SKIN HYDROLYSIS BY TWO NEWLY ISOLATED FACULTATIVELY ANAEROBIC ORGANISMS

R. SAMBASIVA RAO, S. C. NANDY, & M. SANTAPPA

Central Leather Research Institute, Madras

Received on December 31, 1976.

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[1, 2, 3] which were found[4] to cause only partial hydrolysis of skin collagen. In certain cases presence of obligate anaerobes was also recorded.[5] Such an anaerobe, *Clostridium capitovale* was found to cause considerable damage to hides. Hydrolysis of skin collagen by certain anaerobes, eg. *Cl. histolyticum* and *Cl. perfringens*, was investigated[6] 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.[4,7] 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.[8] 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 be putrefied. These organisms, yet to be idendified up to the species level, were found to grow under both aerobic and anaerobic conditions. Nutrient agar was used as the basal medium. Anaerobic condition was created with the help of McIntosh & Fildes 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.[9] 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 10ml. 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[10] 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.[11]

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 inoculated 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 unaffec-

aed whether the organisms are grown aerobically or anaerobically.

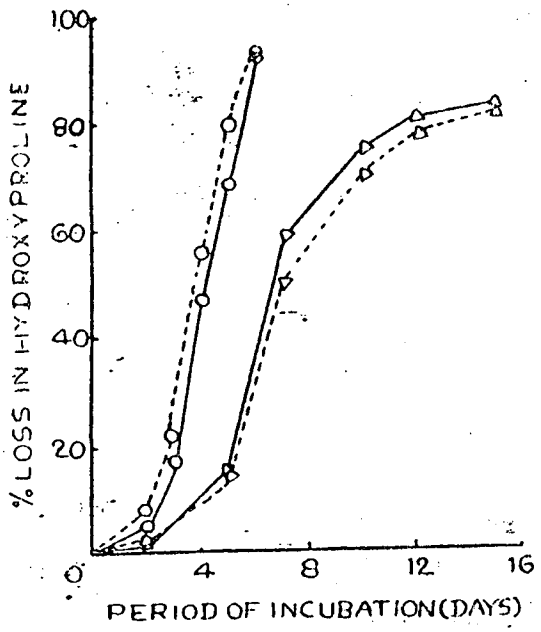

Fig. 1 : Effect of period of incubation on the hydrolysis of skin collagen by bacillus and pseudomonas strains under aerobic and anaerobic conditions.

○——○ *Bacillus* sp. in aerobic condition
○ - - - ○ *Bacillus* sp. in anaerobic condition
△——△ *Pseudomonas* sp. in aerobic condition
△ - - - △ *Pseudomonas* sp. in anaerobic condition

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.

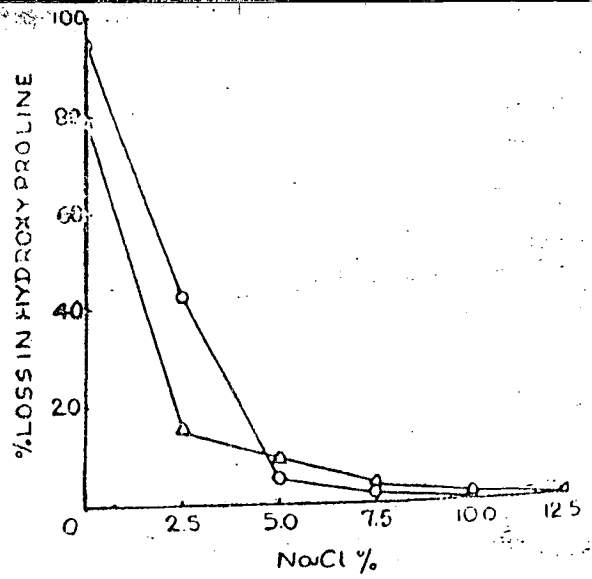

Fig. 2 : Effect of NaCl on the hydrolysis of skin collagen in aerobic condition

○——○ *Bacillus* sp. 10 days after incubation
△——△ *Pseudomonas* sp. 15 days after incubation

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

TABLE 1

Time (days) taken for complete hydrolysis of skin by the *Bacillus* sp. and *Pseudomonas* sp in the presence of collagenase inhibitors

Inhibitors	*Bacillus sp.*				*Pseudomonas sp.*	
	Inhibitor con. (M).				*Inhibitor con. (M).*	
	.00001	.00005	.0001	.0005	.00001	.00005
O – Phenanthroline	5	7	*	*	*	*
8 – Hydroxyquinoline	6	8	9	20	*	*
2 : 2' – Dipyridyl	5	6	9	*	*	*
P – Chloromercuribenzoic acid	7	8	*	*	*	*

*No complete hydrolysis within 1 month of incubation

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 0.0000 1M 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 carbonato-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.

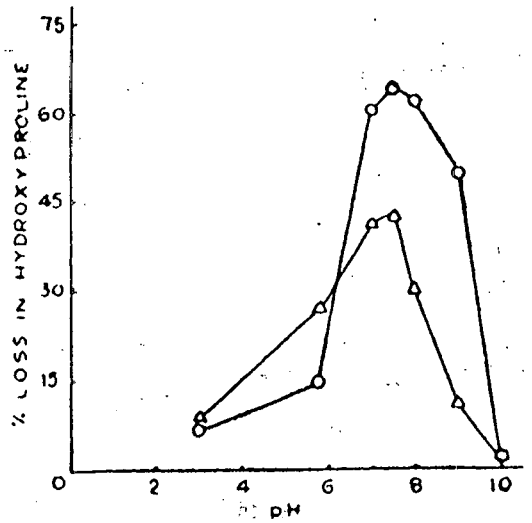

Fig. 3 : Effect of pH on the hydrolysis of skin collagen in aerobic condition

O——O *Bacillus* sp. 4 days after incubation

Δ——Δ *Pseudomonas* sp. 10 days after 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.

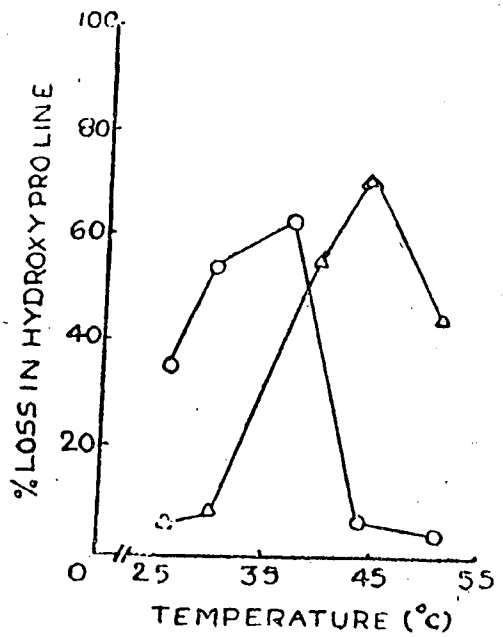

Fig. 4: Effect of temperature on the hydrolysis of skin collagen in aerobic condition

O—O *Bacillus* sp. 4 days after incubation
Δ—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.

Effect of UV irradiation on Bacillus sp. on subsequent hydrolysis of skin collagen

48 hrs. old cell suspension of *Bacillus* sp. in sterile normal saline solution was exposed to ultra-violet irradiation for different periods upto 2 hrs. Irradiated cells were then inoculated to buffer tubes containing sterile skin pieces. Tube cultured with non-irradiated cells was taken as control. Skin hydrolysis was observed for a period of one month. Extent of collagen hydrolysis was determined as before and the results obtained are given in Table 2.

TABLE 2

Effect of UV irradiation on the hydrolysis of skin collagen by *Bacillus* sp.

Time of exposure in minutes	*% loss in hydroxy—proline after 30 days*
0	98.77
30	97.12
60	94.83
90	70 00
120	54.19

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 upto 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

Strains	*Unhairing index after*			
	1 day	*2 days*	*3 days*	*4 days*
Uninoculated (control)	0	1	2	2
Bacillus sp.	2	4	6	6
Pseudomonas sp.	4	5	6	6

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.

Fig. 5 : Hydrolysis of fresh sterilized goat skin by *Bacillus* sp. in aerobic condition of growth

Discussion

The collagenase activity of two facultatively anaerobic strains of the genus Bacillus and Pseudomonas has been reported earlier.[5] 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 *Cl. histolyticum*[6] was found to require 4 days. *Pseudomonas* sp., however, takes a longer time, although the rate of hyrdolysis is considerably rapid upto 7 or 8 days, when 60-65% of the hyrdoxyproline 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