SKIN HYDROLYSIS BY TWO NEWLY ISOLATED FACULTATIVELY
ANAEROBIC ORGANISMS

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Two facultatively anaerobic organisms (one Bacillus sp. and one Pseudomonas sp.) which were earlier found to possess collagenolytic activity have been examined more closely for their hydrolytic action on skin collagen. Bacillus sp. appeared to possess strong hydrolytic action whereas Pseudomonas sp. is much weaker in its action at 37°C under both aerobic and anaerobic conditions. Collagen hydrolysis by both these organisms is appreciably retarded above 5% NaCl concentration. Pseudomonas sp. is more sensitive to collagenase inhibitors than the Bacillus sp. The optimum pH and temperature for skin hydrolysis by both the organisms have been found out. UV irradiation of the inoculum affects the collagenolytic activity of the Bacillus sp. to a considerable extent. Both the organisms are capable of unhairing the skin completely within 3 days. Bacillus sp is responsible for skin degradation to a greater extent compared to that of Pseudomonas sp.

Introduction

Raw hides and skins are associated in most occasions with aerobic and facultatively anaerobic organisms which were found to cause only partial hydrolysis of skin collagen. In certain cases presence of obligate anaerobes was also recorded. Such an anaerobe, Clostridium capitolave was found to cause considerable damage to hides. Hydrolysis of skin collagen by certain anaerobes, eg. Cl. histolyticum and Cl. perfringens, was investigated and it was observed that the former organism could completely hydrolyse the skin taken in phosphate buffer within 4 days. In practice, a skin might putrefy completely within few days even if it is not contaminated with anaerobic organisms. It was thus presumed that aerobic or facultatively anaerobic organisms could do such degradation when they grow in association but this could not be proved definitely by experiments. Two facultatively anaerobic organisms, one Bacillus sp. and the other Pseudomonas sp, were isolated in this laboratory only recently which were found to possess collagenolytic activity. Hydrolysis of skin collagen by these two organisms has been studied and results obtained are presented in this communication.
Materials and Methods

Organisms

One Bacillus sp. and one Pseudomonas sp. were isolated in this laboratory from a soak liquor where a piece of goat skin was allowed to putrefy. These organisms, yet to be identified up to the species level, were found to grow under both aerobic and anaerobic conditions. Nutrient agar was used to identify up to the condition was created with the help of McIntosh & Field's anaerobic jar using methylene blue as indicator.

Hydrolysis of skin collagen

Fresh goat skin was collected from abattoir, cleaned, fleshed, washed and the hairs were shaved off with a safety razor. Small skin pieces, weighing exactly 1 g each, were taken in a petridish and then sterilized by fumigating them with ethylene oxide. After exposing to ethylene oxide for a period of 18 to 20 hrs, the petridish was kept outside at room temperature for one day to remove the ethylene oxide if any. Each skin piece was aseptically transferred to a sterilized tube containing 10 ml of 0.1 M phosphate buffer (pH 7.4) and incubated at 37°C for 4 days. Tubes showing no contamination were taken for further experiments.

After 48 hrs of growth in nutrient agar, the organisms were cultured into tubes containing skin pieces and the cultured tubes were incubated at 37°C aerobically as well as anaerobically. The hydroxyproline content in fresh skin was determined according to Neuman & Logan's method and the average value was noted. Skin sample left after hydrolysis was taken out and analysed for hydroxyproline. The amount of hydroxyproline lost from fresh skin was then calculated and the values are expressed as percent loss in hydroxyproline.

Unhairing index

Unhairing of skin was examined by plucking out the hairs. The values for unhairing index indicate the extent of unhairing and was determined on the basis of the following criteria.

- 0 - no unhairing,
- 1 - very slight unhairing,
- 2 - slight unhairing
- 3 - slight to moderate unhairing,
- 4 - moderate unhairing,
- 5 - moderate to easy unhairing, and
- 6 - easy unhairing.

UV irradiation

A Hanovia 'Chromatolite' lamp with a wavelength of 237 nm was used for UV irradiation. Bacterial suspension taken in petriplates after removing the lids were placed at a distance of 10 cm at the time of exposure. In order to avoid temperature effect, UV irradiation was not continued for more than 30 minutes at a time.

Results

Effect of incubation period on the hydrolysis of skin collagen

The tubes containing sterile skin pieces in phosphate buffer were incubated with Bacillus sp. and Pseudomonas sp. and incubated aerobically and anaerobically for a period of 16 days at 37°C. The extent of collagen hydrolysis caused by these organisms is presented in Fig. 1.

Fig. 1 indicates that hydrolysis of skin collagen is more rapid in case of Bacillus sp. compared to Pseudomonas sp. The rate of collagen hydrolysis is practically unafte-
Effect of NaCl on the hydrolysis of skin collagen

Sodium chloride was added to phosphate buffer in increasing concentrations (upto 12.5%) and sterilized skin pieces were added to the sterile tubes. Bacillus and pseudomonas strains were then inoculated to the tubes containing different salt concentrations and incubated aerobically at 37°C for 10 and 15 days respectively. Hydroxyproline content of the residue was estimated in each case and the results obtained are presented in Fig. 2.

The growth, as well as, collagen hydrolysis by the two organisms are appreciably retarded by increasing NaCl concentrations.

Effect of collagenase inhibitors on the hydrolysis of skin

Collagenase inhibitors viz. o-phenanthroline, 8-hydroxyquinoline, 2:2'-dipyridyl and p-chloromercuribenzoic acid were added in different concentrations to different test tubes containing phosphate buffer. To these tubes, sterile skin pieces were added aseptically and inoculated with Bacillus sp and Pseudomonas sp. Cultured tubes were then incubated at 37°C in aerobic condition for a period of one month. The time taken for complete hydrolysis of the skin sample was noted and is presented in Table 1.

It is apparent from Table 1, that all the inhibitors studied are capable of preventing
complete collagen hydrolysis by the *Pseudomonas* sp. even at the lowest concentration (0.00001 M) examined even after 30 days. *Bacillus* sp. appears to hydrolyse skin collagen at 0.00001 M concentration of all the inhibitors tested. O-phenanthroline and p-chloromercuribenzoic acid at 0.0001 M, and 2:2'-Dipyridyl at 0.005 M; concentration are found to be effective.

**Effect of pH on the hydrolysis of skin collagen**

Media were adjusted using different buffers. pH 3 was adjusted with citrate buffer, pH 5.8 with phosphate buffer, pH 7 to 9 with barbitol buffer and pH 10 with carbonate-bicarbonate buffer. 10 ml. of sterilized buffer solution were distributed in tubes and skin pieces were added as before. The tubes were inoculated with *Bacillus* sp. and *Pseudomonas* sp. and incubated aerobically at 37°C for 4 and 10 days respectively. The residues in the tubes were collected after centrifuging and analysed for hydroxyproline. Fig. 3 indicates the loss in hydroxyproline content at different pH concentrations after specified period of incubation.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th><em>Bacillus</em> sp. Inhibitor con. (M)</th>
<th><em>Pseudomonas</em> sp. Inhibitor con. (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0001</td>
<td>0.0005</td>
</tr>
<tr>
<td>O-Phenanthroline</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>2:2’-Dipyridyl</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>P-Chloromercuribenzoic acid</td>
<td>7</td>
<td>*</td>
</tr>
</tbody>
</table>

*No complete hydrolysis within 1 month of incubation*
It may be observed from Fig 3 that both the Bacillus sp. and the Pseudomonas sp. hydrolyse skin collagen optimally at pH 7.5.

**Effect of temperature on the hydrolysis of skin collagen**

Tubes cultured with the Bacillus sp. and Pseudomonas sp. were incubated aerobically for a period of 4 and 10 days respectively at different temperature levels. Loss in hydroxyproline content in each tube was determined and the results obtained are presented in Fig. 4.

![Graph showing the effect of temperature on hydrolysis of skin collagen by Bacillus sp.](image)

**Fig. 4**: Effect of temperature on the hydrolysis of skin collagen in aerobic condition

- **O** Bacillus sp. 4 days after incubation
- **Δ** Pseudomonas sp. 10 days after incubation

It is evident from Fig. 4, that the optimum temperature for the hydrolysis of skin collagen by Bacillus sp. is about 37°C and by the Pseudomonas sp. is 43°C.

**Table 2**

<table>
<thead>
<tr>
<th>Time of exposure (in minutes)</th>
<th>% loss in hydroxyproline after 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98.77</td>
</tr>
<tr>
<td>30</td>
<td>97.12</td>
</tr>
<tr>
<td>60</td>
<td>94.83</td>
</tr>
<tr>
<td>90</td>
<td>70.00</td>
</tr>
<tr>
<td>120</td>
<td>54.19</td>
</tr>
</tbody>
</table>

In the control tube, skin piece was found to be hydrolysed completely within 7 days of incubation whereas for 30 min. exposure it took 18 days and for 60 min. exposure 27 days. Table 2 further indicates that UV irradiation affects the collagenolytic activity of the Bacillus sp.

It may be pointed out that the loss in collagenase activity is not due to irreversible spore formation. This was examined by subculturing the organisms to nutrient broth after irradiation, allowing them to grow well and once again studying their ability to hydrolyse skin collagen. But
cells irradiated earlier for 1½ and 2 hrs. could not hydrolyse collagen within 30 days.

Unhairing action of bacillus and pseudomonas strains

Fresh goat skin pieces (about 5 cm. square) were cleaned, fleshed and taken in petriplates, sterilized by exposing them to ethylene oxide and then inoculated on the flesh side under aseptic conditions, with the suspensions of bacillus and pseudomonas strains. The petriplates were incubated at room temperature (30°C) inside a closed moist desiccator. The extent of unhairing was determined up to 5 days of incubation and the observations are recorded in Table 3.

**Table 3**

Unhairing of skin by Bacillus sp. and Pseudomonas sp. in aerobic condition

<table>
<thead>
<tr>
<th>Strains</th>
<th>Unhairing index after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>2</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>4</td>
</tr>
</tbody>
</table>

Data presented in Table 3 indicate that both these strains are capable of unhairing the skin completely under aerobic conditions within 3 days. Slight unhairing may be noted, even in the case of control skin, probably due to the action of autolytic enzyme.

It was further noted that the Bacillus sp. could hydrolyse the skin pieces in the inoculated spot resulting in small holes (Fig. 5) whereas no such effect was noted in the case of the Pseudomonas sp.

**Discussion**

The collagenase activity of two facultatively anaerobic strains of the genus Bacillus and Pseudomonas has been reported earlier. In the present paper the hydrolysis of skin collagen by these organisms has been studied quantitatively. The Bacillus sp. hydrolyses skin collagen completely within 6 days (Fig 1) whereas *Clostridium histolyticum* was found to require 4 days. *Pseudomonas* sp., however, takes a longer time, although the rate of hydrolysis is considerably rapid up to 7 or 8 days, when 60-65% of the hydroxyproline content is found to be lost.

Fig. 2 indicates that at 2.5% NaCl concentration, the collagenase secreted by the Bacillus sp. is more resistant compared to...