

Halotolerant Collagenolytic Activity of *Achromobacter iophagus*

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(Received 23 October 1972)

SUMMARY

A strain of *Achromobacter iophagus* isolated from cured hides was shown to lyse collagen rapidly under aerobic conditions. Changes of temperature, pH, NaCl or collagen concentration affected either the maximum rate of collagen lysis or the lag before this rate was attained, or both. Collagenolytic activity occurred in 0.85 to 10% (w/v) NaCl.

INTRODUCTION

Anaerobic collagenolytic activity by bacteria has been demonstrated in four *Clostridium* spp., three *Bacterioides* spp. and one strain of *Staphylococcus aureus* (Waldvogel & Swartz, 1969). Exhaustive studies have been made of the collagenase from *Clostridium histolyticum* (Kono, 1968; Schaub & Strauch, 1968). Recently, aerobic collagenolytic activity was demonstrated in two strains of *Pseudomonas*, three of *Bacillus* and eight of *Achromobacter* (Adamcic & Clark, 1970; Thomson, Woods & Welton, 1972). There are no reported investigations of the aerobically produced collagenases.

Collagenolytic activity by hide bacteria is regarded as the cause of leather decay and thus a knowledge of the conditions under which these bacteria can degrade collagen is important to the hide and skin industry. The present study of a vigorously collagenolytic hide bacterium aimed to determine the optimal conditions for collagenolytic activity with a view to purification of the collagenase.

METHODS

All percentage compositions are w/v and all nutrients are Difco (Detroit, Michigan, U.S.A.) unless stated otherwise.

Source and identification. The bacterium was isolated from a batch of South African cured hides whose bacterial population possessed a high collagenolytic activity (Woods, Welton, Thomson & Cooper, 1972). It was identified according to *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) and *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (Skerman, 1967).

Media. The bacterium was maintained on a complex medium containing (g/l): casein hydrolysate (British Drug Houses, Poole, Dorset and Merck AG, Darmstadt, Germany), 17.0; glucose, 5.0; glycerol, 10.0; NaCl, 23.4; Na₂SO₃, 0.1; nutrient broth, 8.0; soytone, 3.0; tryptone, 0.5; vitamin-free Casamino acids, 0.5; yeast extract, 2.0; and agar, 15.0. The pH was adjusted to 7.6 with NaOH. Owing to the rapid spreading of the bacterium, this medium was not suitable for isolating clones. Discrete colonies were obtained on a medium consisting of: minimal salts solution, 125 ml; glucose, 2 g; agar, 15 g; distilled water to 1 l. The minimal salts solution contained (g/l): KH₂PO₄, 8; K₂HPO₄, 24; MgSO₄.7H₂O, 0.8; Na₂SO₄ (anhydrous), 16; NH₄Cl, 40; and NH₄NO₃, 8.

Table 1. *Characteristics of collagenolytic Achromobacter iophagus*

Gram-negative rod	+
Length (μm)	2.2 to 7.2
Width (μm)	1.0 to 1.8
Flagella	Many, peritrichous
Pigmentation	—
Endospores	—
Capsule	—
Facultative aerobe	+
Autotrophic	—
Halotolerant (NaCl range 0.85 to 10%; optimal growth at 2.34%)	+
Indole produced	+
Catalase produced	+
Oxidase produced	—
Nitrates reduced to nitrites	+
Acid or alkali produced from litmus milk	—
Acid, but no gas, produced from glucose, mannose, sucrose and glycerol	+
Acid from lactose	—
Hydrolysis of gelatin and starch	+
Hydrolysis of cellulose and chitin	—

+, —, Positive or negative reaction.

Collagenolytic activity assays. Collagenolytic activity was measured by determining released amino acids, expressed as $\mu\text{moles glycine/ml}$, by the method of Adamcic & Clark (1970). Collagen reaction mixtures consisted of a suspension of bovine Achilles tendon collagen (Boehringer, Mannheim, Germany) in 6 ml of tris-HCl or tris-maleate buffer containing NaCl and 0.002 M-CaCl₂, at concentrations of 0.2, 0.5, 1.5, 3.5 and 5.5 mg/ml with NaCl concentrations of 0.85, 2.34, 4.0, 7.0, 10.0 and 13.0%. Tris-maleate buffer was used to achieve pH values of 6.0 to 7.6 while tris-HCl was used for values of 7.2 to 9.2. The final concentration of tris in all buffers was 0.1 M. From an overnight plate culture of the bacterium a buffered, washed suspension containing approximately 10⁹ bacteria/ml was prepared. Samples (0.2 ml) of this suspension were added to reaction mixtures and incubated aerobically at 15, 20, 25, 30 and 35 °C. Experimental controls comprised collagen suspensions alone and bacteria in buffer without collagen. All reaction mixtures and controls were duplicated and two assays were made from each at the various times.

RESULTS

Identification

The characteristics of the bacterium are shown in Table 1 (identified as *Achromobacter iophagus*).

Collagenolytic activity

Control values shown in Fig. 1 to 4 are the highest values at each time interval. For clarity, all time zero symbols have been omitted. The least significant difference (L.S.D.) values for Fig. 1 and 3 are based on data for 4 days while those for Fig. 2 and Fig. 4 are based on data for 5 and 3 days respectively.

Effect of temperature. The effect of incubation at different temperatures is shown in Fig. 1. There was no significant difference between 30° and 35 °C. These temperatures produced the

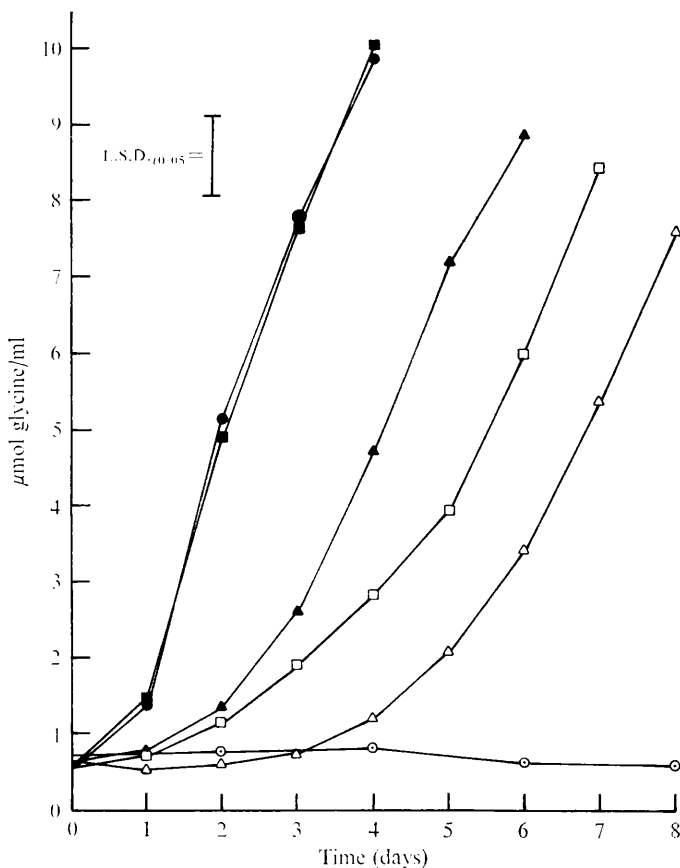


Fig. 1. Collagenolytic activity of *Achromobacter iophagus* at different temperatures. Reaction mixtures contained 2.34% NaCl and 1.5 mg collagen/ml of buffer at pH 7.6. Δ — Δ , 15 °C; \square — \square , 20 °C; \blacktriangle — \blacktriangle , 25 °C; \blacksquare — \blacksquare , 30 °C; \bullet — \bullet , 35 °C; \circ — \circ , control. The activity is shown as amino acids (expressed as μmol glycine) released from collagen under the conditions described in the text.

highest maximum rate of collagen lysis and the shortest lag period before this rate was attained. As the temperature was reduced the maximum rate of lysis decreased and the lag period increased. Even at 15 °C, however, a rapid rate of collagen breakdown was reached within approximately 6 days.

Effect of NaCl concentration. These results are presented in Fig. 2. Collagenolytic activity occurred between 0.85% and 10.0% NaCl but was inhibited by 13.0% NaCl. The maximum rate of collagen degradation occurred at 2.34% NaCl and the least lag occurred at 2.34% NaCl. At NaCl concentrations above 2.34% the maximum rate decreased and the lag period increased. The collagenolytic activities at 0.85% and 4.00% NaCl were not significantly different.

Effect of pH. Fig. 3 shows the effect of pH over the range pH 6.0 to 9.2. No significant differences were discerned in the overall data for pH 7.2, 7.6 and 8.0, at which values the maximum rate of collagen breakdown was highest and the lag period was shortest. With increasing deviation from pH 7.2 to 8.0 the maximum rate of collagen lysis decreased and the lag period increased.

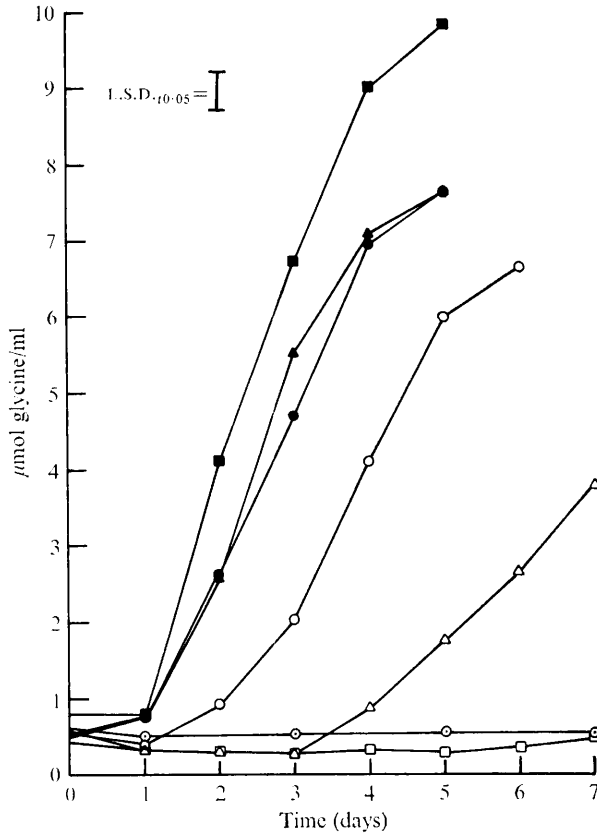


Fig. 2. Collagenolytic activity of *Achromobacter iophagus* at different NaCl concentrations. Reaction mixtures, incubated at 30 °C, contained 1.5 mg collagen/ml of buffer at pH 7.6. \blacktriangle — \blacktriangle , 0.85%; \blacksquare — \blacksquare , 2.34%; \bullet — \bullet , 4.0%; \circ — \circ , 7.0%; \triangle — \triangle , 10.0%; \square — \square , 13.0%; \odot — \odot , control. For method see Fig. 1 and text.

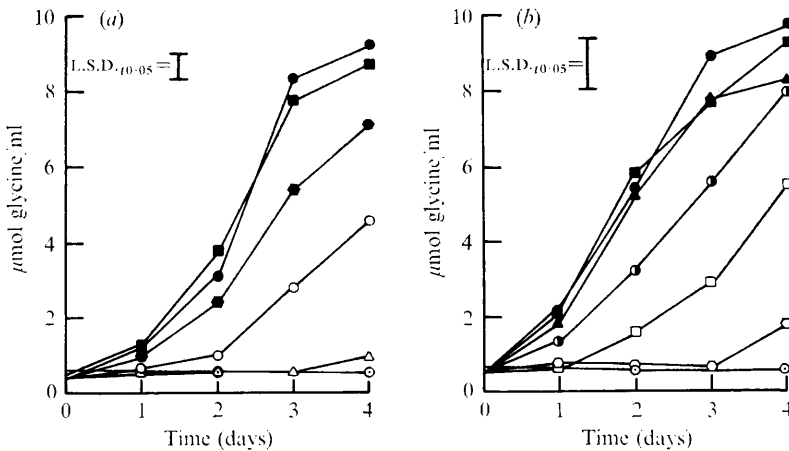


Fig. 3. Collagenolytic activity of *Achromobacter iophagus* at different pH values. Reaction mixtures, containing 2.34% NaCl and 1.5 mg collagen/ml, were incubated at 30 °C. \triangle — \triangle , pH 6.0; \circ — \circ , pH 6.4; \bullet — \bullet , pH 6.8; \bullet — \bullet , pH 7.2; \blacksquare — \blacksquare , pH 7.6; \blacktriangle — \blacktriangle , pH 8.0; \bullet — \bullet , pH 8.4; \square — \square , pH 8.8; \circ — \circ , pH 9.2; \odot — \odot , control. For method see Fig. 1 and text. Tris-maleate buffer was used for pH values 6.0 to 7.6 (a), and tris-HCl for pH values 7.2 to 9.2 (b).

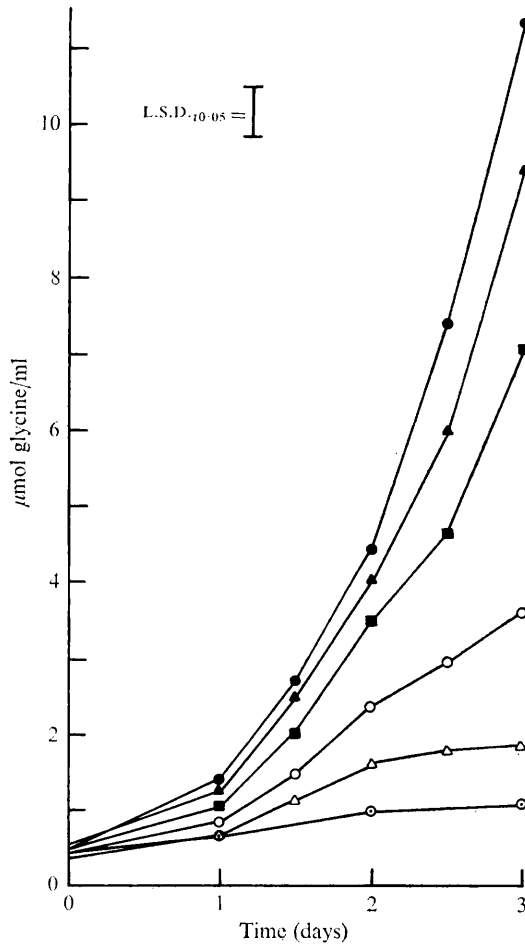


Fig. 4. Collagenolytic activity of *Achromobacter iophagus* at different collagen concentrations. Reaction mixtures, incubated at 30 °C, contained 2.34% NaCl and were adjusted to pH 7.6. Δ — Δ , 0.2 mg/ml; \circ — \circ , 0.5 mg/ml; \blacksquare — \blacksquare , 1.5 mg/ml; \blacktriangle — \blacktriangle , 3.5 mg/ml; \bullet — \bullet , 5.5 mg/ml; \odot — \odot , control. For methods see Fig. 1 and text.

Effect of collagen concentration. Activity at different collagen concentrations is shown in Fig. 4. Collagenolytic activity increased as the collagen concentration was raised from 0.2 to 5.5 mg/ml.

DISCUSSION

Achromobacter spp. isolated from hides are among the few bacteria which produce collagenase aerobically (Thomson *et al.* 1972). Under optimal conditions the present strain of *Achromobacter iophagus* exhibited an extremely rapid aerobic degradation of collagen with a short lag period. Deviation from optimal conditions resulted in either an increased lag or a reduced activity or both. When only one parameter was varied the bacterium was collagenolytic at 15 to 35 °C at 0.85 to 10.0% NaCl and at least pH 6.4 to 8.8. The activity increased with collagen concentration over the range studied. It is therefore feasible that this strain could be responsible for significant decay in a hide poorly cured or subjected to an extended period of delayed curing (Cooper, Galloway & Woods, 1972).

This is the first report of a hide bacterium with collagenolytic activity at high NaCl concentrations, which implies a halotolerant collagenase.

This work was supported by a research grant from the South African Council for Scientific and Industrial Research and R. L. W. wishes to acknowledge a research bursary from the Livestock and Meat Industries Control Board.

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