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THE PRODUCTION OF HYALURONIDASE (HYALURONATE LYASE) BY *CORYNEBACTERIUM ACNES**

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ABSTRACT

This paper presents evidence of the production of hyaluronidase by *Corynebacterium acnes*. In a study of seven random isolates of *C. acnes* from lesions of cystic acne and from normal skin, hyaluronidase activity ranged from less than one turbidity reducting unit (TRU) to 72 TRU per mg of extracellular protein. These findings raise the interesting possibility that *C. acnes* hyaluronidase may play a role in the development of acne by contributing to the inflammatory phase through an increased permeability of the follicular epithelium to free fatty acids and other irritants.

Corynebacterium acnes is the predominant bacterium found in sebaceous gland-rich areas of normal human skin (1). It is also one of the two bacteria characteristically found in lesions of acne vulgaris (2). Numerous investigations have characterized many of the biochemical and physiological properties of this organism (3, 4, 5, 6), however, the production of hyaluronidase (hyaluronate lyase) by *C. acnes* has only been reported once previously in a brief technical note describing a new screening method for detecting hyaluronidase producing organisms (7).

MATERIALS AND METHODS

Five isolates of *C. acnes* were obtained from lesions of acne vulgaris in patients with cystic acne attending the Acne Clinic at the University of California Center for Health Sciences, Los Angeles, California. Two isolates of *C. acnes* were obtained from facial skin of individuals without acne. The source of isolation of each strain is listed in Table I. All isolates conformed to the accepted characteristics of this species (i.e., anaerobic, catalase positive, liquefied gelatin) and all were sensitive to *C. acnes* bacteriophage.

For production of hyaluronidase, cells were inoculated from log phase cultures into 85% saline solutions in dialysis bags immersed in brain heart infusion broth (Difco) supplemented with 1% dextrose. Anaerobiasis was achieved by constant bubbling of a mixture of 90% N and 10% CO_2 into the dialysis bags. Incubation was carried out at 37° C for 72 hours. The bacterial cells were removed from the supernates by high speed centrifugation for 20 minutes. Two liter batches of culture supernates were dialysed against distilled water for 72 hours at 4° C. Following this the protein in the supernates was precipitated by dialysis against four volumes of saturated ammonium sulfate solution. The precipitated extracellular proteins (ECP) were separated by high speed centrifugation, redissolved in distilled water and dialysed against distilled water to eliminate the ammonium sulfate. The protein solutions were concentrated by lyophilization and the lyophilized ECP was used for hyaluronidase assays. Viscosimetric and turbidimetric assays were used to demonstrate hyaluronidase activity.

Viscosimetric assay. The viscosimetric assay is based on the reduction of viscosity of hyaluronic acid substrate by the action of hyaluronidase. For all viscosity determinations an Ostwald viscosimeter in a thermo-regulated constant temperature glass water bath at 25° C was used. The flow time of distilled water in the Ostwald viscosimeter used was 58.7 seconds. Potassium hyaluronate (Grade 111-P from human umbilical cord, lot number 30C-2180, Sigma Chemicals, St. Louis, Missouri) was used as the substrate throughout. Preliminary experiments measuring the flow time of varying concentrations of hyaluronic acid (HA) solutions in 0.1 M citrate buffer established that 0.1% HA concentrations gave flow times close to 100 seconds. Since frequent repeated readings were to be taken in subsequent experiments, this concentration was considered optimal. More concentrated HA solutions had increasingly longer flow times. 0.4% HA solution for example, had a flow time of 317.4 seconds. All flow times were determined using a Heuer stop-watch which gave readings to the nearest one tenth of a second.

The optimal pH of *C. acnes* hyaluronidase activity in the viscosimetric assays was determined by measuring the reduction of flow times of 0.1% HA solutions in 0.1 M citrate and phosphate buffers ranging in pH from 4.0 to 8.0 in the presence of 250 μg of *C. acnes* ECP per ml of substrate. 0.4% HA solutions were prepared in each buffer tested. 10 mg lyophilized *C. acnes* ECP was dissolved per ml of distilled water. One ml of 0.4% HA solution was added to 2.9 ml of corresponding buffer and 0.1 ml of enzyme solution added at zero time. Flow times were measured after 15 minutes at 25° C. The initial viscosity of the HA solution without enzyme was determined by measuring the flow time of 1 ml of 0.4% HA mixed with 2.9 ml of corresponding buffer and 0.1 ml of distilled water.

The effect of heat on *C. acnes* hyaluronidase activity was determined by heating 10 mg of enzyme per ml of citrate buffer at varying temperatures for 5 minutes. The hyluronidase activity of the heated samples was determined by testing the effect of 0.1 ml of each on the flow time of 1.0 ml of 0.4% HA mixed with 2.9 ml of 0.1 M citrate buffer, pH 5.6.

For estimation of hyaluronidase activity of the different *C. acnes* isolates, 10 mg of *C. acnes* ECP was dissolved in 1 ml of citrate buffer (0.1 M, pH 5.6) and a series of 10-fold dilutions were made. Flow time deter-

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TABLE I

Reduction in specific viscosity of 0.1% HA in the presence of 25 μg of C. acnes ECP per ml of substrate*

C. acnes strain no.	Source	Viscosity (% reduction)		
		5 min.	10 min.	15 min.
6	normal skin	0.13 (90)	0.09 (93)	0.08 (93)
18	"	0.09 (93)	0.07 (94)	0.06 (95)
8a	cystic acne	1.09 (16)	101 (22)	0.93 (27)
38	"	0.71 (45)	0.57 (56)	0.49 (62)
79	"	0.48 (63)	0.34 (73)	0.27 (77)
142	"	0.70 (46)	0.53 (59)	0.43 (67)
143	"	0.62 (52)	0.48 (63)	0.40 (69)

* specific viscosity = $t_{sample}/t_{buffer} - 1$ where t is flow time in seconds viscosity of 0.1% HA without enzyme was 1.30

minations of 3.9 ml of 0.1% HA solutions in citrate buffer were determined at 5, 10 and 15 minutes after the addition of 0.1 ml of each dilution of ECP. The flow time of 3.9 ml of 0.1% HA solution with 0.1 ml of buffer without enzyme was determined as the control for initial viscosity of the HA solution. In all viscosity determinations the stock HA solution was stored at 4° C during the experiment. Maintenance of the HA at room temperature for several hours resulted in a slight spontaneous decrease in viscosity. The reduction in specific viscosity was calculated using the formula:

$$\text{specific viscosity} = \frac{t_s}{t_b} - 1,$$

where t_s = flow time of the sample and

t_b = flow time of the buffer (8).

Turbidimetric assay. An alternative procedure to demonstrate hyaluronidase acitvity is the turbidimetric assay which is based on the formation of a colloidal suspension when HA is mixed with serum albumin at an acid pH. Depolymerization of HA by hyaluronidase prior to the addition of albumin reduces the turbidity of the HA-albumin mixutre since only HA units of 6000 to 8000 molecular weight or more, react with the acid albumin to produce colloid formation (9). The reduction in turbidity can be measured spectrophotometrically. One turbidity reducing unit (TRU) is defined as the amount of enzyme which in 30 minutes at 37° C reduces the turbidity of 0.2 mg of HA solution to that of 0.1 mg of HA solution.

0.1 M phosphate buffer, pH 5.3 in 0.15 M NaCl was used as the diluent for both the HA and the ECP solution. Bovine serum albumin (Fraction V, Nutritional Biochemicals, Ohio) was prepared as suggested by Dorfman (9), by dissolving 10 g of albumin, 32.6 g of sodium acetate, 45.6 ml of glacial acetic acid in 10 liters of water. The pH of the 0.1% albumin solution was brought to 3.72 with concentrated HCl. HA standards were prepared by adding 0.1 to 0.6 ml of a solution of 0.4 mg HA/ml to a series of duplicate colorimeter tubes, and adding buffer to 1.0 ml. Blanks contained 1.0 ml buffer. The tubes were heated in a boiling water bath for 5 minutes, and cooled for 5 minutes in an ice bath. Next 4.0 ml of acid albumin solution was added and the tubes gently mixed. After 10 minutes the turbidity was determined in a Coleman Jr. spectrophotometer at 600 mμ. Milligrams of HA substrate were plotted against optical density readings and a straight line standard curve was obtained.

For the assay of the unknowns, 0.5 ml of a solution of 0.4 mg HA/ml were added to a series of duplicate colorimeter tubes. *C. acnes* ECP was dissolved in phosphate buffer in concentrations of 1 mg/ml and a series of 2-fold dilutions prepared. 0.5 ml of each enzyme dilution was added to each tube of substrate and incubation was carried out for exactly 30 minutes at 37° C. The reaction was stopped by heating the tubes in a boiling water bath for 5 minutes, followed by cooling in an ice bath for 5 minutes. 4.0 ml of acid albumin solution was then added to each tube, and after 10 minutes, the turbidity of each tube was measured in the same way as described above for the standards. A complete series of standards was included for each series of unknowns tested. The HA solutions and ECP solutions were prepared fresh on the day that they were to be used. The optical densities of the unknowns were plotted on the standard curve and the TRU/mg of ECP calculated.

RESULTS

The optimal pH for *C. acnes* hyaluronidase activity was between pH 4.8 and 6.4. Hyaluronidase activity was inhibited at pH 4.0 and 8.0. The reduction in flow times of HA solutions in the presence of *C. acnes* ECP at different pHs is shown in Table II.

C. acnes hyaluronidase was heat labile and could be 90% inactivated by heating at 65° C for 5 minutes, and completely inactivated by heating at 80° C for 5 minutes. Storage at 4° C for seven days did not affect the hyaluronidase activity. Prolonged storage at 4° C was not attempted.

The reduction of viscosity of HA by *C. acnes* ECP was proportional to the concentration of ECP/ml of substrate. Figures 1 and 2 demonstrate the reduction of viscosity of 0.1% HA by different concentrations of ECP from *C. acnes* strain 38 and *C. acnes* strain 6. The specific viscosity of 0.1% HA solution prior to the addition of enzyme was 1.30. As shown in Figure 2, addition of 25 μg of *C. acnes* 6 ECP per ml of substrate,

TABLE II

Reduction in flow time of 0.1% hyaluronic acid in different buffers after 15 minutes of incubation with 250 μg of C. acnes 79 ECP/ml of substrate

0.1 M buffer	pH	Flow time of HA without enzyme	Flow time of HA with enzyme	Reduction in flow time (secs.)
Citrate	4.0	100.5	100.6	—
"	4.6	103.8	83.3	20.5
"	4.8	99.8	72.2	27.6
"	5.0	97.2	69.4	27.8
"	5.6	98.3	70.0	28.3
"	6.0	102.1	71.1	31.0
Phosphate	6.4	95.4	67.4	28.0
"	7.0	95.1	74.2	20.9
"	7.5	98.5	85.2	13.3
"	8.0	97.6	94.5	3.1

reduced the viscosity by 93% in 15 minutes; addition of 2.5 μg, by 83% in 15 minutes; and addition of 0.25 μg, by 44% in 15 minutes.

As can be seen from Table I and from a comparison of Figures 1 and 2, there was great variation in the amount of hyaluronidase activity exhibited by the different *C. acnes* isolates. The lowest activity was present in *C. acnes* 8a, isolated from a lesion of cystic acne. 25 μg of *C. acnes* 8a ECP per ml of substrate reduced the viscosity of 0.1% HA by only 27% in 15 minutes. By contrast, the highest activity was exhibited by *C. acnes* 6 and 18, both isolated from normal skin. 25 μg of ECP from these strains per ml of substrate reduced the viscosity of 0.1% HA by 90 and 93% respectively in 5 minutes.

Figure 3 demonstrates the reduction of viscosity of HA by 25 μg of *C. acnes* 142 ECP per ml of substrate as a function of time of incubation. The initial phase of the reaction is extremely rapid, with 46% reduction in viscosity produced in the first 5 minutes. Incubation of the substrate beyond 60 minutes did not decrease the viscosity further.

Results of the turbidimetric assays of hyaluronidase activity are listed in Table III. The TRU values ranged from less than 1 TRU/mg of ECP to 72 TRU/mg of ECP. There was good correlation between the results of the viscosimetric and the turbidimetric assays. *C. acnes* 6 and 18 had the highest TRU values, while *C. acnes* 8a had an activity of less than one TRU/mg of ECP.

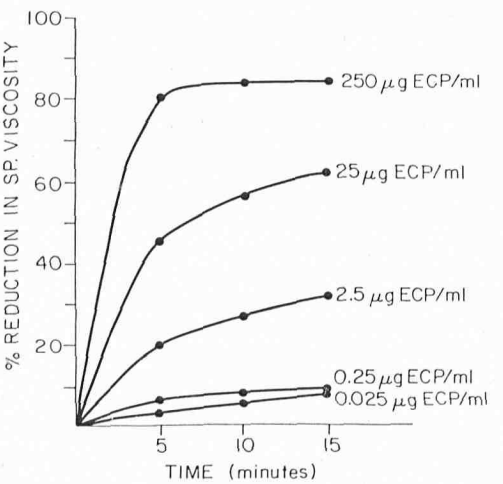

FIG. 1. Reduction in specific viscosity of 0.1% HA solution by different concentrations of *C. acnes* 38 ECP.

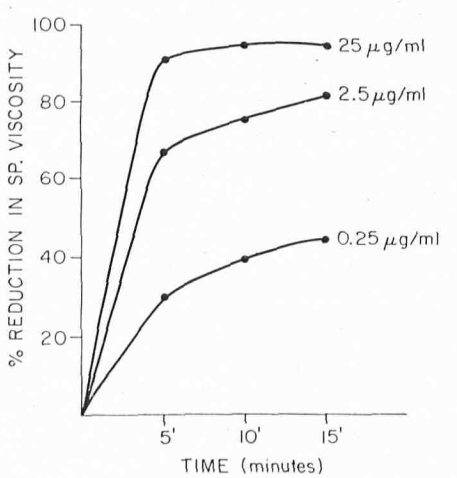

FIG. 2. Reduction in specific viscosity of 0.1% HA solution by different concentrations of *C. acnes* 6 ECP.

DISCUSSION

Hyaluronidases belong to the mucopolysaccharidase group of enzymes. Numerous bacteria including pneumococci, staphylococci, clostridia, proteus and flavobacteria produce this enzyme (10). *C. acnes* hyaluronidase appears to be similar to other bacterial hyaluronidases in that the optimal pH of its activity is in the acid range and it is heat labile.

All methods of assaying hyaluronidase activity

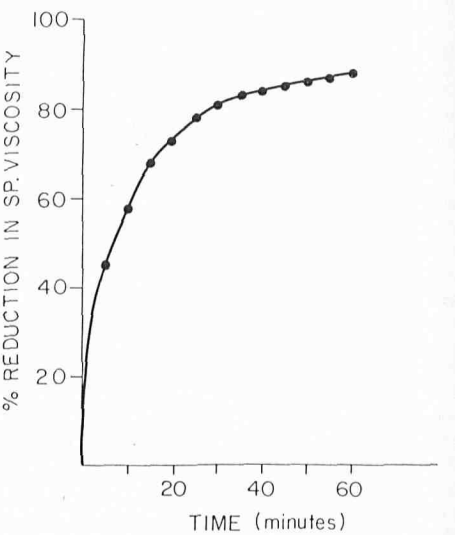

FIG. 3. Reduction in specific viscosity of 0.1% HA solutions by 25 μg of *C. acnes* 142 ECP per ml of substrate over a period of 60 minutes.

TABLE III

Hyaluronidase activity of C. acnes isolates expressed in turbidity reducting units (TRU)/mg of C. acnes ECP

C. acnes strain no.	Source of isolation	TRU/mg of ECP
6	normal skin	72
18	"	64
8a	cystic acne	less than 1
38	"	4
79	"	13
142	"	4
143	"	4

are based on the reduction of hyaluronic acid to small subunits. In contrast to testicular hyaluronidases, which are heat stable and whose major end products are tetrasaccharides, bacterial hyaluronidases are heat labile and have as their major end product disaccharides containing 4,5-desaturated uronides (10).

The levels of hyaluronidase activity of the isolates in the present study ranged from very weakly active to moderately active. Both the viscosimetric and turbidimetric assays are widely used accepted techniques for measuring hyaluronidase activity (11, 12). All hyaluronidase assays are affected by the physico-chemical properties of the substrate since no standardized hyaluronic acid is available. Results within any one laboratory may be internally consistent, but it is difficult to accurately compare bacterial hyaluronidase activity determined in one laboratory to that determined in another. Comparisons of activity are further confused by numerous different units which have been used to express enzyme activity. Furthermore, publications which have used identical units to express hyaluronidase activity (e.g. TRU) have often expressed activity in terms of non-comparable parameters such as ml of culture supernate (13), hours of incubation (14), or mg of protein at various stages of enzyme purification (15). Thus, it is difficult to compare the results of the assays in the present paper with those of others. For example, Abramson and Freidman (15), in a study on the purification of staphylococcal hyaluronidase from what they described as an active hyaluronidase producing strain of *Staphylococcus aureus* found hyaluronidase activity in the range of 1.1 TRU/mg of protein. Their protein was obtained by lyophilizing the precipitate obtained by 100% ammonium sulfate saturation of culture supernates. The culture medium used was very similar to the one used in the present paper, and consisted of saline which had been dialysed against brain heart infusion broth supplemented with dextrose. The enzyme was then further concentrated more than 4000 fold by sequential ammonium sulfate fractionation and Sephadex gel filtration, so that the final purified protein preparation had an activity of 4380 TRU/mg. Thus the stage of purification of the enzyme significantly affects final reported activity.

The role of microbial hyaluronidases in disease has not been definitely established. Even though hyaluronidase production is a characteristic of many pathogenic bacteria, known non-pathogenic organisms can also produce this enzyme. Several studies which have attempted to correlate hyaluronidase production *in vitro* with invasiveness *in vivo*, have resulted in negative findings (16, 17). Yet in the case of staphylococci, for example, there is an almost 94% correlation between the production of coagulase and the production of hyaluronidase. Coagulase negative staphylococci do not produce this enzyme (18).

In certain disease conditions it appears that bacterial hyaluronidases, even from normally saprophytic bacterias may have a significant effect on the pathogenesis of the disease. A prime example of this is in the pathogenesis of gingivitis. Studies have suggested that the hyaluronidases from the microbial flora of dental plaques may affect the permeability of gingival epithelium and be a contributing factor in the development of gingivitis (19). In this connection, Schultz-Haudt et al have demonstrated that epithelial cell bridges are reduced and intercellular cement substance is removed by the application of testicular hyaluronidase preparations to gingival epithelium (20). Thilander has observed the widening of intercellular spaces of gingival epithelium after applications of hyaluronidase *in vivo* to the gingiva (21).

The demonstration of hyaluronidase production by *C. acnes in vitro* may provide new insights into the effect of this organism *in vivo*. *C. acnes* is numerically the predominant bacterium in the sebaceous follicles of normal human skin. Together with *S. epidermidis* it can also be routinely isolated from lesions of acne vulgaris. The role *C. acnes* in the pathogenesis of acne has been investigated intensively during the past decade. Recent studies have suggested that lipases derived from *C. acnes* in the sebaceous follicles are primarily responsible for the hydrolysis of triglycerides of "virgin" sebum to release free fatty acids which are known to be the major inflammation producing substances in sebum (22, 23, 24).

Hyaluronic acid, the substrate of hyaluronidase activity is known to be the prime constituent of the ground substance of the dermis (25). It has also been shown in human gingival epithelium that the intercellular cementing substance consists in part of hyaluronic acid which is removable *in vitro* by hyaluronidase (26). While the composition of the intercellular cement substance in human pilosebaceous follicular epithelium has not yet been elucidated, it is conceivable that the breakdown of this intercellular substance by *C. acnes*, hyaluronidase might be a factor in increasing the permeability of the follicular epithelium and facilitating the diffusion of irritating free fatty acids into perifollicular areas of the dermis.

In the present study of only seven isolates of *C. acnes* it was interesting that the two most active hyaluronidase producing strains were both isolated from normal skin and some of the strains from cystic acne were relatively poor hyaluronidase producers under the conditions of the present experiment.

Further studies are in progress to determine whether there are statistically significant differences in the production of hyaluronidase by isolates of *C. acnes* from both obstructive and inflammatory acne lesions as compared to isolates from normal skin, and what role *C. acnes* hyaluronidase may play in the pathogenesis of acne.

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