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# Streptococcal Sialidase

## I. Isolation and Properties of Sialidase Produced by Group K Streptococcus

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A survey has been made of the activity of a wide variety of standard strains of streptococci against bovine submaxillary mucin. Strain 6646 (group K) and strain D 168A "X" (group M) completely broke down and strain H 60R (group F) incompletely broke down bound sialic acid of bovine submaxillary mucin added to the growth medium. Among these strains, strain 6646 (group K) produced sialidase in the cell and in the culture fluid. An appropriate amount of glucose in the culture medium stimulated growth and the production of enzyme, but an excess of glucose in the culture medium caused abundant growth without production of the enzyme. The streptococcal sialidase was precipitated from the culture fluid by ammonium sulfate at 50% saturation, and further purification was achieved by diethylaminoethyl cellulose chromatography. $Ca^{++}$ and $Co^{++}$ stimulated the sialidase activity, and $Mn^{++}$, $Zn^{++}$, and ethylenediaminetetraacetate inhibited it. With acetate buffer, the optimal *p*H lay between 5 and 6. Sialic acid was detected in the reaction product of the streptococcal sialidase and bovine submaxillary mucin.

Sialidases are distributed in a variety of living organisms: myxoviruses, bacteria, and animals. To date, in the bacterial domain, *Vibrio cholerae* (4), *Clostridium perfringens* (9), *Corynebacterium diphtheriae* (15), and *Diplococcus pneumoniae* (6) are known to produce sialidases. As regards streptococci, Stewart, Steele, and Martin (11) reported that some of the streptococcal cultures possess receptor-destroying activity, but its nature has not been clearly defined. It seems worthwhile to study the action of streptococci on sialomucoid, an important component of animal organs and fluids.

### MATERIALS AND METHODS

*Streptococcus strains.* Strains used are shown in Table 1; they were donated by the Type Collection Laboratory of Institute for Infectious Diseases, Tokyo University, and the National Institute of Public Health of Japan. Group K strains, D34 and 3742, were donated by H. D. Slade (Division of Bacteriology, Northwestern University Medical School, Chicago, Ill.).

*Sialomucoid.* Bovine submaxillary mucin was prepared according to the method of Blix, Svennerholm, and Werner (3) and was treated with alkali by the method of Gibbons (8). As reported by Faillard (7), the alkali-treated bovine submaxillary mucin was more sensitive to hydrolysis by sialidase than was the original material; it was used as substrate throughout the study. This material contained 7.74% of sialic acid, which was assayed with *N*-acetylneuraminic acid as standard.

*Culture medium.* Todd-Hewitt medium was used throughout this study. The medium was adjusted to *pH* 7.5 with NaOH solution, dispensed into 1-liter flasks, and sterilized by autoclaving. Sterilized glucose was added to each flask to a final concentration of 0.25%.

*Preparation of enzyme.* After 18 hr of growth at 37 C, the culture fluid was separated from the cells by centrifugation. The supernatant fluid was brought to 50% saturation with solid ammonium sulfate. Virtually all of the enzymatic activity resided in the fraction precipitated at 50% saturation. The precipitate was harvested, dissolved in distilled water, and dialyzed against distilled water for 4 days at 4 C. The dialyzed solution was centrifuged to remove precipitate and was then lyophilized. Further purification of the enzyme was achieved by diethylaminoethyl (DEAE) cellulose column chromatography. The column (1.0 by 15 cm) of DEAE cellulose (Serva; 0.53 meq/g) was prepared by eluting with 0.02 M ammonium carbonate. The sialidase preparation (40 units/mg) was dissolved in 10 ml of distilled water and was pumped into the column. After application of the sample, the column was washed with distilled water, and then the following solvents were applied: 0.02 and 0.05 M ammonium carbonate, and 0.05, 0.1, 0.2, and 0.4 M sodium chloride in 0.05 M ammonium carbonate. Fractions were collected at a flow rate of 1 ml/10 min at 30-min intervals. A typical chromatogram of the enzyme is shown in Fig. 1. The fractions rich in sialidase, between 0.2 and 0.4 M sodium chloride, were pooled, dialyzed against distilled water, and lyophilyzed. With this procedure, 40-fold purification was achieved.

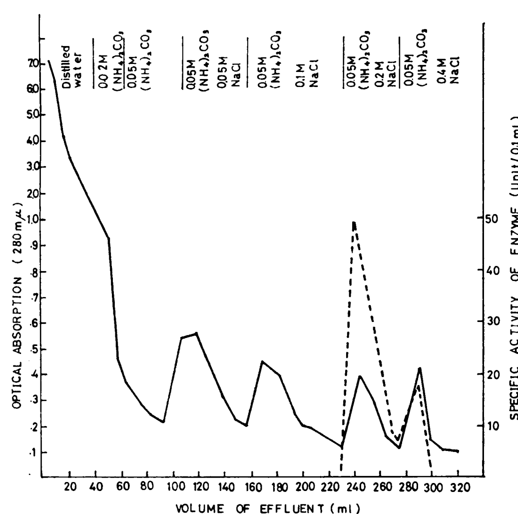

FIG. 1. *Chromatographic separation of the streptococcal sialidase on DEAE cellulose column. The preparation obtained with ammonium sulfate fractionation was applied. Solutions used in elution are indicated in figure. Solid line and the ordinate on the left show the protein concentration as determined by the absorption at 280 mμ. Dotted line and the ordinate on the right refer to the number of enzyme units per 0.1 ml.*

*Assay procedure for sialidase.* The standard reaction mixture contained the following substances in 0.5 ml of 0.1 N sodium acetate buffer (*p*H 5.5): sialidase, 1 mg; bovine submaxillary mucin, 1 mg; $CaCl_2$, 0.5 mg. The reaction mixture was put into a stoppered glass tube and was incubated for 90 min at 37 C. After incubation, the released sialic acid was determined by the thiobarbituric acid method of Aminoff (2), taking as a standard authentic *N*-acetyl-neuraminic acid. Crystallized *N*-acetylneuraminic acid was donated by T. Yamakawa (16). The molar extinction coefficient of the colored complex was $70.7 \times 10^3$ at 549 mμ (14). A unit of enzyme is that amount which will release 1 μg of free sialic acid from 1 mg of bovine submaxillary mucin in 90 min at 37 C. Readings of optical density of the colored complex were carried out with a spectrophotometer (Hitachiseisakujo Co., Tokyo, Japan).

Optical density of culture growth was read at 660 mμ with the spectrophotometer.

## RESULTS

*Screening of the strains which break down sialomucoid.* The stock strains were inoculated into the Todd-Hewitt medium supplemented with 0.1% of bovine submaxillary mucin. After incubation for 18 hr at 37 C, the growth culture was divided into two portions, each portion containing 0.5 ml of specimen. To one portion, an equal amount of 0.2 N HCl was added, and the mixture was heated in a boiling bath for 10 min; to the other an equal amount of distilled water was added before boiling. After treatment, each solution was centrifuged at 2,000 × *g* for 30 min, the supernatant fluid was separated, and the released sialic acid was estimated. None of the strains produced significant amounts of free sialic acid in the culture fluid (Table 1), whereas in the hydrolyzed fluids various amounts of sialic acid were found. These data suggest that strain 6646 (group K) and strain D168"X" (group M) completely break down, and H60R (group F) incompletely break down bound sialic acid of bovine submaxillary mucin. Some strains of group A streptococci, T-3, -12, and -42, slightly decomposed sialic acid of bovine submaxillary mucin. Among these strains, only strain 6646 produced potent sialidase in the culture fluid.

*Influence of glucose on the production of sialidase.* Glucose and hydrogen ion concentrations of the culture medium had a significant effect upon the elaboration of sialidase (Table 2).

TABLE 1. *Screening of strains which break down sialomucoid*[a]

| Strain | Free sialic acid in culture fluid | Bound sialic acid[b] |
|---|---|---|
| | (μg/ml) | (μg/ml) |
| *Streptococcus pyogenes* | | |
| T-1 (group A, type 1) | 2.5 | 24.9 |
| T-3 (group A, type 3) | — | 19.1 |
| T-4 (group A, type 4) | — | 24.1 |
| T-6 (group A, type 6) | — | 25.1 |
| T-12 (group A, type 12) | — | 21.5 |
| T-42 (group A, type 42) | — | 17.2 |
| T-44 (group A, type 44) | — | 21.8 |
| T-46 (group A, type 46) | — | 26.2 |
| *Streptococcus* spp. | | |
| O9OR (group B) | 2.9 | 32.5 |
| K64 (group C) | 2.6 | 30.3 |
| SS498 (group D) | 2.7 | 42.1 |
| 6642 (group E) | 2.8 | 26.6 |
| H6OR (group F) | 2.4 | 13.2 |
| F68A (group G) | 2.6 | 35.6 |
| F9OA (group H) | 2.8 | 34.7 |
| 6646 (group K) | 2.4 | 3.6 |
| 3472 (group K) | 2.2 | 18.9 |
| D34 (group K) | 2.8 | 19.8 |
| D167B (group L) | 2.6 | 34.2 |
| D168 "X" (group M) | 2.8 | 3.6 |
| B357 (group O) | 2.6 | 34.7 |
| *Streptococcus lactis* | | |
| 7963 (group N) | 2.7 | 35.5 |

[a] Experiment was carried out as described in the text. Initially, the culture medium contained 1 mg of bovine submaxillary mucin per ml. If the bovine submaxillary mucin was intact during cultural incubation, hydrolyzed specimen should contain 38.7 μg of free sialic acid.

[b] Amount of free sialic acid released by hydrolysis with 0.1 N HCl.

TABLE 2. *Effect of glucose on the production of sialidase*

| Content (%) of glucose | End *p*H | Specific activity (units per 0.1 ml)[a] | Cell growth (optical density)[b] |
|---|---|---|---|
| 0 | 8.5 | 0.85 | 0.28 |
| 0.25 | 6.1 | 9.15 | 2.54 |
| 0.5 | 4.8 | 0 | 4.0 |

[a] Unit is defined as amount (micrograms) of sialic acid released in the reaction system containing 1 mg of bovine submaxillary mucin and 0.1 ml of the culture fluid. Other conditions were the same as those described in the text.

[b] Growth of the cells was represented by optical density at 660 mμ.

Glucose at 0.25% stimulated growth and the production of sialidase, and at 0.5% it yielded abundant growth of cell but no sialidase. This may be because of the high concentration of hydrogen ion caused by the excess glucose.

*Presence of sialidase in the cell.* The cells were harvested from 390 ml of the growth culture which contained 240 units of sialidase per ml, washed, and suspended in 10 ml of distilled water. The suspension was treated in a sonic oscillator at 20 kc for 30 min and was centrifuged at 10,000 × *g* for 30 min. The supernatant fluid contained 708 units of sialidase per ml. The cells contained 7.5% of the amount of sialidase excreted into the culture fluid.

*Characteristics of the streptococcal sialidase.* When the preparation of streptococcal sialidase was incubated with *N*-acetylneuraminic acid in 0.1 M phosphate buffer (*p*H 7.0) for 18 hr at 37 C, no change was observed in the amount of *N*-acetylneuraminic acid. This suggests that the enzyme preparation does not contain any agent which attacks *N*-acetylneuraminic acid.

The rate of enzymatic activity was linearly proportional to incubation time for at least 30 min. This experiment was carried out on a scale 10 times as large as that described in the assay procedure for sialidase in Materials and Methods. At intervals, 0.5-ml samples were withdrawn and assayed for the released sialic acid.

The enzyme activity was linear with respect to the concentration of enzyme. The reaction mixtures contained doubling concentrations of enzyme, ranging from 0.0625 to 1 mg/ml.

*Heat stability.* The enzyme preparation was dissolved in distilled water and then dispensed into test tubes. The tubes containing 0.1 ml (1 mg) of enzyme were heated for 2 min in a water bath at a variety of temperatures, ranging from 50 to 80 C. The activity of the enzyme was destroyed rapidly at above 60 C (Table 3).

TABLE 3. *Heat stability of streptococcal sialidase*[a]

| Temperature (C) | Specific activity (units per mg) of treated enzyme | Per cent of activity |
|---|---|---|
| Control | 63.0 | 100 |
| 50 | 57.0 | 95.7 |
| 60 | 1.28 | 2.2 |
| 70 | 0 | 0 |

[a] Each test tube containing 1 mg of enzyme was heated at indicated temperature for 2 min; the other conditions were the same as those described in the text. Activity of the enzyme was determined by the standard procedure.

*Effect of pH.* Enzymatic activities at several *p*H values were determined under the standard conditions except for the use of various buffers as follows; acetate buffer, *p*H 3.5 to 5.5; phosphate buffer, *p*H 5.5 to 7.5; tris(hydroxymethyl)aminomethane (tris) chloride buffer, *p*H 7.5 to 9.0; veronal buffer, *p*H 7.0 to 9.0. Each reaction mixture contained 0.1 M of respective buffer and 0.01 M of $CaCl_2$, except for the phosphate buffer. The effect of *p*H on the enzymatic activity varied with respect to buffers (Fig. 2). With acetate buffer, the optimal *p*H lay between 5 and 6.

*Effect of cations and ethylenediaminetetra-*

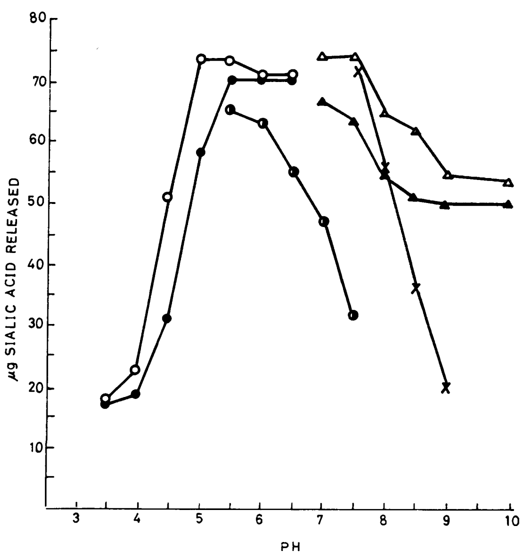


FIG. 2. *Effect of pH on sialidase activity. The reaction mixtures were those described in the text. Each reaction mixture was made to 0.5 ml with 0.1 M of respective buffer. Symbols: ○, acetate buffer with $CaCl_2$ (0.01 M); ●, acetate buffer with $CoCl_2$ (0.01 M); ◑, phosphate buffer; ×, Trischloride buffer with $CaCl_2$ (0.01 M); △, Veronal buffer with $CaCl_2$ (0.01 M); ▲, Veronal buffer with $CoCl_2$ (0.01 M).*

TABLE 4. *Effect of cations and EDTA on sialidase activity*

| Test substance | Concn | Sialic acid released | Per cent of activity[a] |
|---|---|---|---|
| | M | μg | |
| $ZnCl_2$ | 0.01 | 15.1 | 38.7 |
| $MgCl_2$ | 0.01 | 33.8 | 86.5 |
| $MnCl_2$ | 0.01 | 17.6 | 44.8 |
| $MnCl_2$ | 0.001 | 30.6 | 78.0 |
| $CoCl_2$ | 0.01 | 39.0 | 99.94 |
| $NiSO_4$ | 0.01 | 31.2 | 79.6 |
| $CaCl_2$ | 0.01 | 39.2 | 100.0 |
| EDTA (*p*H 5.5) | 0.01 | 18.1 | 46.2 |
| None (control) | — | 33.2 | 85.0 |

[a] The activity of enzyme when $CaCl_2$ was applied as the activator was assumed as 100. The standard assay procedure was used.

*acetate* (*EDTA*). The reaction system contained, in 0.5 ml of acetate buffer (*p*H 5.5), two-thirds mg of bovine submaxillary mucin, 1 mg of the sialidase, and various amounts, specified in Table 4, of cation or sodium EDTA (*p*H 5.5). The sialidase in this experiment was dialyzed against 0.001 M sodium EDTA for 2 days prior to being lyophilized. The sialidase was stimulated by $Ca^{++}$ and $Co^{++}$ and was inhibited by $Zn^{++}$, $Mn^{++}$, and sodium EDTA. The divalent cations were used as chloride salts, except for $NiSO_4$.

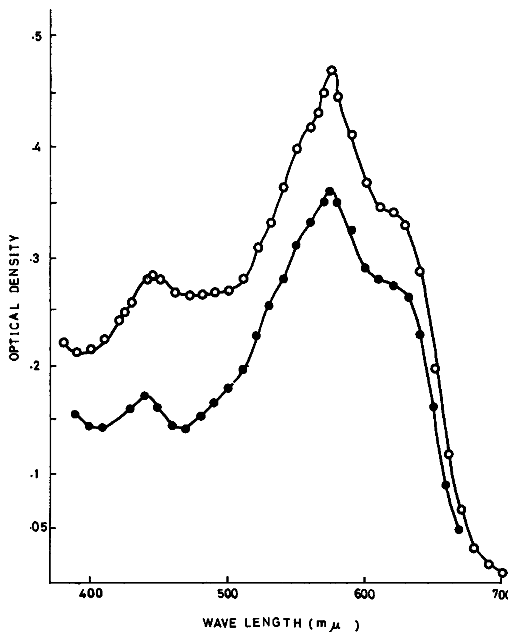


FIG. 3. *Absorption spectra of the colored complexes obtained with the sialidase reaction product* (○) *and with N-acetylneuraminic acid* (*12 μg*) (●) *in the resorcinol procedure.*

*Characterization of the reaction product of sialidase.* To identify the reaction product released from bovine submaxillary mucin by the sialidase, the reaction product was separated from the reaction mixture by dialysis. Dialysis was carried out against a small amount of distilled water, by use of cellulose tubing (Visking Co., Div. of Union Carbide Corp., Chicago, Ill.). The dialysate was lyophilized and was used to make the colored complexes by the thiobarbituric acid method (2), the alkali Ehrlich method (2), and the resorcinol method (12). Figure 3 shows optical absorption spectra of the colored complexes with the resorcinol reagent. With some variance, spectra of the colored complexes of the sialidase reaction product and of *N*-acetylneuraminic acid were identical with those of the resorcinol reagent.

## DISCUSSION

This report describes the occurrence and the characteristics of a sialidase produced by one strain of group K *Streptococcus*. Previously, Stewart, Steele, and Martin (11) found in many bacterial cultures an agent which they described as follows, "An agent capable of modifying red cells (CMA) antigenically so that these become agglutinable by immune serum prepared against influenza virus treated cells has been identified in cultures of pneumococci and of many strains of streptococci, particularly streptococci of viridans type. This agent is believed to be the receptor-destroying enzyme." They demonstrated receptor-destroying activity in 1 out of 27 CMA-positive streptococcal cultures with $FM_1$ strain (influenza virus Type A) as test strain; with PR8 strain, they found receptor-destroying activity in 7 of these cultures. Roberts and Stewart (10) found 5 CMA-positive strains out of 14 strains of group K streptococci and 20 CMA-positive strains out of 36 Viridans group streptococci. In our experiments, the strains which destroyed bound sialic acid of bovine submaxillary mucin were found in Lancefield group A, F, K, and M streptococci, but sialidase was found in the culture fluid of only strain 6646 (group K). In the culture fluids of the other strains of group K streptococci, sialidase was not found. At present, we cannot say whether these strains which destroy the bound sialic acid of bovine submaxillary mucin produce sialidase or not, nor whether these strains produce the CMA and the receptor-destroying enzyme or not.

Thonard, Hefflin, and Steinberg (13) demonstrated sialidase activity in culture fluids of oral

bacteria, but did not identify the bacteria. The fact that group K streptococci and Viridans group streptococci are oral bacteria is very interesting and suggests that the affinity to sialic acid may have some relationship to the ecology of these bacteria. Streptococcal sialidase resembles *Vibrio cholerae* sialidase in respect to the requirement of $Ca^{++}$ for activation of $Zn^{++}$, $Mn^{++}$, and EDTA (1) for inhibition, but the former is less susceptible to the activator and to the inhibitor than the latter. It has been reported that the sialidases of *Corynebacterium diphtheriae*, *Clostridium welchii*, and *Diplococcus pneumoniae* do not require the divalent cations for activation and are not inhibited by EDTA (5, 15).

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