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Reduction of Disulfide Bonds by *Streptomyces pactum* during Growth on Chicken Feathers

BRIGITTE BÖCKLE† AND RUDOLF MÜLLER*

Department of Biotechnology II, Technical Biochemistry, Technical University of Hamburg-Harburg, 21073 Hamburg, Germany

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For disintegration of chicken feathers by *Streptomyces pactum*, keratinolytic proteinases and extracellular reduction of disulfide bonds were necessary. Conditions for disulfide reduction were examined with oxidized glutathione as model substrate. The reduction of glutathione depended on the presence of metabolically active cells. The mycelium also reduced tetrazolium dyes and cystine.

Characteristics of keratin are its high mechanical stability and resistance to proteolytic degradation due to tight packing of the protein chains through intensive interlinkage by cystine bridges. Cysteine is the major amino acid in keratins (18). Several keratinolytic microorganisms have been characterized, mostly bacteria of the genus *Bacillus* (12, 23) or *Streptomyces* (14, 17) and saprophytic and dermatophilic fungi (19, 21). However, a distinction should be made between initial disintegration of complex keratinous organs, such as chicken feathers, into smaller substructures and the complete dissolution of the molecular keratin. The former may be caused by proteases acting on the interkeratin matrix, whereas the attack on the almost crystalline keratin needs additional degradative mechanisms. Most of the investigations focused on the action of keratinolytic proteinases. However, the cleavage of the cystine bonds may also have a significant influence on keratin degradation (6–9, 17, 19, 20). This reduction is poorly understood for most keratinolytic microorganisms so far. Due to its ability to degrade chicken feathers better than other strains, *Streptomyces pactum* DSM 40530 was selected for this study. *S. pactum* was grown on feather medium (3) containing 2.5 or 5 g of washed whole chicken feathers per liter. During growth, proteinase activity was determined by the method of Kunitz (10). Soluble thiol groups were determined by the method of Ellman (5). Feather degradation by *S. pactum* was optimal at 33°C and pH 7.5. However, the highest proteinase production was observed at 28°C. Feather concentrations from 1.7 to 6.7 g/liter were degraded within 4 days. While proteinase activity in the culture filtrates was not significantly influenced by the keratin concentration, extracellular free thiol concentration correlated strongly with the amount of feathers. Since *S. pactum* produces the antibiotic pactamycin (2), clean but nonsterile native chicken feathers could be added after 2, 2.8, and 4.5 days without leading to bacterial contamination. In all cultures, an initial disintegration of the added feathers was observed within 6 h. A slight initial decrease of thiol concentration was observed as a result of the feather addition (Fig. 1). The further degradation and thiol formation were similar to those in cultures with autoclaved feathers. The addition of sodium azide together with feathers to 4-day-old cultures did not affect proteinase activity. However, no release of thiol groups was observed, and after initial disintegration of the feathers, no keratin degradation occurred.

The ability of *S. pactum* to reduce glutathione (GSSG) or cystine (2 mM) (Fig. 2) was tested with 2- to 3-day-old cultures grown in feather medium. Thiol release was highest at pH 7 and at temperatures around 33°C. Thiol release was independent of the amount of oxygen supplied as long as some aeration was provided. When the flasks were no longer aerated, thiol release stopped within 1 h. Addition of 0.05% sodium azide caused an inhibition of thiol release within 2 h. EDTA addition (10 mM) resulted in an immediate inhibition of thiol release (Fig. 3). The addition of divalent metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Mo^{2+}), carbohydrates (fructose and galactose), different substrates of biochemical redox reactions (isocitrate, malate, malonate, 2-oxo-glutarate, succinate, glycerol-phosphate, lactate, and pyruvate), proteins or amino acids (casein, cystine, aspartate, and glutamine), and other substances (NADH, NADPH, $NaNO_2$, and NH_4Cl) had no effect on GSSG reduction. Glucose, glutamate (Fig. 4), and Ni^{2+} and Zn^{2+} caused an inhibition of GSSG reduction. No substance that had a positive effect on GSSG reduction was found. To obtain information on the localization of the disulfide reducing

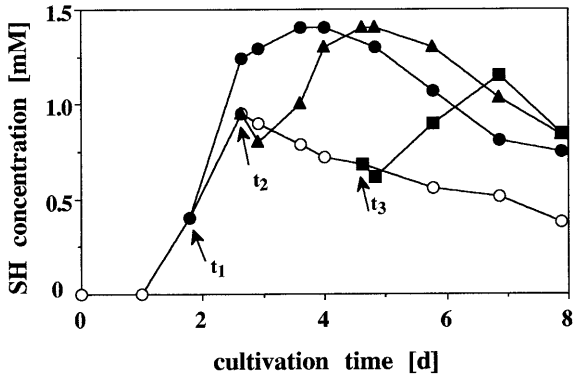

FIG. 1. Formation of extracellular thiol groups after addition of native chicken feathers (5 g/liter) to different growth states of *S. pactum* in feather medium (mineral medium with 2.5 g of chicken feathers per liter). ○, without further addition of chicken feathers (control); ●, addition of native chicken feathers at t_1; ▲, addition of native chicken feathers at t_2; ■, addition of native chicken feathers at t_3. d, days.

* Corresponding author. Mailing address: Department of Biotechnology II, Technical Biochemistry, Technical University of Hamburg-Harburg, Denickestrasse 15, 21073 Hamburg, Germany. Phone: 0049-40-7718-3118. Fax: 0049-40-7718-2127. E-mail: ru.mueller@tu-harburg.d400.de.

† Present address: Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, E-28006 Madrid, Spain.

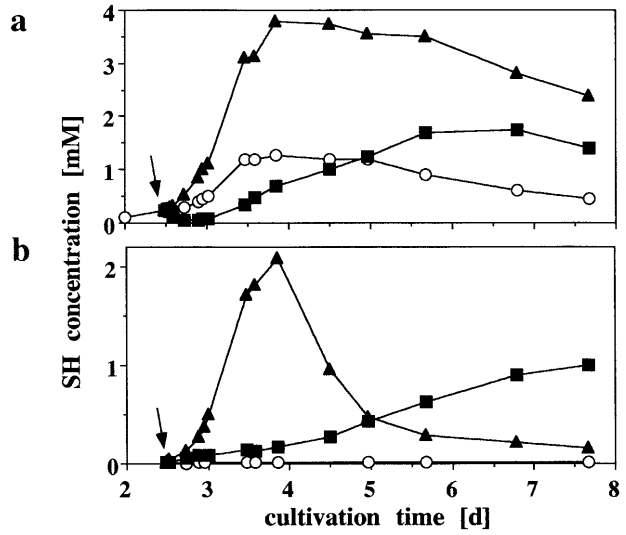

FIG. 2. Formation of extracellular thiol groups after addition of disulfide-containing substrates to *S. pactum* cultures. (a) Addition of cystine or oxidized GSSG (final concentration, 2 mM) to 2-day-old cultures of *S. pactum* in feather medium (mineral salts medium with 5 g of chicken feathers per liter). (b) Addition of cystine or GSSG to 2-day-old cultures of *S. pactum* in mineral salts medium with starch (2.5 g/liter) and ammonium sulfate (1.5 g/liter). The arrows indicate the times at which native chicken feathers, cystine, or GSSG was added to the cultures. GSSG concentration was 2 mM. ○, control (mineral salts medium with chicken feathers [a] or mineral salts medium with starch and ammonium nitrate [b]); ▲, with GSSG; ■, with cystine. d, days.

system of *S. pactum*, different fractions of cultures were incubated with GSSG. With fresh culture filtrate, no GSSG reduction was detected (Fig. 5a). Incubation of washed cells with GSSG resulted in an immediate increase of thiol concentration (Fig. 5b). However, the homogenate of the same mycelium showed no reduction. NADH or NADPH had no effect. It can be concluded that the reducing power was maintained only in the presence of metabolically active cells and that the electron donors had to be produced permanently.

Several extracellular keratinases from other keratinolytic microorganisms have been described elsewhere (13–16, 22, 25). These enzymes were exclusively hydrolytic. With the keratinases from *Streptomyces fradiae* and *Bacillus licheniformis*, the release of SH groups from keratin was tested but could not be detected, in contrast to whole cultures (11, 15, 16).

Several keratinase assays with natural keratin substrates have been described elsewhere (4, 14, 17, 24, 25). However,

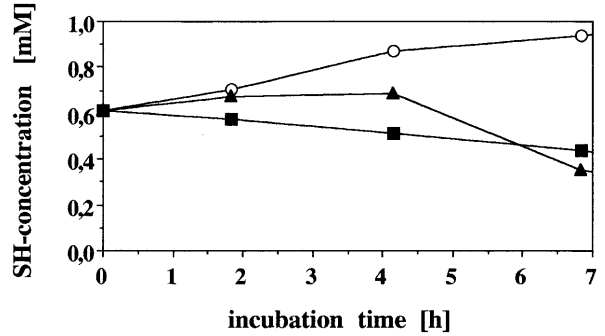

FIG. 3. Effect of the addition of sodium azide (0.05%) or EDTA (10 mM) on the reduction of GSSG in 3-day-old cultures of *S. pactum* in feather medium. ○, culture plus GSSG (control); ▲, with 0.05% sodium azide; ■, with 10 mM EDTA.

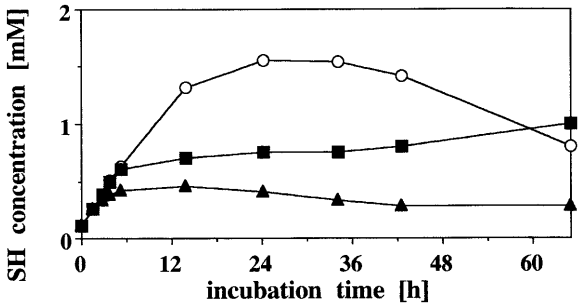

FIG. 4. Effect of the addition of glutamate (5 mM) or glucose (20 mM) on the reduction of GSSG in 3-day-old cultures of *S. pactum* in feather medium. GSSG concentration was 2 mM. ○, culture plus GSSG (control); ▲, with 5 mM glutamate; ■, with 20 mM glucose.

only the initial release of peptides, the disintegration of the multicellular keratin structures, or the degradation of denatured keratin was tested. The extracellular enzymes of *S. pactum* caused disintegration of native feathers but were not able to cause a significant degradation of keratin as calculated from the dry weight. The main proteolytic enzyme, a serine proteinase, was purified (3) and had a substrate specificity similar to that of the keratinolytic proteinases from *S. fradiae* or the commercially available proteinase K (13). For the degradation of keratin with *S. pactum* culture filtrate or with the purified proteinase, the addition of the reducing agent dithiothreitol was necessary.

The involvement of disulfide cleavage in keratin degradation has been described for a few microorganisms. Several keratinolytic fungi cause sulfitolysis by excreting sulfite and by producing an acid pH at the mycelial surface (6–9, 19, 20). Intracellular disulfide reductases have been described for a

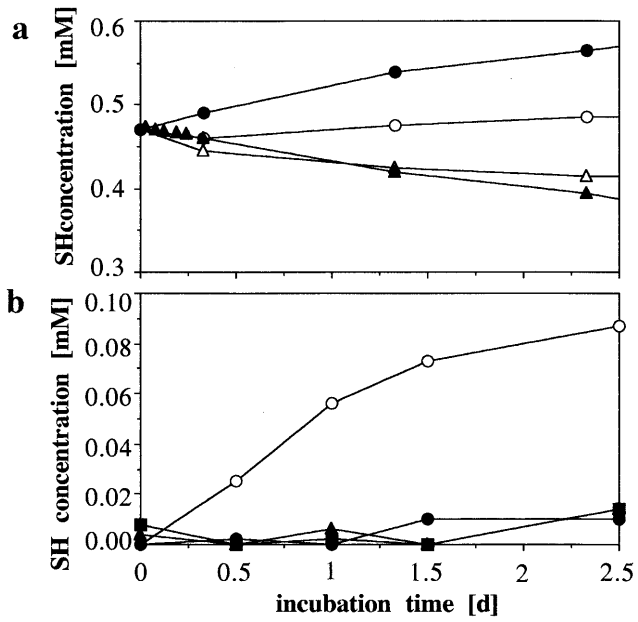

FIG. 5. Release of free SH groups by different fractions of an induced culture of *S. pactum*. (a) Incubation of complete culture (circles) and of filtrate (triangles) with 2 mM GSSG (closed symbols); controls were without addition of GSSG (open symbols). (b) Incubation of mycelium with 4 mM GSSG (○) and of cell extract with 4 mM GSSG without (●) and with NADH (▲) or NADPH (■) (2 mM each).

Streptomyces sp. (1). However, the degradation of insoluble keratin must occur outside of the cell at the keratin particles. This can be achieved by a cell-bound redox system at the surface of the cells or by a soluble reducing component excreted into the medium. In the case of keratin degradation by *S. pactum*, no permanent contact between mycelium and particles was observed, making direct reduction at the substrate surface unlikely. However, it cannot be excluded that reduction occurs by short contacts between mycelium and substrate.

S. pactum grown on feathers immediately reduced tetrazolium salts, which are common substrates for the demonstration of low membrane potentials. Nitroblue tetrazolium chloride and 2,3,5-triphenyltetrazolium chloride were reduced within seconds. This was visible by a deep blue or red color of the mycelium surface. In culture filtrates, no reduction was observed. This membrane potential may play an important role in keratin degradation by reducing the disulfide linkages in the keratin or by producing soluble reducing agents that react at the keratin surface and make the protein chains available for cleavage by proteinases.

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REFERENCES

1. **Aharonowitz, Y., Y. Av-Gay, R. Schreiber, and G. Cohen.** 1993. Characterization of a broad-range disulfide reductase from *Streptomyces clavuligerus* and its possible role in β-lactam antibiotic biosynthesis. J. Bacteriol. **175:** 623–629.
2. **Bhuyan, B. K., A. Dietz, and C. G. Smith.** 1962. Pactamycin, a new antitumor antibiotic. I. Discovery and biological properties, p. 184–190. Antimicrob. Agents Chemother. 1961.
3. **Böckle, B., B. Galunsky, and R. Müller.** 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum*. Appl. Environ. Microbiol. **61:**3705–3710.
4. **Carter, T. P., D. J. Best, and K. J. Seal.** 1988. Studies on the colonization and degradation of human hair by *Streptomyces fradiae*, p. 171–179. *In* D. R. Houghton, R. N. Smith, and H. O. W. Eggins (ed.), Biodeterioration 7. Elsevier Applied Science, London, United Kingdom.
5. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82:** 70–77.
6. **Kunert, J.** 1972. Keratin decomposition by dermatophytes: evidence of the sulphitolysis of the protein. Experientia **28:**1025–1026.
7. **Kunert, J.** 1973. Keratin decomposition by dermatophytes: I. Sulfite production as a possible way of substrate denaturation. Z. Allg. Mikrobiol. **13:**489–498.
8. **Kunert, J.** 1989. Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporum gypseum*: a comparison. J. Basic Microbiol. **29:**597–604.
9. **Kunert, J., and Z. Stransky.** 1988. Thiosulfate production from cystine by the keratinophilic prokaryote *Streptomyces fradiae*. Arch. Microbiol. **150:**600–601.
10. **Kunitz, M.** 1947. Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. **30:**291–310.
11. **Lin, X., C.-G. Lee, E. S. Casale, and J. C. H. Shih.** 1992. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. Appl. Environ. Microbiol. **58:**3271–3275.
12. **Molyneux, G. S.** 1959. The digestion of wool by a keratinolytic *Bacillus*. Aust. J. Biol. Sci. **12:**274–281.
13. **Morihara, K., and K. Oda.** 1992. Microbial degradation of proteins, p. 293–364. *In* G. Winkelmann (ed.), Microbial degradation of natural products. VCH-Verlagsgesellschaft mbH, Weinheim, Germany.
14. **Mukhopadyay, R. P., and A. L. Chandra.** 1990. Keratinase of a streptomycete. Indian J. Exp. Biol. **28:**575–577.
15. **Nickerson, W. J., and S. C. Durand.** 1963. Keratinase. II. Properties of the crystalline enzyme. Biochim. Biophys. Acta **77:**87–90.
16. **Nickerson, W. J., J. J. Noval, and R. S. Robison.** 1963. Keratinase. I. Properties of the enzyme conjugate elaborated by *Streptomyces fradiae*. Biochim. Biophys. Acta **77:**73–86.
17. **Noval, J. J., and W. J. Nickerson.** 1959. Decomposition of native keratin by *Streptomyces fradiae*. J. Bacteriol. **77:**251–263.
18. **Papadopoulos, M. C.** 1986. The effect of enzymatic treatment on amino acid content and nitrogen characteristics of feather meal. Anim. Feed Sci. Technol. **16:**151–156.
19. **Rajak, R. C., H. K. Malviya, H. Deshpande, and S. K. Hasija.** 1992. Keratinolysis by *Absidia cylindrospora* and *Rhizomucor pusillus*: biochemical proof. Mycopathologia **118:**109–114.
20. **Ruffin, P., S. Andrieu, G. Biserte, and J. Biguet.** 1976. Sulphitolysis in keratinolysis. Biochemical proof. Sabouraudia **14:**181–184.
21. **Safranek, W. W., and R. D. Goos.** 1982. Degradation of wool by saprophytic fungi. Can. J. Microbiol. **28:**137–140.
22. **Wawrzkiewicz, K., J. Lobarzewski, and T. Wolski.** 1987. Intracellular keratinase of *Trichophyton gallinae*. J. Med. Vet. Mycol. **25:**261–268.
23. **Williams, C. M., and J. C. H. Shih.** 1989. Enumeration of some microbial groups in thermophilic poultry waste digesters and enrichment of a feather-degrading culture. J. Appl. Bacteriol. **67:**25–35.
24. **Young, R. A., and R. E. Smith.** 1975. Degradation of feather keratin by culture filtrates of *Streptomyces fradiae*. Can. J. Microbiol. **21:**583–586.
25. **Yu, R. J., S. R. Harmon, and F. Blank.** 1972. Hair digestion by a keratinase of *Trichophyton mentagrophytes*. J. Invest. Dermatol. **53:**166–171.