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Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity

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Abstract Several thermophilic anaerobic bacteria with keratinolytic activity growing at temperatures between 50°C and 90°C were isolated from samples collected on the island of São Miguel in the Azores (Portugal). On the basis of morphological, physiological, and 16S rDNA studies, the isolate 2KXI was identified as a new species of the genus *Thermoanaerobacter*, designated *Thermoanaerobacter keratinophilus*. This strain, which grows optimally at 70°C, pH 7.0, and 0.5% NaCl, is the first member of the genus *Thermoanaerobacter* that has been described for its ability to degrade native keratin. Around 70% of native wool was solubilized after 10 days of incubation under anaerobic conditions. The strain was shown to possess intracellular and extracellular proteases optimally active at 60°C, pH 7.0, and 85°C, pH 8.0, respectively. Keratin hydrolysis was demonstrated in vitro using a sodium dodecyl sulfate gel containing feather meal. The extracellular protease responsible for breaking down keratin fibers was purified to homogeneity in only one step by applying hydroxyapatite column chromatography. The enzyme belongs to the serine-type proteases and has a molecular mass of 135 kDa.

Key words Keratin degradation · Wool · Feather · *Thermoanaerobacter keratinophilus* · Thermoactive proteases · Purification

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Introduction

Keratin is the most abundant structural protein in skin, horn, hair, wool, and feathers. The most prominent mechanical characteristic of keratin is its stability against chemical reagents (acids and alkalis). Stability is caused by a high degree of cross-linkage via disulfide and hydrogen bonds and by hydrophobic interactions (Arai et al. 1996). Commonly known proteases (e.g., trypsin, pepsin) are not able to attack keratin to a large degree (Goddard and Michaelis 1934). Several microorganisms such as fungi (Tsuboi et al. 1989; Malathi and Chakraborty 1991; Dozie et al. 1994; Siesenop and Böhm 1995; Santos et al. 1996; Oyeka and Gugnani 1997; Singh 1997; Mignon et al. 1998; Gradisar et al. 2000), actinomycetes (Kunert 1989; Sinha et al. 1991; Böckle et al. 1995; Ignatova et al. 1999; Mohamedin 1999), and *Bacillus* species (Takami et al. 1989; Williams et al. 1990; Takami et al. 1992b; Atalo and Gashe 1993; Varela et al. 1997) are known to degrade keratinaceous substrates. However, only one report is available on the degradation of native keratin by a thermophilic anaerobic microorganism, *Fervidobacterium pennivorans* (Friedrich and Antranikian 1996).

In recent years there has been a great demand for a thermostable keratinolytic enzyme in various industries such as the textile industry. The finding of new enzymes that modify fibers such as wool and silk is necessary for the development of new enzyme-based, environmentally friendly technologies particularly attractive to the textile industry (Nolte et al. 1996). A variety of new enzyme-based products and processes utilizing cellulases, amylases, and lipases as well as proteases have already been introduced to the market (Fornelli 1993). However, there is still a need to find more robust and specific thermostable keratinolytic enzymes that can be applied in the textile (modification of wool fibers), food (hydrolysis of proteins derived from animals and plants), and pharmaceutical (production of bioactive peptides) industries.

In the present study, we report on the isolation and identification of a new thermophilic anaerobic microorganism with the ability to attack native keratin. Furthermore, we

present data on the characterization and purification of the enzyme responsible for keratin degradation.

Materials and methods

Sample collection

Mixed water and sediment samples were obtained from geothermally heated vents on the island of São Miguel in the Azores. These samples were collected in 100-ml glass bottles. The bottles were completely filled, sealed with gas-tight butyl rubber stoppers, and then the samples were reduced by adding 1 ml of 5% (wt/vol) $Na_2S{\cdot}9H_2O$ to the bottles. The new isolate, 2KXI, was collected at Furnas, a site with diverse geothermally heated waters. The sample site had a temperature of 74°C and a pH of 6.0. Only few hours after collecting the samples, inocula were transferred to 100-ml vials containing 50 ml of anaerobically prepared medium (see below) and keratin fibers.

Culture conditions

Isolation and enrichment of thermophilic anaerobic keratin-degrading microorganisms was performed in a complex medium (CM). The medium was prepared by following the anaerobic techniques described by Balch and Wolfe (1976), with N_2 in the gas phase. The CM-medium contained (per liter): K_2HPO_4, 1.6 g; NaH_2PO_4, 1.0 g; NaCl, 3.0 g; $(NH_4)_2SO_4$, 1.5 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; $CaCl_2{\cdot}2H_2O$, 0.05 g; $FeCl_3{\cdot}6H_2O$, 0.01 g; $SrCl_2{\cdot}6H_2O$, 0.03 g; H_3BO_3, 0.03 g; $NaWO_4$, 0.03 g; yeast extract, 0.1 g; tryptone, 0.05 g; trace element solution (Balch et al. 1979), 10 ml; vitamin solution (Wolin et al. 1964), 10 ml; resazurin, 0.001 g; $NaHCO_3$, 1.0 g; and $Na_2S{\cdot}9H_2O$, 0.5 g. The pH of the medium was adjusted to 6.8 with 1 N NaOH. Each culture tube contained one of the following insoluble polymers as the major carbon source: chicken feathers, merino wool, or human hair. Tyndallization was performed at 100°C for 60 min in order to reduce thermal degradation of the fibers. Incubation was performed anaerobically at 70°C, 80°C, and 90°C. Positive enrichment cultures were subcultured at least three times in the same medium prior to culture purification. Cultures were purified by using the end-point dilution technique after spreading on agar plates containing complex medium with 2% (wt/vol) agar. Single colonies from the most diluted tubes were picked, and the process of end-point dilution was repeated at least three times before the culture was considered to be pure. A phase-contrast microscope (Axioplan, Zeiss, Jena, Germany) was used for routine examination of the cultures.

Growth determination

Growth experiments were performed in triplicate in CM-medium containing 5 g/l each of yeast extract and tryptone. Due to difficulties monitoring growth with insoluble keratinaceous substrates, the soluble proteins yeast extract and tryptone were used. Growth was monitored by determining the cell number in a Neubauer counting chamber.

Antibiotic susceptibility

Antibiotics were added from filter-sterilized stock solutions to sterile prereduced medium to give a final concentration of 10 μg/ml.

Substrate utilization

Experiments were performed in 10-ml Hungate tubes containing CM-medium with 0.05% (wt/vol) yeast extract and 0.05% (wt/vol) tryptone. Major carbon sources were prepared separately in stock solutions. The end concentration of the substrates in the test tubes was 0.5% (wt/vol). The tubes were inoculated (5%, vol/vol) with an exponentially growing culture and incubated at 70°C. After three transfers, the final cell count was measured and compared with the control (no carbon source).

Isolation of DNA

Cells were disrupted with a French pressure cell, and DNA was purified on a hydroxyapatite column (Cashion et al. 1977). The DNA was hydrolyzed with P1 nuclease, and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Meshbah et al. 1989). The resulting deoxyribonucleosides were analyzed by high-performance liquid chromatography.

G+C content and 16S rDNA sequence

The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) by the method of Meshbah et al. (1989). For the experiments, a high-pressure pump, a UV detector model LKB 2151 connected with a Shimadzu CR-3A integrator (Shimadzu, Kyoto, Japan), and a Nucleosil 100-C18 column (250 by 4 mm) equipped with a Nucleosil 100-C18 precolumn (20 by 4 mm; VDS Optilab, Berlin, Germany) were used. The chromatographic conditions were 26°C, 0.6 M $(NH_4)\,H_2PO_4$/acetonitrile, 80/6 (vol/vol) (pH 4.4), as the solvent, and a flow rate of 0.7 ml/min (Tamaoka and Komagata 1984). The instrument was calibrated with nonmethylated Lambda-DNA (Sigma, Steinheim, Germany) with a GC content of 49.858 mol%. Analysis of 16S rDNA was performed by the method of Rainey et al. (1994) using a Taq Dideoxy Terminator Cycle Sequencing kit (Applied Biosystems, Weiterstadt, Germany).

Enzyme preparation

After 24 h of growth, the cells of strain 2KXI were harvested by centrifugation at 26,000 *g* for 30 min, and the pel-

lets were washed twice with 50 mM sodium phosphate buffer, pH 6.8. Ten grams of cells were resuspended in 50 ml of the same buffer and sonicated with a Branson Sonifier model 450 (Dietzenbach, Germany) in 1-s bursts for 5 min at an output of 20 W; the procedure was repeated three times. After centrifugation at 35,000 *g* for 30 min, the cell-free crude extract was used for further investigations. The supernatant containing the keratinolytic enzyme was concentrated 50-fold by ultrafiltration with an Amicon system (models 8400 and 8050; Amicon, Lexington, MA, USA) using a 10 kDa cut-off membrane. All samples were dialyzed in a 50-fold volume of 50 mM sodium phosphate buffer, pH 6.8.

Protease assay

Proteolytic activity was measured by a modified method of Kunitz (1947). Enzyme fractions (5–50 µl) were incubated with 450–495 µl of 0.5% (wt/vol) casein (Hammersten; Merck, Darmstadt, Germany) in 120 mM universal buffer (Britton and Robinson 1931) to give a final volume of 500 µl. Samples were incubated in a water bath at optimal temperature and pH for 15 to 30 min. The reaction was stopped by cooling on ice and the addition of 500 µl of 10% (wt/vol) trichloroacetic acid. After 10 min of incubation at room temperature, the reaction mixture was centrifuged at 13,000 rpm (Heraeus Sepatech, Osterode, Germany) for 10 min and the absorbance was measured at 280 nm. Sample blanks were used to correct for the nonenzymatic release of aromatic amino acids. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of aromatic amino acids (with tyrosine as the standard) per minute under the defined assay conditions. The protein content was measured by the method of Lowry et al. (1951). Microassays were performed with bovine serum albumin as the standard.

Thermostability

The thermostability profile was studied by incubating the dialyzed enzyme preparations in sodium phosphate buffer, pH 6.8, at various temperatures (from 60°–100°C). The residual proteolytic activity was measured at various time intervals at optimal temperature of the enzyme.

Effects of inhibitors and metal ions

Effects of different inhibitors and metal ions on the activity of the proteases were determined by preincubating the enzyme with the reagents for 1 h at room temperature. The final concentrations of the reagents in the protease assay were 1 to 5 mM. The reaction was started by the addition of 0.5% (wt/vol) casein giving a final volume of 500 µl.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 9% (wt/vol) gels (Laemmli 1970), and protein bands were detected by silver staining (Blum et al. 1987). Proteolytic activity in gels was detected with azocasein as the substrate. After electrophoresis, the gels were rinsed in 0.25% (wt/vol) Triton X-100 for 30 min and further incubated in 1.5% (wt/vol) azocasein in distilled water for 30 min. The gels were incubated under optimal assay conditions until clear activity bands were visible (5 min) in the orange-stained gel. The protein bands in the gels were then stained with Coomassie brilliant blue according to the method of Weber and Osborn (1969). To determine the molecular mass of the proteins, a broad-range molecular weight marker (Bio-Rad, Hercules, CA, USA) was used as the standard.

Zymogram with feather meal

SDS-PAGE was performed in 9% gels (see above) containing 0.2% feather meal. In order to prevent a quick sedimentation of the feather meal, the components without ammonium persulfate (APS) were preincubated in a 60°C water bath for 5 min. APS was added while stirring, and the solution was immediately transferred to the gel chamber system. Because of the preheated solution, polymerization started immediately, and the distribution of feather meal was nearly homogeneous. Protein bands possessing keratinolytic activity were visualized by staining the gel with Coomassie brilliant blue. Clear bands were visible at the positions where protein bands showed keratinolytic activities.

Enzyme purification

All purification steps were conducted at room temperature. Fiftyfold concentrated supernatant (4.36 mg/ml) was dialyzed against 10 mM sodium phosphate buffer, pH 7.2. The enzyme fraction was separated by hydroxyapatite column chromatography (column material, Boehringer Mannheim, Mannheim, Germany). After previously equilibrating the column with 10 mM sodium phosphate buffer pH 7.2. The column was washed with 150 ml of equilibration buffer. The proteins were eluted by increasing the ionic strength of the buffer stepwise (0, 50, 125, and 500 mM). The pH decreased from 7.2 to 6.8 (elution buffer: 500 mM sodium phosphate buffer, pH 6.8). Fractions with proteolytic activity were collected (2.5 ml per tube) at a flow rate of 0.8 ml/min, pooled, and concentrated by ultrafiltration (Amicon chamber, Beverly, MA, USA) using a 10 kDa cut-off membrane.

Degradation of wool

Cells were incubated in 50 ml of CM-medium containing 0.1% (wt/vol) yeast extract, 0.1% (wt/vol) tryptone, 0.25% (wt/vol) $Na_2S_2O_3$, and 0.5 g wool per vial. The vials were inoculated with 1 ml of an exponentially growing culture and incubated at 70°C in a nonshaking water bath for 10 days. At various time intervals, two of the vials were used

to determine the dry weight of the wool. Membrane filters (pore size, 0.2 µm) were incubated at 100°C for 24 h. As a control, wool was incubated without any inoculum under the same conditions. The proteolytic activity in the culture broth was also detected by protease assay (see above) applying 50 µl of culture broth to the assay at 85°C and pH 8.0 for 30 min.

Results

Enrichment and isolation of keratin-degrading bacteria

Enrichment cultures were obtained when samples from hydrothermal vents were incubated at 70°C with wool and feathers. Interestingly, no growth was obtained in Hungate tubes that were incubated at 80°C and 90°C. Keratinolytic activity of the cultures was evident by observing broken keratin fibers after 2 days of cultivation. Only wool and feathers were attacked; human hair was not degraded and no significant growth on this substrate was observed. Out of 12 samples, 4 pure cultures of anaerobic microorganisms with keratinolytic activity were obtained. The most active isolate on wool, 2KXI, was chosen for identification and further characterization of its proteolytic enzyme systems.

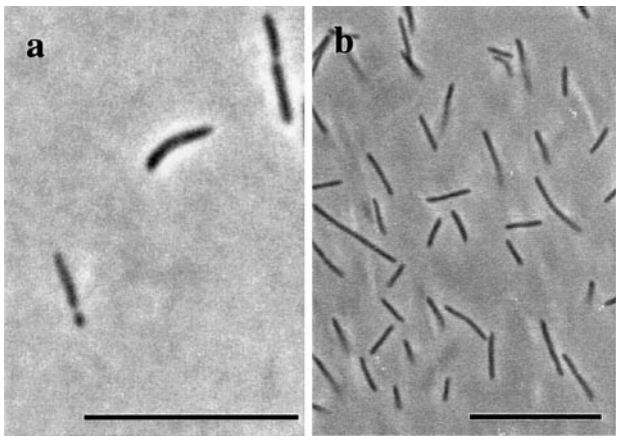

Fig. 1a,b. Phase-contrast micrographs of cells of the new strain 2KXI, *bar* 5 µm. Coccoid cell (**a**) and cells in exponential growth phase (**b**)

Cell morphology

Isolate 2KXI is a rod-shaped bacterium that occurs singly, in pairs, or in short chains. The rods are straight to slightly curved and motile. Rods with pointed ends and coccoid cells are frequently observed (Figs. 1a,b). The cell length ranges from 1 to 3 µm and the width from 0.2 to 0.3 µm in the exponential growth phase grown on medium with 0.5% (wt/vol) yeast extract and 0.5% (wt/vol) tryptone. In the stationary growth phase, pleomorphic forms, including filaments up to 10 or 15 µm were seen. Under our conditions, no spores were observed. The Gram-stain reaction was negative regardless of the growth phase.

Physiological and biochemical properties

Optimal temperature for growth was 70°C. No growth occurred below 50°C or above 80°C (Fig. 2a). The highest growth rate was obtained at pH 7.0, whereas only low growth rates were measured at pH 9.0 (Fig. 2b). Very long cells were observed under alkaline conditions. Optimal NaCl concentration was 0.5% to 1% (wt/vol) (data not shown). Further increase in the NaCl concentration led to a rapid decrease in the growth rates. The generation time of strain 2KXI at 70°C and pH 7.0 in a medium composed of 5 g/l yeast extract and 5 g/l tryptone was 67 min. All growth experiments were performed with sulfate as the electron acceptor. Substitution of sulfate by thiosulfate resulted in a threefold increase in cell yield. The final cell density in batch cultures with 2.5 g/l thiosulfate reached 3×10^9 cells/ml.

Substrate utilization

The new isolate was able to use casein, bactopeptone, yeast extract, tryptone, collagen, gelatin, starch, pectin, glucose,

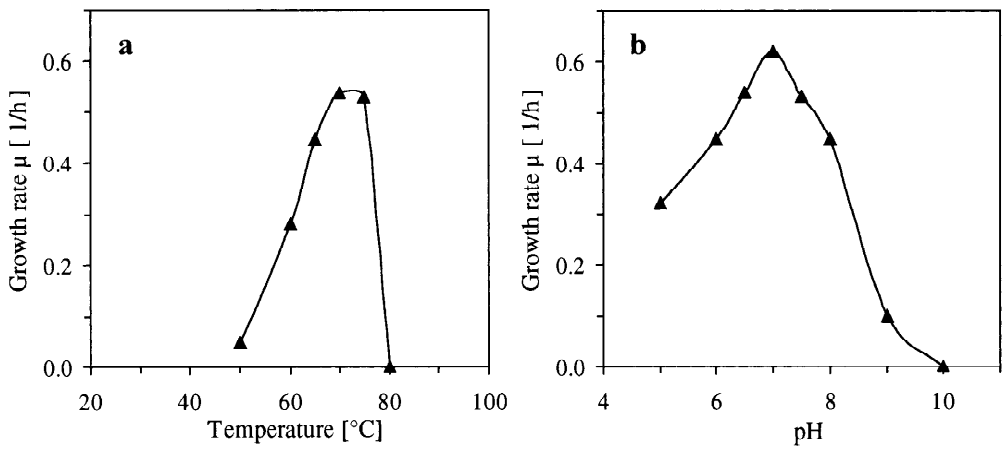

Fig. 2a,b. Influences of temperature and pH on growth of the isolated thermophilic strain 2KXI. **a** temperature was varied at pH 6.8; **b** pH was changed at 70°C

fructose, galactose, mannose, pyruvate, maltose, and cellobiose as growth substrates. The presence of 0.05% (wt/vol) yeast extract and 0.05% (wt/vol) tryptone is essential for growth. In addition to those substrates, the strain was able to grow solely on native wool and feather. The substrates that were not utilized by the new isolate include xylan, CM-cellulose, pullulan, xylose, arabinose, lactose, and olive oil. The new isolate was fermentative and strictly anaerobic.

Antibiotic susceptibility

Growth of strain 2KXI was completely inhibited by 10 µg/ml ampicillin, streptomycin, or kanamycin. Growth was not significantly inhibited by 10 µg/ml chloramphenicol or cycloheximide.

16S rDNA sequence analysis

Phylogenetic analysis of 16S rDNA sequences placed strain 2KXI in the cluster comprising members of the genus *Thermoanaerobacter*, with a maximal sequence identity to *Thermoanaerobacter brockii* subsp. *brockii* of 93.7%. The 16S rDNA sequence of strain 2KXI exhibited levels of similarities ranging from 93.7% to 84.4% with the sequences currently assigned to the genus *Thermoanaerobacter*. The G+C content of the new isolate was 37.6 ± 0.4 mol%. On the basis of physiological and morphological studies, strain 2KXI was characterized as a new species of the genus *Thermoanaerobacter* and named *Thermoanaerobacter keratinophilus* (Fig. 3).

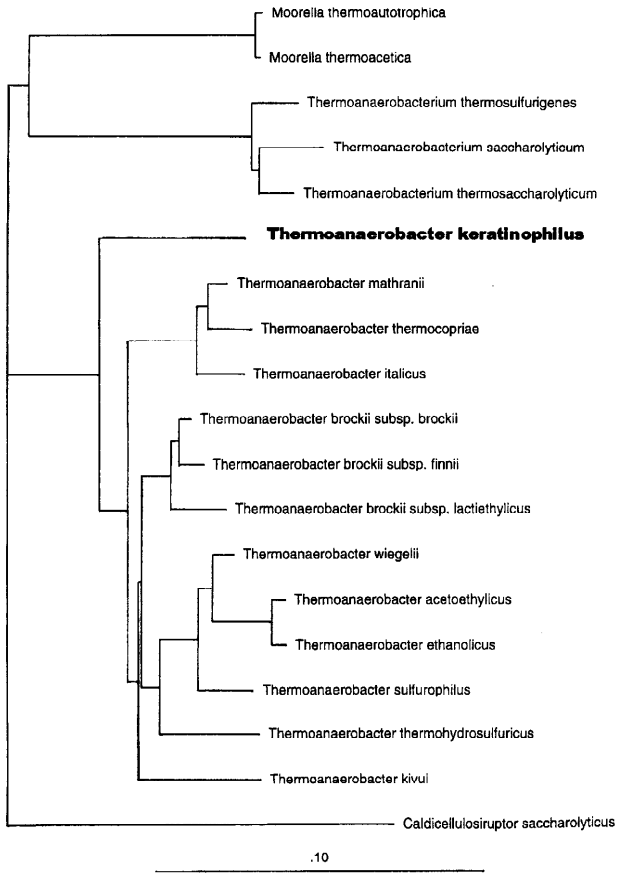

Fig. 3. Phylogenetic dendrogram based on the 16S rDNA sequence comparison indicating the position of strain 2KXI within the radiation of members of the genus *Thermoanaerobacter* and related taxa. *Scale bar* corresponds to 10 nucleotide changes per 100 nucleotides

Degradation of wool

When wool fiber was used as a carbon source, the new isolate could attack this substrate so that a 60% decrease in dry weight was measured after 6 days of growth. As shown in Fig. 4, nearly 70% of the wool was solubilized after 10 days. The protease activity in the culture fluid increased substantially from 10 U/l to 230 U/l from the second to the fourth day and remained constant until the end of fermentation. In the control experiment without inoculum, the wool dry weight was reduced by approximately 10% due to thermal degradation.

Enzyme characterization

T. keratinophilus possesses an intracellular protease and an extracellular keratinolytic enzyme that exhibit different physiochemical properties. The enzyme of the crude extract (intracellular fraction) was found to be active at a neutral pH of 6.5 to 7.0 (Fig. 5b). Since casein showed instability at pH values lower than 6.0, the proteolytic activity in acidic ranges with casein could not be tested. No enzyme activity was measured using hemoglobin or bovine serum albumin at pH 3.0 to 5.0. The extracellular enzyme was optimally active at pH 8.0 and was active over a broad pH range from 6.0 to 11.0 (Fig. 5b). The optimal temperatures for intracel-

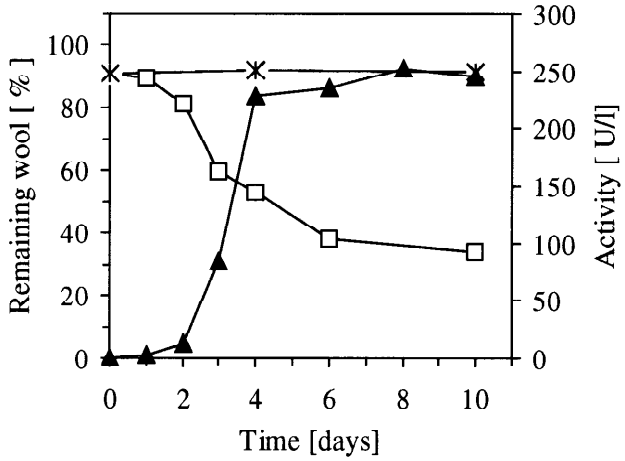

Fig. 4. Time course of the protease activity and solubilization of wool during growth of *Thermoanaerobacter keratinophilus* at 70°C, pH 7.0, on wool. Protease activity (*triangles*) was determined by standard protease assay at 85°C, pH 8.0. Decrease in wool dry weight in percent (*squares*). Control wool concentration without inoculum (*asterisks*)

lular and extracellular proteases were completely different. The intracellular protease showed optimal activity at 60°C. The keratinolytic extracellular enzyme was optimally active at 85°C. Both enzymes retained nearly 40% of their maximal activity at 100°C (Fig. 5a).

Investigations regarding the thermostability of the extracellular protease at neutral pH showed that no significant loss of activity was measured after 24 h of incubation at 70°C. The half life at 80°C, 90°C, and 100°C was 6 h, 1.5 h, and 5 min, respectively. By comparison, the intracellular protease of the same microorganism had a half life of about 2 h at 60°C and at 80°C.

The activity of the extracellular protease was completely reduced by serine-, metallo-, and cysteine-proteinase inhibitors (Table 1). Unlike the extracellular enzyme, the intracellular protease was not inhibited by these inhibitors. A slight inhibition (20%) was observed using the acid protease inhibitor pepstatin (Table 1).

Divalent metal ions Mg^{2+}, Ca^{2+}, and Mn^{2+} in concentrations up to 5 mM caused a slight activation of the extracellular protease (10%–20%) The activity of the intracellular protease on the other hand was reduced by those ions (data not shown).

Keratinolytic activity in SDS-PAGE

To identify the protein band responsible for keratin hydrolysis, SDS-PAGE including feather meal was performed. Both the intracellular and the extracellular enzyme fractions were applied to the gel. After incubating the gel at 60°C and pH 7.0 for 2 h, one broad activity band was visible for the extracellular protease (Fig. 6, *lane 1*). In the crude extract, no keratinolytic activity could be detected (Fig. 6, *lane 2*), indicating that only the extracellular enzyme fraction was active on keratinaceous substrates.

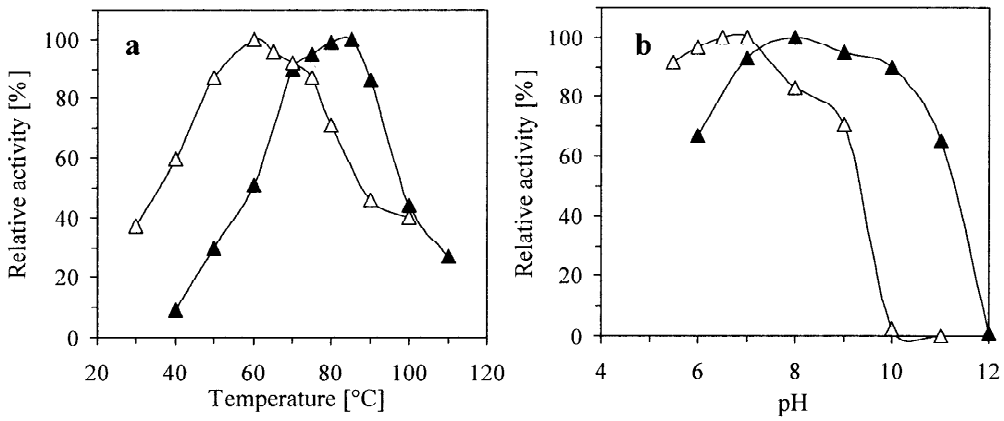

Fig. 5a,b. Characteristics of intracellular (*open triangles*) and extracellular (*solid triangles*) proteases of *Thermoanaerobacter keratinophilus* determined by casein hydrolysis. **a** Effect of temperature on intracellular (at pH 7.0) and extracellular (at pH 8.0) protease activities; **b** effect of pH at a temperature of 60°C for intracellular and 85°C for extracellular protease activities

Table 1. Effects of inhibitors on the activity of *T. keratinophilus* proteases

Inhibitor	Concentration (mM)	Class of inhibitor	Remaining protease activity (%)	
			Intracellular	Extracellular
None			100	100
PMSF[a]	1	Serine	ND	1
	5	Serine	101	ND
	10	Serine	103	ND
Pefabloc SC[b]	1	Serine	ND	1
	5	Serine	ND	0
Iodoacetate	1	Cysteine	ND	0
	5	Cysteine	95	ND
	10	Cysteine	110	ND
EDTA[c]	1	Metal chelator	ND	0
	5	Metal chelator	98	ND
	10	Metal chelator	103	ND
Pepstatin	0.3	Acidic	92	ND
	0.6	Acidic	78	ND

ND, not determined
[a] phenylmethylsulfonyl fluoride
[b] Boehringer Mannheim, Mannheim, Germany
[c] ethylenediaminetetraacetate

Table 2. Purification of the extracellular protease of *T. keratinophilus* by hydroxyapatite (HA) column chromatography

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (-fold)
Supernatant[a]	62.3	109	1.8	100	1
HA	4.5	79	17.5	72	10

[a] Cell-free supernatant of *T. keratinophilus* was concentrated 50-fold and dialyzed against 10 mM sodium phosphate, pH 7.2

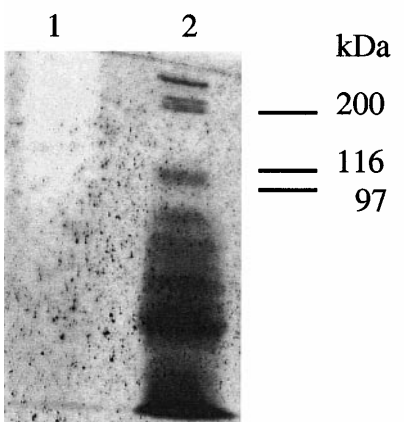

Fig. 6. SDS zymogram pattern of extracellular (supernatant) and intracellular (cell-free crude extract) enzyme fractions of *Thermoanaerobacter keratinophilus* in a 9% polyacrylamide gel containing feather meal. *Lane 1*: concentrated supernatant; *lane 2*: cell-free crude extract

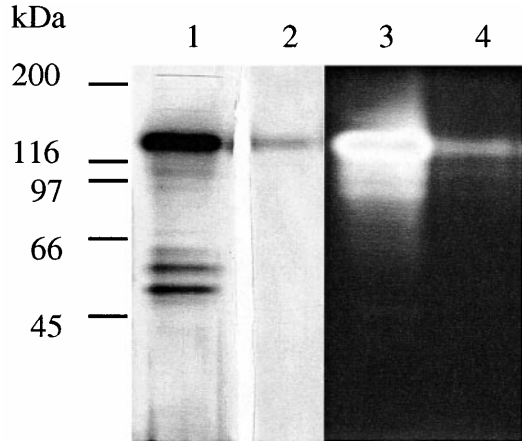

Fig. 7. Electrophoretic analysis of samples before and after purification on hydroxyapatite using 9% (wt/vol) SDS polyacrylamide gel. In *lanes 1* and *2*, protein bands were detected by silver staining. In *lanes 3* and *4,* activity bands were made visible after incubation of the gel in azocasein at 80°C and pH 8.0. *Lanes 1* and *3*: concentrated supernatant before purification (20 µg of protein); *lanes 2* and *4*: protease fraction after purification on hydroxyapatite (0.9 µg of protein)

Purification of the extracellular protease

The extracellular protease, which showed keratinolytic activity, was purified from the supernatant by hydroxyapatite column chromatography. The proteins were eluted by increasing the ionic strength of the elution buffer stepwise. The keratinolytic enzyme was eluted with 125 mM sodium phosphate buffer. The extracellular protease of *T. keratinophilus* was purified tenfold in a single step with 72% recovery (Table 2). The purified enzyme had a molecular mass of 135 kDa (Fig. 7) and a specific activity of 17.5 U/mg. No significant changes in temperature and pH optima between purified and nonpurified enzyme samples were observed.

Discussion

Earlier studies showed that the ability of extremely thermophilic, anaerobic microorganisms to degrade native keratin is rare among known Archaea and Bacteria. None of the 18 strains tested by Friedrich and Antranikian (1996) were able to attack native feathers. Only one strain, *Fervidobacterium pennivorans,* could hydrolyze chicken feathers within 3 days. In this study, four additional strains were isolated that were able to attack native wool and feathers. One of these isolates with the highest activity on wool was further described. Data obtained from the phylogenetic analysis designated the new isolate 2KXI as a member of cluster V of the genus *Clostridium*. We concluded that the new isolate belongs to a new *Thermoanaerobacter* species, for which we propose the name *Thermoanaerobacter keratinophilus*.

Apart from the Gram-positive stain reaction, all morphological characteristics described for the genus *Thermoanaerobacter* (Wiegel, 1986), such as formation of irregular, non-spore-forming rods, are similar to the morphology of the new isolate 2KXI. Although members of the genus *Thermoanaerobacter* are Gram-positive, negative Gram-staining was also described for *Thermoanaerobacter wiegelii* (Cook et al. 1996). Similar to other *Thermoanaerobacter* species (Schmid et al. 1986; Lee et al. 1993; Cayol et al. 1995; Cook et al. 1996; Liu et al. 1996; Bonch-Osmolovskaya et al. 1997; Larsen et al. 1997; Slobodkin et al. 1999), the G+C content of 37.6 mol % is low. *T. keratinophilus* reduces thiosulfate to hydrogen sulfide as has been described for *Thermoanaerobacter brockii*, *Thermoanaerobacter ethanolicus,* and *Thermoanaerobacter thermohydrosulfuricus* (Lee et al. 1993). A substantial improvement of growth of *Thermoanaerobacter* species in the presence of thiosulfate was also reported by Faudon et al. (1995).

T. keratinophilus produces at least two proteases that have different physicochemical properties. The intracellular protease is optimally active at pH 7.0 and 60°C and shows optimal activity in the range of optimal growth conditions. Since the enzyme was active only on soluble proteins, it can be speculated that the intracellular protease is responsible

for degrading peptides that are formed during the course of keratin hydrolysis. On the other hand, the extracellular protease is responsible for the degradation of keratin fibers. Similar catalytic keratinase activities at temperatures above 70°C have been described only for *Chrysosporium keratinophilus* (Dozie et al. 1994), *Bacillus* sp. strain AH-101 (Takami et al. 1989; Takami et al. 1992a, 1992b), and *Fervidobacterium pennivorans* (Friedrich and Antranikian 1996). Most of the other keratinases from bacteria and fungi (Böckle et al. 1995; Cheng et al. 1995; Santos et al. 1996; Singh 1997; Chitte et al. 1999; Vignardet et al. 1999; Gradisar et al. 2000) are active at alkaline pH but show optimal activity at least 20°C below the temperature optimum of the enzyme in the extreme thermophilic bacterium *T. keratinophilus*.

It is interesting to note that this is the first report on keratinase production by *Thermoanaerobacter* species. Even the most closely related strains, *Thermoanaerobacter brockii* and *Thermoanaerobacter finnii,* do not show keratin degradation (Friedrich and Antranikian 1996). Compared to other thermophilic protease producers, the strain 2KXI secretes a high level of enzyme into the culture supernatant (up to 90%) (Blumentals et al. 1990; Eggen et al. 1990; Lin and Tang 1990; Friedrich 1994; Choi et al. 1999). In the case of *Thermococcus stetteri,* only 5% of the total protease is released to the culture fluid (Klingeberg et al. 1995).

Similar to the results reported for *P. furiosus* and *T. stetteri,* multiple protease bands were observed in SDS gels, most probably due to autolysis (Blumentals et al. 1990; Klingeberg et al. 1995).

As described earlier, keratin hydrolysis is enhanced by reducing the disulfide bonds in keratin (Takami et al. 1992b; Böckle et al. 1995; Ignatova et al. 1999). Due to the reduced conditions of the growth medium in the presence of sodium sulfide, it can be speculated that these conditions favor wool degradation by *T. keratinophilus*. The detailed molecular action of the enzyme still remains to be elucidated.

Inhibition studies of the extracellular and intracellular proteases did not lead to a final conclusion regarding their catalytic action. Acidic amino acids may play a role in the catalytic center of the intracellular protease, which was slightly inhibited by 580 µM pepstatin (Table 1), whereas the extracellular protease of *T. keratinophilus* was completely inhibited in the presence of phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid, and ethylenediaminetetraacetate (EDTA). Since the complete inhibition by PMSF could not be made reversible by the addition of dithiothreitol, the enzyme cannot be classified as a cysteine proteinase. The enzyme belongs to the metalloserine proteases.

The high concentration in the supernatant and the thermostability of the extracellular keratinase would allow the enzyme to be used in industrial processes running at elevated temperatures. The high thermostability at 70°C (no loss of activity within 24 h) combined with low thermostability at 100°C ($t_{1/2}$ = 5 min) would allow a process to be controlled by varying the temperature.

Description of *Thermoanaerobacter keratinophilus* sp. nov.

Thermoanaerobacter keratinophilus Riessen and Antranikian (ke.ra.ti.no'phil.us. Gr. n. *keras* keratin; Gr. adj. *philos* loving; M.L. adj. *keratinophilus* keratin-loving). The cells are rod-shaped and occur singly, in pairs, or in short chains. The rods are straight to slightly curved and frequently form coccoid cells. The cell length ranges from 1 to 3 µm and the width from 0.2 to 0.3 µm in the exponential growth phase. Obligately anaerobic. Growth occurs above 50°C and below 80°C, with an optimum temperature of 70°C. Optimal pH for growth is 7.0 (range 5.0–9.0), and optimal NaCl concentration is 5–10 g/l (range 0–30 g/l). Utilizes casein, bactopeptone, yeast extract, tryptone, collagen, gelatin, starch, pectin, glucose, fructose, galactose, mannose, pyruvate, maltose, and cellobiose as substrates. Yeast extract and tryptone are essential for growth on saccharolytic substrates. No growth occurs on xylan, CM-cellulose, pullulan, xylose, arabinose, lactose, or olive oil. Thiosulfate is not required for growth but significantly stimulates growth. Growth is inhibited by ampicillin, streptomycin, and kanamycin. The G+C content of the type strain is 37.6 ± 0.4 mol%. Type strain 2KXI was isolated from hydrothermal vents in the area of Furnas on the Azorean island São Miguel and has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen under accession number DSMZ 14007.

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