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Purification and Characterization of a Hyaluronidase Associated with a Temperate Bacteriophage of Group A, Type 49 Streptococci

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Urea treatment of a temperate bacteriophage from a type 49 strain of group A streptococcus (*Streptococcus pyogenes*) followed by ammonium sulfate fractionation, ion exchange, and affinity chromatography of solubilized proteins provided for the recovery (12%) and purification (44-fold) of the phage-associated hyaluronidase. The molecular weight of the homogeneous, purified enzyme was estimated to be 71,000 by polyacrylamide gel electrophoresis (in the presence of sodium dodecyl sulfate) and 75,000 by gel filtration with Sephacryl S-200. The enzyme has a pH optimum of 5.5, a V_{max} of 0.1 absorbance unit/min per μg of protein, and a K_m of 4.8×10^{-2} mg/ml with umbilical cord hyaluronic acid as substrate. Of the cations tested, calcium and magnesium were the only effectors of the enzyme. The enzyme is a glycoprotein (7.25% carbohydrate) containing glucose, galactose, and glucosamine. Analysis of the amino acid composition revealed a predominance of acidic amino acids and a relatively high content of cysteine. The partial specific volume, estimated from the amino acid and sugar analyses, was 0.725 cm^3/g.

Previous studies have shown that a hyaluronidase (HAase) activity, which has been demonstrated to function by a beta-elimination mechanism, is associated with streptococcal bacteriophages (1, 15, 25). The apparent function of the phage enzyme is to allow the penetration of the mucoid hyaluronic acid capsule of the streptococcal cell by the bacterial viruses (1, 15, 21). In the absence of an exogenous source of HAase, strains of group A streptococci that possess a mucoid character are resistant to infection by virulent phages (21), whereas such strains are naturally susceptible to infection by most temperate bacteriophages (15, 25). By the use of a sensitive assay for the determination of HAase activity (2), it has been shown that both virulent and temperate streptococcal bacteriophages produce HAase, but differences of several orders of magnitude separate the low levels of enzyme in virulent phages that require the addition of HAase for plaque formation and the high levels in temperate phages that do not (1).

Although the presence of HAase associated with streptococcal bacteriophages has been well established, no study of enzyme purification has been reported. We report here the purification to homogeneity of the HAase associated with a temperate bacteriophage from a group A, type 49 strain and some characteristics of this enzyme. In the purification, advantage is taken of the glucose content by using a specific affinity adsorbent for glucose- or mannose-containing glycoproteins.

MATERIALS AND METHODS

Strains. The temperate phage used in this study was obtained from a group A, M type 49 strain (University of Minnesota strain no. GT 8760) by induction with mitomycin C as described previously (32). The group A, M type 12 strain designated as K56 was originally supplied by E. Kjems (15) and was used for the growth of the bacteriophage. Phages were assayed as reported earlier (31).

Materials. Reagents and materials were from sources described previously (1, 2). In addition, diethylaminoethyl-cellulose (DEAE-cellulose, DE 52) and carboxymethyl-cellulose (CM-cellulose, CM 52) were from Whatman Ltd., Maidstone, England, and all reagents for polyacrylamide gel electrophoresis (PAGE) were from Eastman Kodak Co., Rochester, N.Y. Lentil lectin was prepared from *Lens culinaris* seeds by the O'Brien modification (K. J. O'Brien, Ph.D. thesis, University of Minnesota, Minneapolis, 1975) of the method of Howard et al. (14). Covalently linked lentil lectin-Sepharose (typically 10 mg of lectin per ml of Sepharose) was prepared for affinity chromatography by the cyanogen bromide method according to Cuatrecasas (6). Sepharose 4B and Sephacryl S-200 are products of Pharmacia, Uppsala, Sweden. Bio-Gel A-5m (agarose with an exclusion limit of approximately

5×10^6 daltons) was obtained from Bio-Rad Laboratories, Richmond, Calif. Membrane filters (HA type, 0.45-μm pore size) were purchased from Millipore Corp., Bedford, Mass. Methyl α-D-mannoside was obtained from Sigma Chemical Co., St. Louis, Mo. Trisyl, a premixed trimethylsilylating reagent, was purchased from Pierce Chemical Co., Rockford, Ill. Hyflo-Super-Cel was from Johns Manville, Denver, Colo.

Culture medium and preparation of bacteriophages. Broth for bacterial growth and agar plates for phage assays have been described (32). Preparation of bacteriophage lysates has been reported earlier (1). Lysates (30 liters; 10^7 plaque-forming units [PFU] per ml) were passed through Hyflo-Super-Cel, and bacteriophages were recovered from the clear lysates (4×10^6 PFU/ml) by using the polyethylene glycol method as described elsewhere (1). Concentrated suspensions of bacteriophages (6×10^8 PFU/ml) were applied to a column of Bio-Gel A-5m (5 by 55 cm) and washed with 10 mM Tris-hydrochloride buffer (pH 7.2) containing 10 mM sodium phosphate and 150 mM NaCl. Bacterial viruses that eluted in the void volume of the column were sterilized by filtration through membrane filters and then concentrated by ultracentrifugation at 100,000 × *g* for 2 h and served as the starting material for HAase purification.

Enzyme assay. HAase activity was measured as described previously (2). The method utilizes a carbocyanine dye, 1-ethyl-2-{3-[ethylnaphtho(1,2d)-thiazolin-2-ylidene]-2-methyl-propenyl} naphtho (1,2d)-thiazolium bromide (Eastman Organic Chemicals, catalog no. 2718) for the spectrophotometric determination of the enzyme activity. It is based on the shift in the wavelength of maximal absorbance in the visible spectrum of the dye toward longer wavelengths (600 to 650 nm) upon interaction with hyaluronic acid (8). Measurement of the absorbance of the dye-polysaccharide complex at 640 nm allows the estimation of the extent of substrate degradation and provides an assay for HAase activity. The assay was modified as follows: hyaluronic acid (25 μg) and HAase preparations were incubated at 37°C in 0.25 ml (final volume) of 20 mM sodium acetate buffer (pH 5.0) containing 10 mM NaCl. Samples (0.04 ml) were withdrawn at desired times and the reaction was terminated by dilution in 0.36 ml of water at 4°C. Dye (0.1 mM in water containing 50% dioxane, 1 mM acetic acid, and 0.5 mM ascorbic acid) was subsequently added to the samples to a final volume of 4.0 ml, and optical densities were read at 640 nm against a blank containing dye (3.6 ml) and water (0.4 ml). The control contained enzyme heated for 10 min at 100°C. The unit of enzyme has been defined as that amount of HAase that results in a 10% decrease in the absorbance of the dye-hyaluronic acid complex after incubation of the polysaccharide for 1 h at 37°C and at pH 5.0; it is equivalent to 0.2 National Formulary unit (2).

Purification of hyaluronidase. A suspension (25 ml) of bacteriophages (0.61 mg of protein per ml) was incubated at room temperature in 8 M urea for 30 min. All subsequent operations were carried out at 4°C. The suspension was then dialyzed against 200 volumes of 10 mM Tris-hydrochloride buffer (pH 7.2) containing 150 mM NaCl with four changes in 48 h. The insoluble material formed during the dialysis was removed by centrifugation at 30,000 × *g* for 1 h; then the resulting supernatant was subjected to ammonium sulfate precipitation. The material precipitating between 30 and 65% saturation of $(NH_4)_2SO_4$ was collected, dissolved in 20 ml of 50 mM sodium phosphate buffer (pH 7.2), and dialyzed against 200 volumes of the same buffer with four changes. A precipitate that formed during the dialysis was removed by centrifugation, and the resulting supernatant was passed through membrane filters (Millipore Corp.) and used for the subsequent chromatography.

(i) DEAE-cellulose chromatography. The clarified enzyme preparation was applied to a DEAE-cellulose column (0.9 by 10 cm) that had been previously equilibrated with 50 mM sodium phosphate buffer (pH 7.2). The column was washed with 30 ml of the phosphate buffer at a flow rate of about 40 ml/h and then eluted with an 80-ml linear gradient of 0 to 0.5 M NaCl in the equilibrating buffer. Fractions of 2 ml were collected. The protein was located by measuring the absorbance at 280 nm, whereas the HAase activity was located by the dye-binding method. Active fractions were pooled, lyophilized, suspended in 2 ml of the equilibrating buffer used with the CM-cellulose column, and dialyzed against 500 volumes of the same buffer.

(ii) CM-cellulose chromatography. The sample was applied to a CM-cellulose column (0.9 by 5.0 cm) that had been equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was washed with 20 ml of phosphate buffer, followed by a 40-ml, linear NaCl gradient (0 to 0.5 M) in the same buffer. Fractions (1 ml each) were collected at a flow rate of approximately 40 ml/h and tested for absorbance at 280 nm and for HAase. Active fractions were pooled and made 0.1 mM with respect to $CaCl_2$ and $MnCl_2$.

(iii) Affinity chromatography. The flow-through fractions from the previous step were applied to a column of lentil lectin-Sepharose (0.5 by 6 cm) that was equilibrated in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The column was washed with the equilibrating buffer until no further material absorbing at 280 nm eluted (<0.005). The enzyme was eluted with the buffer solution containing 200 mM methyl α-D-mannoside and 1 M NaCl. Fractions (1 ml) were assayed for protein and enzyme content.

PAGE and molecular weight of purified HAase. The degree of purification was routinely monitored by sodium dodecyl sulfate (SDS)-PAGE according to the procedure of Weber and Osborn (33). The method was also used to evaluate the molecular weight of the enzyme.

The molecular weight of the enzyme in its native form was also determined by gel filtration in a column (2.5 by 33 cm) of Sephacryl S-200 equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 10 mM NaCl. The column was run at a flow rate of approximately 120 ml/h, and the fraction size was 2.2 ml. The molecular weight of the enzyme was calculated by plotting the logarithm of the molecular weight against the distribution constants (K_{av}) of the standards (16).

Kinetics. The kinetic behavior of purified streptococcal bacteriophage HAase was analyzed in the linear range of the assay procedure described, with hyalu-

ronic acid (umbilical cord) as substrate. Double-reciprocal plots of velocity versus hyaluronic acid concentration according to Lineweaver and Burk (18) provided for the determination (by extrapolation) of apparent K_m and V_{max} values.

Amino acid analysis. Samples (147 μg each) of purified HAase were hydrolyzed under vacuum at 110°C ± 2°C for 24, 48, and 72 h with 6 N HCl, and each hydrolysate was analyzed. Amino acid analyses were performed on a Beckman 119 amino acid analyzer equipped with an Infotronics CS210 integrator. The partial specific volume ($\bar{v}$) of the phage enzyme was calculated from its amino acid and carbohydrate composition according to the method of Schachman (30). Values for specific volumes of neutral and amino sugars were taken from the literature (10).

Carbohydrate analysis. Carbohydrate was determined by gas chromatographic analysis of the methyl glycosides as trimethylsilyl derivatives by the method of Bhatti et al. (3). Samples were analyzed on a Hewlett-Packard model 5700A gas-liquid chromatograph in a column of 3% silicone rubber SE30 on chromosorb-HP (Hewlett-Packard) and a temperature increase program of 100 to 200°C at 1°C per min. Amounts were calculated by an internal standardization method with a Hewlett-Packard model 3380A computing integrator.

Preparative purification of HAase. Preparative purification of the enzyme was carried out with ion-exchange celluloses and affinity chromatography adsorbent by using batch techniques as follows. A 50-ml portion of a well-dispersed suspension of DEAE-cellulose in equilibrating buffer (containing 60 g [wet weight] of the ion exchanger) was added to 60 ml of bacteriophage HAase preparation [1.5 mg of protein per ml after $(NH_4)_2SO_4$ treatment], and the mixture was stirred at 4°C for 1 h. After removal of the cellulose by centrifugation at 500 × *g* for 10 min the supernatant was dialyzed against 50 mM sodium phosphate buffer (pH 6.0) and then added to 25 ml of a CM-cellulose suspension (3 g [wet weight]). After stirring at 4°C for 1 h, the CM-cellulose was removed by low-speed centrifugation. Thereafter the supernatant was made 0.1 mM with respect to $CaCl_2$ and $MnCl_2$, mixed with 10 ml of a suspension of lentil lectin-Sepharose in equilibrating buffer (12 g [wet weight]), and stirred at 4°C for 1 h. The affinity chromatography adsorbent was removed by centrifugation and washed twice with equilibrating buffer. HAase was eluted by washing the affinity adsorbent with buffer containing 200 mM methyl α-D-mannoside and 1 M NaCl and was used after determination of the purity by SDS-PAGE.

RESULTS

Enzyme purification. In confirmation of the studies of Kjems (15) on other temperate phages of group A streptococci, we have shown previously (1) that a major portion of the HAase associated with a temperate bacteriophage from a strain of group A, type 49 streptococcus (University of Minnesota strain no. GT 8760) is tightly bound to the phage particle. We were able to release the active enzyme by treatment with urea. This method results in good recovery (87%) of enzymatic activity (1). Table 1 summarizes the procedure used to purify the streptococcal bacteriophage-associated HAase.

As expected, relatively strong affinity of HAase for the covalently linked lentil lectin-Sepharose was observed. This procedure removed two contaminant proteins that were present after CM-cellulose chromatography. Table 1 shows that the enzyme at this final stage was purified about 44-fold with a final yield of 12%. The specific activity of HAase in each of the fractions collected upon affinity chromatography was constant across the peak, suggesting homogeneity (Fig. 1).

This homogeneity was confirmed by PAGE in the presence of SDS. This gel system revealed a single protein (Fig. 2) with a mobility corresponding to a molecular weight of 71,000 (Fig. 3) as calculated according to Weber and Osborn (33). A molecular weight of 75,000 for the enzyme was estimated by gel filtration through Sephacryl S-200. It thus appears that the HAase in its active form consists of a single polypeptide with an approximate molecular weight of 73,000, a mean value from these two methods.

Kinetics. The enzyme was incubated with different concentrations of umbilical cord hyalu-

TABLE 1. *Purification of the streptococcal bacteriophage-associated HAase*

Fraction	Total protein[a] (mg)	Total activity (U × 10^{-3})	Sp act[b] (U/mg of protein)	Yield (%)	Purification (fold)
Phage suspension	152	9.3	62	100	1
Urea supernatant	41	7.8	190	84	3
$(NH_4)_2SO_4$ precipitate (30–65%)	16.4	6.2	380	67	6.1
Removal of insoluble material	9	4.2	470	45	7.6
DEAE-cellulose	2.9	2.6	900	28	15
CM-cellulose	0.65	1.6	2,500	17	40
Affinity chromatography	0.41	1.1	2,700	12	44

[a] Protein concentrations were determined by the method of Lowry et al. (19) using crystalline bovine serum albumin as standard.

[b] One unit is equivalent to 0.2 National Formulary unit (2).

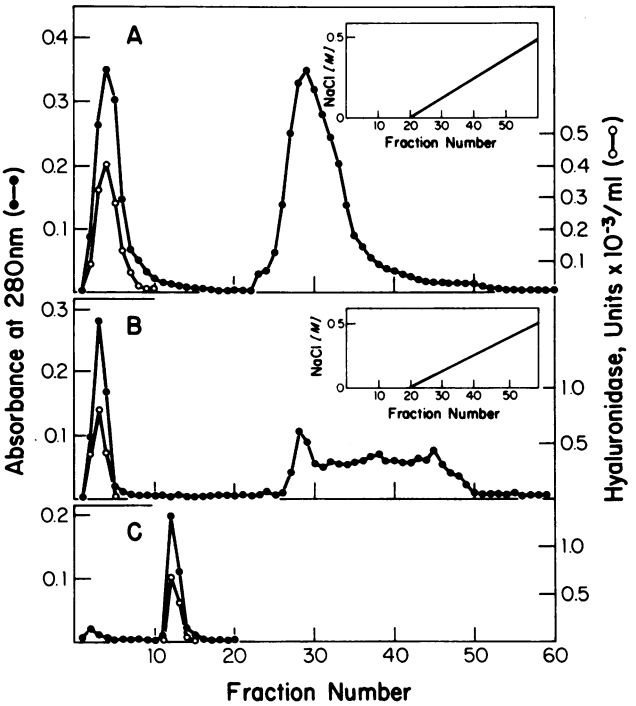

FIG. 1. *Purification of bacteriophage-associated HAase from a strain of group A, type 49 streptococci. See text for details. (A) DEAE-cellulose chromatography. Fractions 2–7 were pooled. (B) CM-cellulose. Fractions 2–4 were pooled. (C) Affinity chromatography.*

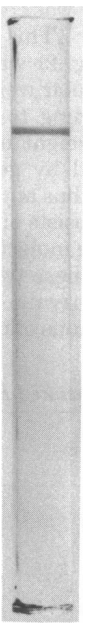

FIG. 2. *SDS-PAGE of phage-associated HAase obtained by affinity chromatography. Gels contained 10% acrylamide and 0.3% cross-linker. Molarity of the gel buffer was decreased by dilution with two volumes of water (33).*

ronic acid purified as described elsewhere (2). An apparent K_m of 4.8×10^{-2} mg/ml was obtained from a double-reciprocal plot of substrate concentration and enzymatic activity (Fig. 4). The V_{max} for phage HAase was estimated to be 0.1 absorbance unit/min per μg of protein.

Effectors. The effect of different salts on HAase was studied. When control, Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Sr^{2+}, and Zn^{2+} ions were added at a final concentration of 1 mM, their enzymatic activities were 100, 104, 88, 101, 87, 99, 91, and 89%, respectively. Figure 5 shows the effect of the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} on HAase. A marked increase of enzymatic activity was observed with $CaCl_2$ at the concentration of 5 mM, whereas less enhancement was observed with $MgCl_2$. $MnCl_2$ was without effect. A mixture of $CaCl_2$ and $MgCl_2$ (at respective levels of 5.0 and 3.0 mM) did not effect an increase in enzyme activity higher than that brought about by $CaCl_2$ alone (5.0 mM).

The enzymatic activity was not stimulated by the addition of thiol reagents such as 2-mercaptoethanol and dithiothreitol at levels of 1.0 mM. Alkylating agents such as iodoacetamide (1.0 mM) and *p*-chloromercuribenzoic acid (1 mM) had no effect on the HAase. These experiments suggest no involvement of free or reduced sulfhy-

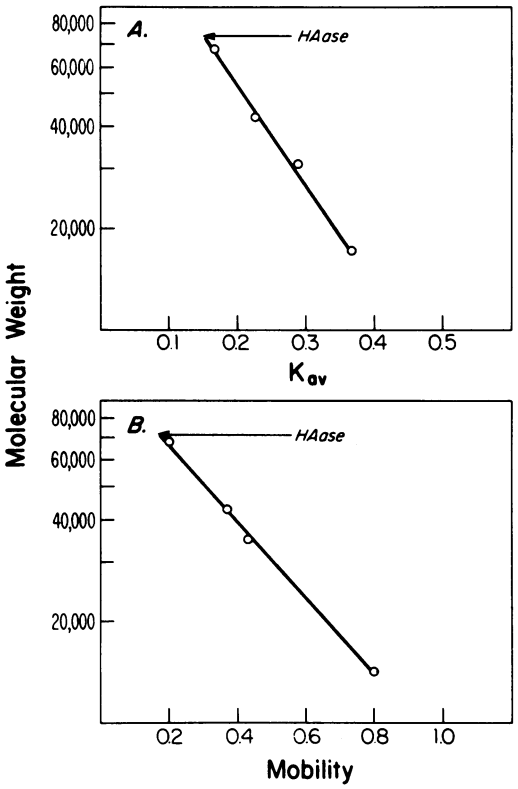

FIG. 3. *Determination of the molecular weight of the bacteriophage-associated HAase enzyme. See text for details. (A) Gel filtration with Sephacryl S-200. (B) SDS-PAGE. Bovine serum albumin (68,000), ovalbumin (43,000), pepsin (35,000), hemoglobin (dimer, 31,000), myoglobin (17,200), and lysozyme (14,300) were used as standards.*

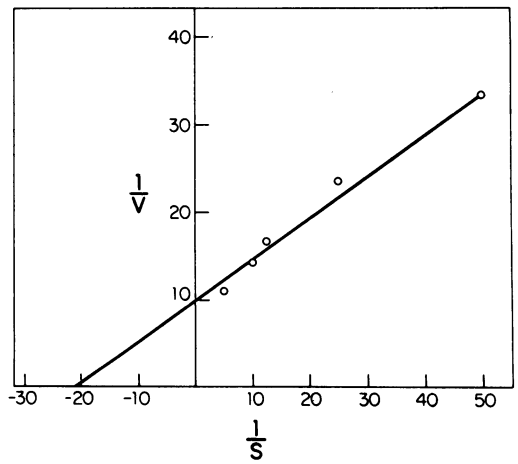

FIG. 4. *Reciprocal (Lineweaver-Burk) plot for bacteriophage-associated HAase. The standard assay was used with 1.0 μg of enzyme and varying amounts of hyaluronic acid (0.02 to 0.2 mg/ml). Velocity of the enzyme reaction was expressed as absorbance at 640 nm/min after 30 min of incubation at 37°C.*

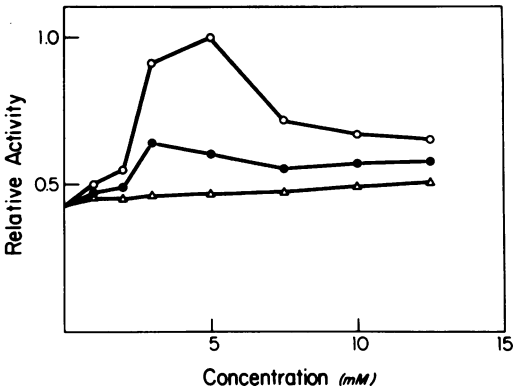

FIG. 5. *Influence of metal ions on the bacteriophage-associated HAase activity. The standard assay was used except that the concentration of metal ions (chloride salts) was varied as shown. Activities are relative to that measured in the presence of 5 mM $CaCl_2$.* ○, *Ca^{2+};* ●, *Mg^{2+};* △, *Mn^{2+}.*

dryl groups in the enzymatic activity, even though the results of the amino acid analysis showed a relatively high amount of half-cystine (Table 2). Ascorbic acid (1 mM) was also without effect on the enzyme.

Effect of pH. The optimum pH of the purified HAase was 5.5 (Fig. 6). The enzyme was exposed to buffer at various pH values at 22°C for 30 min. Thereafter, activities were measured at pH 5.5. Figure 6 shows that the enzyme was stable over a wide range of pH (5.0 to 8.0).

Amino acid composition. Table 2 shows the amino acid composition of the bacteriophage HAase. None of the common amino acids was missing and no unusual amino acids were identified. The protein was relatively rich in acidic amino acids (aspartic and glutamic acids). A partial specific volume ($\bar{v}$) of 0.725 cm^3/g was determined for the enzyme.

Carbohydrate composition. The results of the carbohydrate analysis are given in Table 2. The glycoprotein contains glucose (4.2%), galactose (2.7%), and glucosamine (0.35%) corresponding to 16, 11, and 1 residues, respectively, per enzyme molecule of 71,000 daltons. The low level of glucosamine in the sample approached the method's limits of sensitivity.

DISCUSSION

The general occurrence of HAase activity in animal tissues and microorganisms has been well documented. Several studies have examined the purification and characterization of bovine testes, lysosome, and bull sperm HAases (23), and HAases of microbial origin have also been described. Enzyme production has been demonstrated in numerous bacteria including corynebacteria (17), clostridia (28), flavobacteria and

TABLE 2. *Amino acid and carbohydrate content of streptococcal phage HAase*

Analysis of:	Content	
	Amt	No. of residues[a]
Amino acid[b]		
Aspartic acid	117.5	53
Threonine[c]	74.4	34
Serine[c]	60.1	27
Glutamic acid	179.6	81
Proline	59.9	27
Half-cystine[d]	69.4	31
Glycine	35.9	16
Alanine	110.9	50
Valine[e]	80.7	36
Methionine	10.9	5
Isoleucine[e]	30.7	14
Leucine[e]	138.7	62
Tyrosine	47.0	21
Phenylalanine	60.0	28
Histidine	37.1	17
Lysine	131.4	59
Arginine	49.9	23
Tryptophan[f]		1-2
Carbohydrate[g]		
Glucose	0.23	16 (16.3)
Galactose	0.15	11 (10.7)
Glucosamine	0.017	1 (1.2)

[a] Number of residues (rounded to nearest integer) for a molecular weight of 71,000 and a sugar content of 7.25%. Numbers in parentheses for carbohydrate content represent actual calculated values.

[b] Average of duplicate analyses after 24, 48, and 72 h of hydrolysis. Values are in nanomoles.

[c] Calculated by extrapolation to initial time of hydrolysis.

[d] Determined as cysteic acid after performic oxidation of duplicate samples and 24 h of hydrolysis in 6 N HCl.

[e] Calculated by assuming complete hydrolysis after 72 h.

[f] Determined by spectrophotometric analysis according to the method of Edelhoch (7).

[g] Values are in micromoles per milligram of protein.

Proteus vulgaris (23), staphylococci (29), and *Streptomyces* species (27). Extracellular HAase activities have been detected during growth of beta-hemolytic streptococci of groups A (9, 12, 13, 24, 34), B, C, and G (34).

Bacteriophages are an important (perhaps the only) mechanism of genetic exchange among group A streptococci and may influence decisive biological properties such as antibiotic resistance (20, 32) and virulence (5, 22). Thus, bacteriophages may be significant determinants of the epidemiology of streptococcal infections and perhaps also the pathogenesis of non-suppurative sequelae. HAase associated with the streptococcal bacteriophages plays an essential role in the initiation of phage infection (1, 15, 21). In an earlier report we examined the HAase activity produced by virulent and temperate phages (1). We report here a procedure for the purification of a calcium- or magnesium-stimulated HAase that is tightly bound to a temperate bacteriophage from a group A, type 49 strain (University of Minnesota strain no. GT 8760). The enzyme was isolated after dissociation of the viral particle by urea. The purified enzyme is homogenous when analyzed by SDS-PAGE and has a molecular weight of approximately 71,000 by the same gel system and 75,000 in its active form by gel filtration on Sephacryl S-200. These results suggest that the phage HAase consists of a single polypeptide with a molecular weight that is very similar to that reported for the type 24 bacterial (non-phage-associated) enzyme (9) and much less than that of the HAase produced by *Streptococcus mitis* (26), one of the species found in the oral cavity (11). The K_m value of the phage HAase (4.8×10^{-2} mg/ml) is similar to that determined for *S. pyogenes* type 24 (9, 12) and *S. mitis* (26) but different from the value observed for the type 4 enzyme (13).

The amino acid analysis suggests that the type 49 streptococcal bacteriophage HAase is very different from the testicular enzyme (4) and from the staphylococcal enzyme (29). The carbohydrate content (7.25%) of the phage enzyme is similar to that of the testicular HAase (7.17%) and much lower than that of the staphylococcal enzyme (17%). Glucose, galactose, and glucosamine are present in the viral enzyme as in the

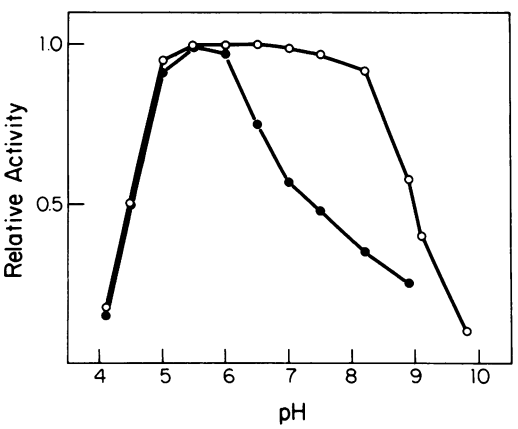

FIG. 6. *Effect of pH on bacteriophage-associated HAase activity* (●) *and stability* (○). *Assays were conducted at 37°C in the following buffers: sodium acetate (20 mM, pH 4.1-6.0), sodium phosphate (10 mM, pH 6.0-7.5), Tris-hydrochloride (10 mM, pH 7.5-8.9), and glycine-Na glycinate (10 mM, pH 8.9-9.8). Sodium chloride (10 mM) was present in all buffers. Activities are relative to that measured at pH 5.5 in acetate buffer.*

staphylococcal enzyme; but in contrast to the two other enzymes, no mannose was found in the phage enzyme preparation. The presence of carbohydrate in the bacteriophage HAase is consistent with its binding to the lentil lectin affinity column. That several other phage proteins were also found to bind to the lectin when a crude enzyme preparation (i.e., after ammonium sulfate precipitation) was applied to the column may point to a glycoprotein nature of these other products; these were removed by the purification procedures (chromatography with DEAE- and CM-cellulose) that preceded the use of the affinity adsorbent.

These studies have been useful in defining the physical, enzymatic, and chemical properties of the purified enzyme, some of which distinguish it from the HAase obtained from other sources. They also provide a homogeneous enzyme preparation for use in future immunological studies.

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