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Characterization of a Keratinolytic Serine Proteinase from *Streptomyces pactum* DSM 40530†

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A serine protease from the keratin-degrading *Streptomyces pactum* DSM 40530 was purified by casein agarose affinity chromatography. The enzyme had a molecular weight of 30,000 and an isoelectric point of 8.5. The proteinase was optimally active in the pH range from 7 to 10 and at temperatures from 40 to 75°C. The enzyme was specific for arginine and lysine at the P_1 site and for phenylalanine and arginine at the P_1' site. It showed a high stereoselectivity and secondary specificity with different synthetic substrates. The keratinolytic activity of the purified proteinase was examined by incubation with the insoluble substrates keratin azure, feather meal, and native and autoclaved chicken feather downs. The *S. pactum* proteinase was significantly more active than the various commercially available proteinases. After incubation with the purified proteinase, a rapid disintegration of whole feathers was observed. But even after several days of incubation with repeated addition of enzymes, less than 10% of the native keratin substrate was solubilized. In the presence of dithiothreitol, degradation was more than 70%.

The microbial degradation of insoluble macromolecules like cellulose, lignin, chitin, and keratin depends on the secretion of extracellular enzymes with the ability to act on compact substrate surfaces. The structural protein keratin can be degraded by some species of saprophytic and parasitic fungi (3, 33, 34), a few actinomycetes (26, 30, 37), some *Bacillus* strains (41), and the thermophilic *Fervidobacterium pennavorans* (13). The mechanical stability of keratin and its resistance to microbial degradation depend on the tight packing of the protein chains in α-helix (α-keratin) or β-sheet (β-keratin) structures and their linkage by cystine bridges. Keratinolytic enzymes, so-called keratinases, which have been purified from different microorganisms and characterized to date (2, 12, 23–25, 28, 36, 39, 42) all act as proteinases and have a high level of activity on insoluble protein substrates such as keratin. Keratinolytic proteinases could play an important part in biotechnological applications like enzymatic improvement of feather meal and production of amino acids or peptides from high-molecular-weight substrates or in the leather industry (9–11, 26, 31, 32).

In our laboratory, in a screening of more than 150 microorganisms for feather-degrading ability, *Streptomyces pactum* DSM 40530 showed the highest level of keratinolytic activity (7). This strain had originally been characterized as a producer of various antibiotics, e.g., pactamycin (5), but not for keratinolytic activities. In this work, the extracellular proteinases were tested for proteolytic and keratinolytic activities and the main extracellular proteinase was purified and characterized.

MATERIALS AND METHODS

Organism and growth conditions. The bacterium used in this study was the strain *S. pactum* DSM 40530. The medium contained the following: 2.5 g of whole chicken feathers per ml, 2 mM potassium phosphate buffer (pH 7.5), 1 mM $MgSO_4$, and 10 ml of a trace element solution containing 27 mM $CaCl_2$, 4 mM Fe(III) citrate, 1.3 mM $MnSO_4$, 0.7 mM $ZnCl_2$, 0.16 mM $CuSO_4$, 0.17 mM $CoCl_2$, 0.10 mM Na_2MoO_4, and 0.26 mM $Na_2B_4O_7$ per liter (40). The medium was sterilized by autoclaving at 121°C for 20 min. For proteinase production, *S. pactum* was grown for 4 days at 28°C with constant shaking (280 rpm).

Enzyme purification. Following centrifugation of the culture (18,000 × *g*, 4°C, 30 min), the supernatant was filtered through a paper filter and concentrated by ultrafiltration (molecular size cutoff, 10 kDa; Amicon, Witten, Germany). The concentrate was dialyzed against 5 mM potassium phosphate buffer, pH 7.5, and applied to a column filled with 4 ml of casein agarose (ICN, Meckenheim, Germany). After washing of the column with 5 mM potassium phosphate buffer, pH 7.5, elution was performed with NaCl gradients from 0 to 0.5 M (160 ml) and 0.5 to 1.0 M (60 ml) at a flow rate of 0.5 ml/min.

Protein concentrations were measured photometrically at 280 nm and with Bradford dye reagent (Bio-Rad, Munich, Germany).

Determination of caseinolytic activity. The caseinolytic activity was determined by a modification of the method of Kunitz (21). The enzyme was incubated with 0.25% (wt/vol) Hammersten casein in 50 mM potassium phosphate buffer, pH 7.5, at 50°C for 20 min. One unit (U) of proteinase activity was defined as the amount of enzyme required to cause an increase of 1.0 A_{280} unit within 1 min.

Electrophoretic methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) and isoelectric focusing with Ampholine PAGE plates (pH 3.5 to 9.5) (Pharmacia LKB, Freiburg, Germany) were used for protein analyses. For zymograms, SDS-PAGE was modified by adding 0.1% gelatin to the gel. Before application, the samples containing 0.03 U of proteinase were mixed with the electrophoresis buffer and incubated at room temperature. After electrophoresis, the gels were washed in 2.5% (vol/vol) Triton X-100 for 1 h at room temperature and then incubated for 30 min at 50°C in 50 mM Tris-HCl buffer, pH 7.5, containing trace elements (40). The reaction was interrupted by incubating the gel in 10% (wt/vol) trichloroacetic acid solution. Staining was performed with amido black (Serva, Heidelberg, Germany).

Influence of pH and temperature on enzyme activity and stability. The pH and temperature optima of the proteinases in the culture medium and of the purified serine proteinase were determined with casein and Azocoll (Calbiochem, Los Angeles, Calif.) as substrates. The pH optimum was studied in the pH range of 5 to 11 with a buffer system of phosphoric acid, acetic acid, boric acid, and NaOH (8) at 50°C. The temperature optimum was studied with casein and Azocoll from 4 to 80°C at pH 7.5, with and without addition of mineral salts (40) or Ca^{2+} (1 and 5 mM). The influence of temperature on keratinolytic activity was studied by incubation of culture filtrate with whole chicken feathers at pH 8 in the temperature range from 20 to 80°C for 24 h. Disintegration of the feather structure was assessed qualitatively.

For stability studies, the culture filtrate was incubated at temperatures from 4 to 60°C at pH 7.8 from several hours to several days. The purified serine proteinase was incubated at pH 5 to 10 and at temperatures from 4 to 50°C. At intervals, samples were tested for residual proteolytic activity with casein as a substrate.

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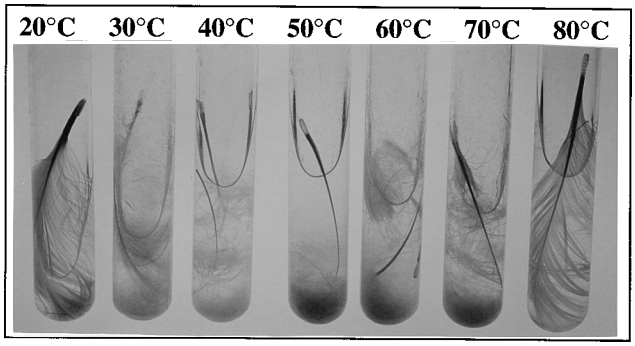

FIG. 1. Disintegration of native chicken feathers by culture filtrate of *S. pactum* at different temperatures.

Effects of proteinase inhibitors, metal ions, chelator, organic solvents, detergents, and reducing agents on the proteinase activity. The following proteinase inhibitors were added to the enzyme: phenylmethylsulfonyl fluoride (PMSF) (0.001 to 0.2 mM), [4-(2-aminoethyl)-benzyl-sulfonylfluoride]hydrochloride (AEBSF) (0.5 and 2.5 mM), elastinal (10 μg/ml), pepstatin (10 and 100 μg/ml), tosyl-L-lysylchloromethylketone (TLCK) (0.1 and 0.5 mM), and tosyl-L-phenylalanylchloromethylketone (TPCK) (0.1 and 0.5 mM). After incubation at room temperature for 30 min, casein was added and the enzyme activity was measured as described above.

EDTA, Ca^{2+}, mineral salts solution (40), dimethyl sulfoxide (DMSO), isopropanol, SDS, Triton X-100, dithiothreitol (DTT), β-mercaptoethanol, and Na-thioglycolate were incubated with the proteinases for 30 min at room temperature (for concentrations, see Table 2). Next, casein was added and enzyme activity was measured as described above. In these assays, potassium phosphate buffer was replaced by 50 mM Tris-HCl buffer, pH 7.5, to avoid precipitations.

Enzyme kinetic measurements with synthetic substrates. The hydrolysis of synthetic chromogenic substrates, amino acid *p*-nitroanilides (*p*NA) and *p*-nitrophenyl esters (ON*p*), was monitored spectrophotometrically at 405 nm by the release of *p*-nitroaniline or *p*-nitrophenol against a blank without enzyme. The reaction buffer (200 mM sodium phosphate, pH 7.8) was thermostated at 50°C. After addition of the enzyme, the reaction was initiated by addition of the substrate (concentrated solution in DMSO; maximal DMSO concentration in the reaction mixtures was 5%). K_m, k_{cat}, and the k_{cat}/K_m ratio were calculated from product accumulation curves, with molar absorption coefficients for *p*-nitroaniline and *p*-nitrophenol determined in the reaction buffer at 50°C. At least six different concentrations were used, and the steady-state kinetic parameters were calculated by using Eadie-Hofstee transformation of the Michaelis-Menten equation. The molar concentration of the enzyme was estimated from the protein content. The transferase-to-hydrolase ratio (k_T/k_H) (16) was measured with benzoyl-arginine-ethylester (BAEE) (5 mM) as the activated substrate and different amino acids, peptides, and amino acid amides as nucleophiles (concentrations, 50 to 200 mM). The occurrence of the hydrolysis product benzoyl-arginine (BA) and the transferase product (BA-X) was monitored by high-performance liquid chromatography. The analysis was performed on an LKB chromatography system (Pharmacia LKB, Bromma, Sweden) consisting of a solvent delivery system, a gradient controller, a UV-Vis detector, and a column oven. An RP 18 (5 μm) column (Merck, Darmstadt, Germany) was used at 56°C. The elution was isocratic with methanol (MeOH) (30%, vol/vol) and 0.067 M potassium phosphate buffer, pH 4.7 (70%, vol/vol), or with step gradients with an MeOH content from 7 to 30% depending on the retention times of the various substrates and products. The amounts of the transferase product BA-X could not be measured directly because standards were not available. Therefore, the concentrations were estimated by the difference between decrease of BAEE and release of BA.

Determination of enzyme activity with insoluble substrates. All assays were performed with the proteinase mixture of the culture medium and with the purified enzyme of *S. pactum.*

The activity with Azocoll was determined by direct spectrophotometric measurement (14) with a UV spectrophotometer (UV-160; Shimadzu, Kyoto, Japan) with an integrated cell stirrer (Spinette electronic cell stirrer SCS 1.22; Starna GmbH, Pfungstadt, Germany). The incubation was performed at 50°C with 2 mg of Azocoll per ml in 50 mM potassium phosphate buffer, pH 7.8, containing trace elements (40).

Native, autoclaved, and milled feather keratin; human hair; native sheep wool; bovine keratin powder (Merck); collagen; elastin (Serva); and gelatin (Merck) were incubated (1% [wt/vol] in the above-mentioned buffer) with 0.25 U of enzyme per ml for 1 to 6 h with constant agitation. Peptide liberation was measured photometrically at 280 nm in the supernatant (after trichloroacetic acid precipitation).

Feather meal (1% [wt/vol]; washed in 70% ethanol, 70°C, 2 h) was incubated with proteinase for 4 days at 37°C with constant agitation in 50 mM potassium phosphate buffer, pH 7.5, with mineral salts (40). Every day, fresh enzyme (0.03 U/ml) was added. After 4 days, the loss of dry weight was determined after filtration through membrane filters (pore size, 0.2 μm), washing, and drying at 105°C for 3 h.

The hydrolysis of keratin azure (Sigma, Munich, Germany) was performed in a solution containing 1% substrate in phosphate buffer, pH 7.8, with mineral salts (40) and 0.04% NaN_3 (to avoid microbial contamination) at 50°C for 5.5 days with constant agitation. A 0.03-U amount of proteinases per ml was added every 24 h. During incubation, liberation of the dye was measured at 620 nm. The residual dry weight of the keratin azure was determined as described above. In addition, the following commercially available proteinases were used: Corolase N (Röhm, Darmstadt, Germany), pronase E (Merck), proteinase from *Streptomyces caespitosus* (ICN), and proteinase K (Merck).

The effect of DTT on keratin degradation was tested with the proteinase mixture and the purified serine proteinase. Autoclaved and native chicken feather downs were incubated with and without 1% (wt/vol) DTT.

RESULTS

Characteristics of the extracellular proteinases of *S. pactum*. The fermentation of *S. pactum* was performed with chicken feathers as the sole carbon source to induce the enzymes responsible for keratin degradation. Zymograms showed that the culture medium contained different proteases in the range of 15 to 30 kDa.

In order to classify the types of proteinases involved, the inhibitory effects of PMSF and EDTA on the enzyme activity were tested with casein as the substrate. Proteinase activity was inhibited up to 70% by PMSF and up to 40% by EDTA. In the presence of both inhibitors, no residual activity was observed. Endoprotease activity was observed with benzoyl (Bz)-Arg-*p*NA, acetyl (Ac)-Lys-*p*NA, succinyl (Suc)-Ala-Ala-Pro-Phe-*p*NA, and Suc-Ala-Ala-Ala-*p*NA. Exoprotease activity with H-Phe-*p*NA and H-Arg-*p*NA was negligible.

The proteinases were active over the pH range from 6 to 11, with maximal activity between pH 7 and 8. The temperature optimum with casein was 55°C. Disintegration of whole chicken feathers by incubation with culture filtrate was optimal in the range of 40 to 70°C (Fig. 1). At 30°C, which is the optimum growth temperature of *S. pactum*, only a slow disintegration was observed. In the culture medium, the proteinases were stable for several weeks at temperatures up to 35°C; at 50°C the half-life was 24 h, and at 60°C the half-life was 6 h.

Purification of the main serine proteinase. The major serine proteinase was purified in one step by casein affinity chromatography (Fig. 2 and 3). The proteolytic activity was separated into two fractions, the proteinases that did not bind to casein agarose and a proteinase which was bound to the column and eluted with 100 mM NaCl. The first fraction represented 29% and the second represented 42% of the proteinase activity

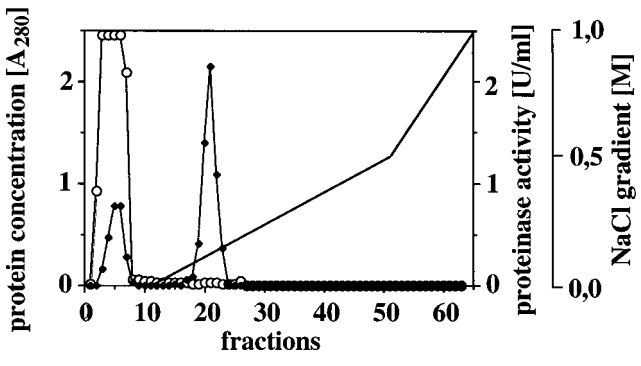

FIG. 2. Purification of *S. pactum* proteinase by affinity chromatography on casein agarose. ○, protein concentration; ●, proteinase activity; ——, NaCl gradient.

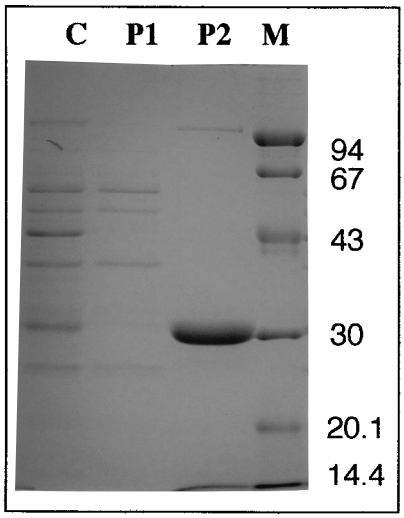

FIG. 3. SDS-PAGE of concentrated culture medium (C) and of the proteinases eluted at the front (P1) and the serine protease peak (P2) after affinity chromatography on casein agarose. Each lane contained 10 μg of protein. M, low-molecular-weight marker proteins (phosphorylase *b*, albumin, ovalbumin, carboanhydrase, trypsin inhibitor, and α-lactalbumin).

TABLE 2. Effect of solvents, detergents, and reducing agents on the activity of purified *S. pactum* proteinase

Substance group	Substance	Concn (%)	Proteinase activity (%)
Control without additives			100
Detergents	SDS	0.1[a]	63
		0.5	59
	Triton X-100	0.1[b]	88
		0.5	88
Organic solvents	DMSO	1[b]	111
		5	118
		10	105
	Isopropanol	1[b]	95
		5	82
Reducing agents	DTT	0.1[a]	113
		0.5	121
	β-Mercaptoethanol	0.1[b]	103
		0.5	100
	Thioglycolate	0.4[a]	66
		0.8	25
		1.2	0

[a] Wt/vol.
[b] Vol/vol.

applied (Table 1). It must be noted that the proteolytic activity in the culture filtrate resulted from different proteinases. Therefore, calculation of the enzyme enrichment is somewhat ambiguous. The second proteinase peak consisted of a single protein band of 30 kDa with an isoelectric point of about 6.0.

Influence of pH and temperature on enzyme activity and stability. The optimum pH for activity of the purified proteinase with casein and Azocoll was 8. In the pH range of 7 to 10, more than 80% of the maximal activity was measured. The proteinase displayed maximal activity with casein, Azocoll, and feather keratin at 60 to 65°C. The purified enzyme was stable for 5 h at temperatures up to 35°C and at pH values from 5 to 10. At 50°C the enzyme was less stable. At pH 5 to 7 the half-life was approximately 5 h; at pH 8 and 9 the half-lives were 2.5 and 1.5 h, respectively; and at pH 10 the proteinase was inactivated within the first minutes of incubation. The stability of the enzyme at temperatures above 50°C could be increased by the addition of the mineral salts solution used in the culture medium or by the addition of Ca^{2+}.

Effect of inhibitors. The purified enzyme was completely inhibited by the serine proteinase inhibitors AEBSF and PMSF. Since the inhibition was not reversible by the addition of DTT, the enzyme is not a cysteine proteinase. None of the other specific serine proteinase inhibitors tested, e.g., elastinal, pepstatin, TLCK, and TPCK, displayed a significant influence on the proteinase activity. The addition of EDTA caused a decrease in proteinase activity up to 30%.

Effect of solvents, detergents, and reducing agents. The proteinase showed a high level of stability with different additives (Table 2). In the presence of SDS and thioglycolate, proteinase activity was reduced. DMSO and DTT had a slightly positive effect on proteinase activity.

Substrate specificity and stereospecificity. P_1 specificity (17, 33) and stereospecificity of the purified enzyme were tested with different synthetic amino acid derivatives (Table 3). The *p*-nitroanilides without amino protection, H-Arg-*p*NA and H-Phe-*p*NA, were hydrolyzed only at a very low rate. The *p*NA of basic amino acids arginine and lysine and longer substrates were preferably cleaved. The k_{cat} values for ON*p* substrates were much higher than those for *p*NA substrates. The proteinase showed a high selectivity for L-enantiomers of amino acid derivatives; D-enantiomers were converted at a much lower rate. With Bz–D-Arg–*p*NA, no hydrolysis could be detected, and *N*-benzyloxycarbonyl (Z)–D-Phe–ON*p* and Z–D-Leu–ON*p* were hydrolyzed at a three- to eightfold-lower rate than the L-enantiomers were.

P_1' specificity and stereospecificity were tested by acyl transfer to different nucleophiles (Table 4). Amides or peptides of the basic or nonpolar amino acids phenylalanine, arginine, alanine, and lysine were accepted as nucleophiles. The peptide Ala-Ala-Ala-Ala was a better substrate than was Ala-NH_2. With the D-enantiomers D-Ala–NH_2 and D-Phe–NH_2, no transferase reaction was observed or the $(k_T/k_H)_{app}$ was much lower.

Liberation of peptides from different soluble substrates (casein and gelatin) and insoluble, high-molecular-weight substrates (native and autoclaved chicken feathers, feather meal, sheep wool, bovine keratin, keratin azure, Azocoll, collagen, and elastin) was observed. The activity level with gelatin and sheep wool was very low; with human hair, it was negligible.

Degradation of keratin azure by proteinases of *S. pactum* and by other commercially available proteinases. The main release of peptides from the insoluble keratin azure was observed in the first 2 days of incubation for all proteinases (Fig.

TABLE 1. Purification of the serine proteinase from *S. pactum*

Purification step	Protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	98.0	64.0	0.65	100	1
Ultrafiltration concentrate	72.8	51.6	0.71	81	1.1
Casein agarose					
Proteinase peak 1	72.0	14.9	0.21	23	0.3
Proteinase peak 2	0.5	21.7	42.1	34	64.8

TABLE 3. Enzyme kinetic parameters for hydrolysis of *p*-nitroanilides and *p*-nitrophenyl esters with different amino acids by the purified serine proteinase of *S. pactum*

Substrate[a]	K_m (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Bz-Arg-*p*NA	0.05	4.6	92
Bz–D-Arg–*p*NA	—[c]	—	—
Ac-Lys-*p*NA	0.42	1.0	2.4
Suc-Ala-Ala-Pro-Phe-*p*NA	0.55	33.0	66
Suc-Ala-Ala-Ala-*p*NA	0.98	0.4	0.4
H-Arg-*p*NA		**	
H-Phe-*p*NA		**	
Z-Phe-*p*NA		*	
Ac-Tyr-*p*NA		*	
Ac-Ala-*p*NA	—	—	—
H-Gly-Glu-*p*NA	—	—	—
Z-Gly-Pro-*p*NA	—	—	—
Suc-Phe-*p*NA	—	—	—
Z-Cys(Bzl)-ON*p*	0.0006	0.3	500
Z-Phe-ON*p*	0.004	4.0	1,000
Z–D-Phe–ON*p*	0.005	1.4	300
Z-Leu-ON*p*	0.008	3.0	400
Z–D-Leu–ON*p*	0.030	1.6	50

[a] *p*NA, *p*-nitroanilide; ON*p*, *p*-nitrophenyl ester; Bz, benzoyl; Bzl, benzyl; Suc, succinyl; Z, *N*-benzyloxycarbonyl.

[b] *, very low activity ($<10^{-5}$ $\Delta A_{405}/s \cdot U \cdot ml^{-1}$); **, low activity (between 10^{-3} and 10^{-4} $\Delta A_{405}/s \cdot U \cdot ml^{-1}$).

[c] —, no hydrolysis detected.

4). Further incubation with addition of fresh enzyme did not result in a significant release of additional degradation products. The keratinolytic activities of the total extracellular proteinases and of the purified serine proteinase of *S. pactum*, however, were significantly higher than were those of the other commercially available proteinases, even higher than that of proteinase K (where K stands for keratin). Nevertheless, the overall loss of dry weight of keratin azure was less than 10% after 6 days of incubation with all enzymes.

Degradation of feather keratin by the proteinase mixture of the culture medium and the purified proteinase of *S. pactum*. Autoclaved and native chicken feather downs and feather meal were incubated with the proteinase mixture of the culture medium and the purified proteinase of *S. pactum*, with repeated addition of enzyme. The dissolution of keratin was estimated by measuring the residual dry weight of nondegraded feathers. Degradation of feather meal was 45% for the proteinase mixture and 40% for the purified proteinase. During disintegration of the whole feathers, the loss of dry weight after filtration was between 10 and 15% (Table 5).

TABLE 4. Transferase-to-hydrolase ratios for different amino acids, amino acid amides, and peptides as nucleophiles and Bz-Arg-OEt as the acyl donor[a]

Nucleophile	$(k_T/k_H)_{app}$
H-Phe-NH_2	10,400
H–D-Phe–NH_2	300
H-Arg-NH_2	7,400
H-Ala-Ala-Ala-Ala-OH	4,700
H-Ala-NH_2	1,500
H–D-Ala–NH_2	No transferase activity observed
H-Lys-NH_2	1,500
H-Arg-OH	No transferase activity observed
H-Asp-Gly-OH	No transferase activity observed
H-Gly-NH_2	No transferase activity observed
H-Gly-Gly-Gly-Gly-OH	No transferase activity observed

[a] Nucleophiles were used at 50 to 200 mM, and Bz-Arg-OEt was used at 5 mM.

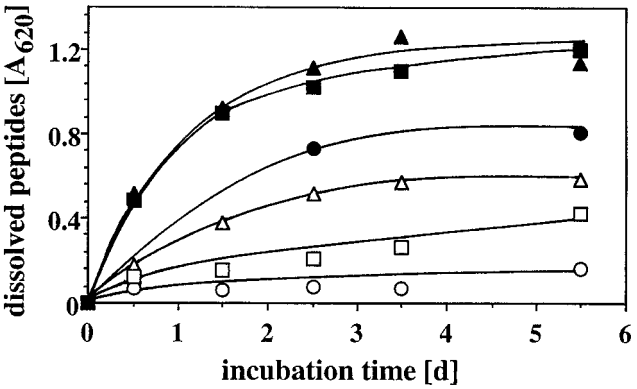

FIG. 4. Dissolution of keratin azure by different proteinases. Assay conditions were as follows: 1% keratin azure in potassium phosphate buffer with trace elements, 0.04% NaN_3, addition of 0.03 U of enzyme per ml every 24 h, and incubation at 50°C with agitation at 1,200 rpm. d, days. ▲, protease mixture from *S. pactum*; △, pronase E; ■, purified proteinase from *S. pactum*; □, Corolase N; ●, proteinase K; ○, proteinase from *S. caespitosus*.

Influence of reducing conditions on feather keratin degradation. The rates of keratin degradation in the presence and absence of oxygen were compared. Culture medium of *S. pactum* containing the proteinase mixture was incubated with whole native chicken feathers under aerobic and anaerobic conditions. The proteinase activity in the culture fluid (0.5 U/ml) decreased to 0.3 U/ml within 20 h. For the following 4 days, the activity remained constant in both assays. The main release of peptides occurred in the first 2 days, and the rates were 0.07 mg/ml in the presence and 0.20 mg/ml in the absence of oxygen. The absolute losses of dry weight after 5 days of incubation were 4 and 7%, respectively.

The addition of DTT showed a supporting effect on the keratinolytic activity of the enzyme mixture and the pure proteinase (Table 5). Native and autoclaved feather downs were degraded to the same degree. While degradation without DTT was limited to about 10%, after addition of 1% DTT about 70% of the keratin was solubilized.

DISCUSSION

The keratinolytic streptomycete *S. pactum* DSM 40530 produces a combination of serine proteinases and metalloprotein-

TABLE 5. Effect of DTT on proteolytic degradation of native and autoclaved chicken feathers by the proteinase mixture and the purified serine proteinase from *S. pactum*[a]

Keratin substrate	Reducing agent	Residual insoluble keratin (%) after incubation with:	
		Proteinase mixture of *S. pactum*	Purified serine proteinase
Native chicken feathers	None	90	89
	1% DTT	34	27
Autoclaved chicken feathers	None	89	85
	1% DTT	32	22

[a] Assay conditions were as follows: 1% keratin substrate in potassium phosphate buffer, pH 7.5, with trace elements, 0.03 U of protease per ml, 0.04% NaN_3, and 1% DTT. Incubation was for 4 days at 37°C with agitation at 1,200 rpm. Every 24 h, 0.03 U of fresh protease per ml was added.

ases which exert an extraordinary activity against insoluble substrates, e.g., keratins. The main component, a serine proteinase, has been purified. The keratinolytic activities of the purified proteinase and the total extracellular proteinases (Fig. 4 and Table 5) were comparable; therefore, the purified enzyme plays a major role in keratin degradation. The production of keratinolytic proteinases has also been described for *Streptomyces fradiae* ATCC 14544 (18, 25, 36). Since the two *Streptomyces* strains belong to the same cluster (15), a high degree of similarity between their enzymes can be expected. Keratinolytic activity has also been shown for a few proteinases from non-keratin-degrading microorganisms and even for proteinases from plants and animals, like papain or pancreatin (27, 35), but in general, outstanding keratinolytic activity is displayed by proteinases from keratin-degrading organisms (2, 12, 13, 23, 39, 42).

The enzymatic cleavage of the peptide bonds of keratin is difficult because of the restricted enzyme substrate interaction on the surface of the keratin particles. The particular ability of the keratinolytic proteinases may be due to a specificity for compact substrates and a more exposed active site. Molecular studies of chitinases, cellulases, and xylanases, which also act on compact substrates, have shown the existence of hydrophobic domains which may facilitate the interaction with different high-molecular-mass substrates (6).

In order to evaluate if keratinolytic enzymes show characteristic substrate specificities, the *S. pactum* proteinase was tested with different synthetic substrates and compared with other proteinases. Like the serine proteinases trypsin and chymotrypsin (1), the *S. pactum* proteinase showed an esterase activity several orders of magnitude higher than its amidase activity. The proteinase displayed strict stereoselectivity and stereospecificity for basic amino acids at the P_1 site of the cleaved peptide bonds with N-protected *p*NA substrates containing one or two amino acids. However, longer substrates, like Suc-Ala-Ala-Ala-*p*NA and Suc-Ala-Ala-Pro-Phe-*p*NA, seemed to be cleaved with minor selectivity for the P_1 site. The enzyme kinetic parameters for Suc-Ala-Ala-Pro-Phe-*p*NA (K_m, 0.55 mM; k_{cat}, 33 s^{-1}; k_{cat}/K_m, 66 $mM^{-1} \cdot s^{-1}$) are on the same order of magnitude for different keratinolytic proteinases, e.g., those of *Trichophyton mentagrophytes* (K_m, 0.35 mM; k_{cat}, 9.46 s^{-1}; k_{cat}/K_m, 27.0 $mM^{-1} \cdot s^{-1}$) (2) and *S. fradiae* (K_m, 0.58 mM; k_{cat}, 75.55 s^{-1}; k_{cat}/K_m, 130.3 $mM^{-1} \cdot s^{-1}$) (18). For Suc-Ala-Ala-Ala-*p*NA, the *S. pactum* proteinase showed kinetic parameters (K_m, 0.98 mM; k_{cat}, 0.4 s^{-1}; k_{cat}/K_m, 0.4 $mM^{-1} \cdot s^{-1}$) on the same order of magnitude as those of the proteinase SFase-2 of *S. fradiae* (K_m, 13.35 mM; k_{cat}, 1.91 s^{-1}; k_{cat}/K_m, 0.1 $mM^{-1} \cdot s^{-1}$). A high degree of specificity for amino acids ($P_n > 2$) was also described for the subtilisin-like keratinolytic proteinase K from *Tritirachium album* (19). The P′ specificity of *S. pactum* proteinase, studied by its transferase activity, also showed the tendency for preferred utilization of longer substrates. Therefore, the presence of amino acids in the more distant vicinity of the cleaved bond seems to be of importance. It would be premature to conclude from the presented results that the serine proteinase from *S. pactum* displayed a specificity for compact proteins, like keratin or collagen. However, the preference for longer substrates at both sides of the peptide bonds may indicate that the proteinase is well suited for the conversion of native and complex substrates.

Further studies of the proteinase specificity include the dissolution of peptides from the surface of protein particles. Azocoll, a common insoluble protein substrate, was solubilized totally by the proteinase (less than 0.03 U/ml) within a few minutes. The degradation of another commercially available substrate, keratin azure, was much slower. The proteinase from *S. pactum*, however, was significantly more active with keratin azure than were other commercially available proteinases which are also highly active with native and insoluble substrates. Nevertheless, our studies showed that the dissolution of keratin azure, as well as of naturally occurring keratins, exclusively by proteolytic attack was limited to 10% of the substrate. Even after repeated addition of fresh enzymes and incubation over several days, no further degradation was achieved. These results indicate that the proteinases could not effect the total degradation of the substrates. A quantitative comparison of the keratinolytic activity with those of other described keratinases is difficult. Most of the keratinase tests described do not give exact data on the absolute dissolution of keratin, only on the initial rate of liberation of peptides.

From our results, we concluded that the cystine bridges, which are an important structural feature of native keratin, prevented the proteolytic degradation of the most compact areas of keratinous tissues. Therefore, an additional cleavage of these disulfide bonds seemed to be indispensable to make the proteins available for the hydrolytic enzymes.

An additional cleavage of the disulfide bonds during microbial growth on keratin has been described for *S. fradiae*, *S. pactum*, *Bacillus licheniformis*, and *Microsporum gypseum* (7, 20, 30, 41). This cleavage can occur directly (the mechanism for which has not been elucidated until now) or by excretion of sulfite, which causes the sulfitolysis of the disulfide bonds. Until now, an ability to reduce disulfide bonds has not been described for any keratinolytic enzyme (7, 23, 29). Culture fluid did not show reducing activity (7, 30). The reduction of disulfide bonds seems to depend on the presence of the whole microorganisms.

The keratin degradation by hydrolytic enzymes in vitro should therefore be accompanied by a simultaneous reduction of cystine bonds. Thioglycolate is a strong disulfide-reducing agent and has been applied for degradation of hair keratin by an alkaline proteinase from the thermophilic *Bacillus* sp. strain AH-101 (38). With 1% thioglycolate at pH 12 and 70°C, the hair was solubilized within 1 h. In the absence of thioglycolate, the proteinase did not show keratinolytic activity. Enhanced keratin degradation after addition of DTT has also been reported for two serine proteinases of *S. fradiae* (36). After addition of 10 mM DTT to a keratinase assay mixture containing keratin azure, peptide dissolution increased twofold. The proteinase of *S. pactum* was not active in the presence of thioglycolate but was active in the presence of DTT. After a single addition of 1% DTT (6.5 mM) to keratinase assay mixtures containing feather keratin, about 70% of the substrate was dissolved, compared with only 10% without reducing agent.

The keratinolytic proteinase of *S. pactum* may therefore be suitable for the processing of keratin under appropriate conditions. The purified serine proteinase was active over a broad range of temperatures (45 to 75°C) and pH values (pH 7 to 10), with optima at 65°C and pH 8. At the optimal growth temperature of *S. pactum* (32°C), the levels of proteolytic activity and disintegration of whole feathers were quite low. Preliminary studies of enzyme activity have shown that a considerably higher rate of keratinolytic activity can be achieved by increasing the incubation temperature and using further additives, like reducing agents or detergents. The stabilizing effect of divalent metal ions may be an aid in long-term applications. A stabilizing effect of Ca^{2+} has already been reported for the *S. fradiae* proteinases (25). Specific Ca^{2+} binding sites that influence proteinase activity and stability apart from the catalytic site are described for several serine proteinases, especially subtilisin-like proteinases, e.g., the commercially available ke-

ratinolytic proteinase K (4). For the evaluation of a biotechnological application of the proteinase of *S. pactum*, a more detailed understanding of the factors that enable this enzyme to act on compact substrates better than comparable enzymes of the same type would be helpful. Therefore, more research on the specific molecular characteristics of this interesting enzyme will be done.

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