feathers at 50 °C. 16S rDNA sequence analysis revealed that T1E is evolutionally distant from the thermophilic, feather-degrading bacteria, but shares high similarity with different *Br. thermoruber* spp. Among these, the most closely related isolate is WR-249, which also produces a thermostable protease, but in contrast with that of T1E, the Lon protease can not be correlated to keratinolysis, since it is a DNA-binding ATPase possessing chaperone-like activity (Lee *et al.*, 2004). Two *Br. thermoruber* strains, B107 and B109, were recently

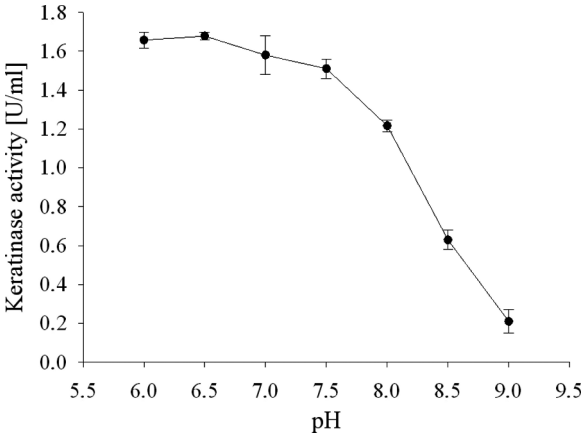

Fig. 4. pH dependence of *Br. thermoruber* T1E keratinase. For the keratinase assay, 48-h cell-free supernatant samples were used.

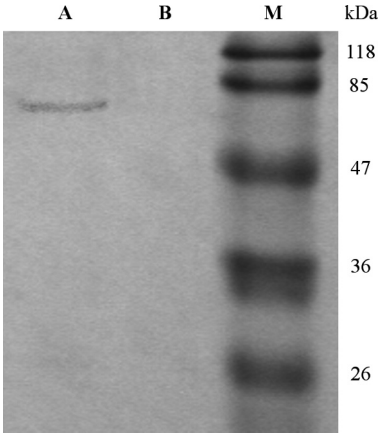

Fig. 5. SDS-PAGE of 48-h cell-free supernatant (A) and abiotic control (B) samples. Lane M, prestained protein molecular weight marker (Fermentas, Vilnius, Lithuania).

identified in hot aerobic poultry manure compost in Taiwan, but their keratin-degrading ability was not investigated (Wang *et al*., 2007). However, Suzuki *et al*. (2009) have demonstrated that another *Br. thermoruber* strain, the Japanese isolate YAS-1, possesses a protease with the ability to decompose fibroin, another hard-to-degrade protein. As illustrated in the examples above, *Br. thermoruber* species may play an important role in the depletion of recalcitrant animal wastes worldwide.

The feather-degrading features of aerobic, thermophilic bacteria are summarized in Table I, but a comparison of the keratin-degradation rates and keratinase activities of the different keratinolytic isolates is rather difficult due to the variety of growth substrates and definitions of enzyme units applied (Gupta and Ramnani, 2006; Suzuki *et al*., 2006). The present study revealed that *Br. thermoruber* T1E can deplete 10 g/l native feathers during 7 days at 50 ºC, which seems to correspond with the keratin-utilization ability of the thermophilic *Bacillus licheniformis* PWD-1 (Williams *et al*., 1990) and *Meiothermus ruber* H328 (Matsui *et al*., 2009). *Streptomyces thermonitrificans* MG104 exhibits a higher feather-consumption rate, but it produces significantly smaller

Table I. Comparison of feather degradation processes by aerobic, thermophilic bacteria.

Conditions	*Brevibacillus thermoruber* T1E[a]	*Bacillus licheniformis* PWD-1[b]	*Streptomyces thermonitrificans* MG104[c]	*Meiothermus ruber* H328[d]
Growth substrate	10 g/l intact goose feathers	10 g/l hammer-milled feathers	10 g/l intact chicken feathers	30 g/l intact chicken feathers
Incubation temperature	50 ºC	50 ºC	50 ºC	55 ºC
Incubation time	7 d	7–10 d	2–3 d	6 d
Maximum concentration of protein released	0.72 mg/ml	not determined	0.42 mg/ml	>10 mg/ml
Average molecular weight of protein products	2.23 kDa	not determined	not determined	<1 kDa
Maximum concentration of amino acid released	0.24 mM	26.45 mM	not determined	37.8 mM
Optimum pH value of keratinase	6.5	7.5[e]	9.0	not determined

Data were taken from: [a]this study; [b]Williams *et al*. (1990); [c]Mohamedin (1999); [d]Matsui *et al*. (2009); [e]Lin *et al*. (1992).

amounts of soluble proteins (Mohamedin, 1999). It is noteworthy that isolates T1E and H328 likewise give rise to short oligopeptides from intact feather substrates. During the fermentation processes, strains PWD-1 and H328 create enormous amounts of free amino acids in different compositions, whilst gas chromatographic amino acid analysis of the T1E culture supernatants detected only aspartic acid in low concentration.

In consequence of the deamination of peptides and amino acids, the intensive process of feather degradation coincides with a significant increase in the pH value of the culture broth. Accordingly, most of the microbes, *e.g. S. thermonitrificans* MG104, and their keratinolytic enzymes are adapted to alkaline circumstances. The optimum pH for the keratinase production of the mesophilic strain *Bacillus* sp. FK 46 grown on 10 g/l intact feathers was also found to be pH 9 (Suntornsuk and Suntornsuk, 2003). With the identical keratinase assay, the maximal keratinase activity in the FK 46 supernatant was found to be only 0.9 U/ml, while it was 1.51 U/ml in the case of *Br. thermoruber* T1E. Similarly as for *B. licheniformis* PWD-1, the keratinolytic protease of T1E proved to be neutrophilic. This enzyme of T1E, exhibiting a pH optimum at 6.5 and strong inhibition in the alkaline pH range, was assumed to correspond to a 75-kDa band detected on SDS-PAGE. To the best of our knowledge, strain T1E is the first characterized keratinolytic, protease-producing, feather-consuming member of the *Brevibacillus* genus.

The keratinolytic ability of thermophilic, aerobic T1E bacteria can be exploited for feather waste recycling. Feather keratin is much more susceptible for degradation at elevated temperatures, and additionally an efficient fermentation process at 50–55 °C is more suitable for sanitary demands. Feather by-products are potential carriers of the H5N1 agent, but the avian influenza virus is known to lose its viability above 50 °C (De Benedictis *et al*., 2007). Furthermore, unlike most of the mesophilic, keratinolytic bacteria, the thermophilic strains are non-pathogenic, *e.g.* isolate T1E is unable to grow on complex media at 37 °C. As feather waste fermentation through the use of *Br. thermoruber* T1E would produce nutritionally rich, harmless single-cell proteins, soluble oligopeptides, and aspartic acid from keratin, the future applications of this isolate appear to be profitable.

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