

CHICK EMBRYO FIBROBLASTS PRODUCE TWO FORMS OF HYALURONIDASE

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ABSTRACT

Cultured chick embryo fibroblasts derived from skin and skeletal muscle exhibit hyaluronidase activity both associated with the cell layer and secreted into the medium. Although both forms of the enzyme have a number of similar characteristics (R. W. Orkin and B. P. Toole, 1980, *J. Biol. Chem.* **255**), they differ in thermal stability at neutral pH and in behavior on ion-exchange chromatography. Both forms of the enzyme are equally stable at acidic pH for long intervals, but the cell-associated hyaluronidase is significantly less stable than the secreted form at neutral pH and at temperatures $\geq 30^{\circ}\text{C}$. Neither the presence of proteases nor inhibitors of hyaluronidase appear to be involved in this thermal lability of the cell-associated enzyme. Chromatography of the two forms of hyaluronidase on carboxymethyl cellulose reveals that most (60–90%) of the secreted form of the enzyme elutes at a lower ionic strength than the cell-associated enzyme. Treatment of the secreted form of hyaluronidase with neuraminidase shifts its elution profile on carboxymethyl cellulose toward that of the cell-associated form, and also decreases its thermal stability at neutral pH. In contrast, treatment of the secreted form of hyaluronidase with alkaline phosphatase has no detectable effect. These data suggest that the secreted hyaluronidase differs from the cellular form in possessing additional sialic acid residues which endow the former with increased stability in the extracellular milieu.

Regulation of hyaluronate levels by hyaluronidase may be critical for specific aspects of morphogenesis and tissue remodelling. Numerous studies have correlated high levels of hyaluronate with cell proliferation and migration and reduced levels of this glycosaminoglycan (apparently resulting from the action of hyaluronidase) with the onset of overt differentiation (see reference 30, for review). However, neither the manner in which hyaluronate exerts its effects on cell behavior nor the mode by which hyaluronidase regulates hyaluronate levels has been well defined.

Until recently, the mechanism of hyaluronidase

action at the cellular level could not be studied directly because the enzyme had not been detectable in cultured cells (3, 15). In preceding papers, we have recently detailed the synthesis and secretion of hyaluronidase by primary and passaged cultures of chick embryo muscle- and skin-derived fibroblasts, as well as its production by a number of mammalian cell lines, and have partially characterized the enzyme (21–23). High levels of enzyme were found to be present both associated with the cells and in the media of these cultures. Both enzyme fractions had the pH optimum of 3.7 and displayed no activity at neutral pH (23), typ-

ical of lysosomal hyaluronidase (4). The secreted forms, but not the intracellular forms, of several lysosomal exoglycosidases have been shown to contain recognition ligands, in most cases a 6-phosphomannosyl moiety, which mediate specific uptake of these enzymes (see references 19 and 26 for review). Thus we compared the cell-associated and secreted forms of hyaluronidase for chemical differences which might relate to this receptor-mediated uptake. We have not found evidence for phosphomannosyl groups on hyaluronidase, but have observed that the secreted form of the enzyme differs from the cellular enzyme both in thermal stability at neutral pH and in behavior on ion exchange chromatography. These differences in the two forms of the enzyme appear to be caused by the presence of additional sialic acid residues in the secreted form of hyaluronidase.

MATERIALS AND METHODS

Preparation and Assay of Hyaluronidase from Cell Cultures

Fibroblast cultures prepared from 10-d chick embryo skin and from 12-d chick embryo thigh muscle (see reference 23) were grown to confluence in complete serum-containing medium (nutrient medium F-12 with 10% fetal calf serum and 1% antibiotic-antimycotic solution, Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) and then incubated for variable periods (at least 48 h) in serum-free culture medium. Some cultures were prepared by the Massachusetts Institute of Technology Cell Culture Center. Hyaluronidase was isolated separately from the cell layer and from the serum-free medium, as previously described (23). Unless otherwise indicated, all manipulations were done at 4°C. Briefly, the medium was collected, concentrated by ammonium sulfate precipitation at 65% saturation, and the precipitate was resolubilized in a small volume of formate buffer (0.1 M sodium formate, 0.15 M NaCl, pH 3.7). The cell layer, after being rinsed with Hanks' Balanced Salt Solution (GIBCO), was mechanically removed from the culture plates, homogenized, and sonicated in the formate buffer, pH 3.7, containing 0.1% Triton X-100. Cell extracts were prepared by high-speed centrifugation of cell homogenates at 80,000 g for 1 h at 4°C. Total enzyme activity in the original cell homogenates and that present in the extracts were the same.

Hyaluronidase activity was assayed in cell- and medium-derived samples by measurement of terminal *N*-acetylglucosamine (24) after incubation with hyaluronate substrate in formate buffer (0.1 M, pH 3.7, containing 0.15 M NaCl and 1.5 mM D-saccharic acid-1,4-lactone), as previously reported (23).

Thermal Stability Studies

Routinely, hyaluronidase-containing samples in formate buffer, pH 3.7, prepared from cell extracts and from serum-free medium, were dialyzed against F-12 medium. Known sample volumes, up to 200 μ l, were dispensed into tubes, gassed for 30 s with a 95% air-5% CO₂ mixture to achieve neutrality, tightly capped, and then incubated either for a fixed time interval

(usually 3 h) over a range of temperatures from 4° to 37°C, or for varying time intervals at a specific temperature. Upon completion of this preincubation at neutral pH, all samples were brought to a total volume of 200 μ l with F-12 medium, and then 50 μ l of a solution of 50–100 μ g hyaluronate substrate in 5 \times formate buffer containing 5 \times saccharolactone was added. The final 250- μ l reaction mixture had a concentration of 0.1 M sodium formate, 0.15 M NaCl, 1.5 mM saccharolactone, at pH 3.7. These samples, containing enzyme and hyaluronate substrate, were then incubated for 8 or 16 h at 37°C, and the hyaluronidase activity was then determined colorimetrically as described above. In those experiments in which the temperature was varied, the hyaluronidase activity remaining after preincubation for 3 h at neutral pH at the indicated temperatures was compared with that value obtained after preincubation at 4°C. The latter value was consistently the same as that present in the original sample in formate buffer, pH 3.7, and was set at 100%. In those studies in which the temperature was held constant but the preincubation time at neutral pH varied, the value obtained from the 0-time sample was set at 100%. In other control experiments, samples were preincubated in formate buffer, pH 3.7, for increasing time intervals at 37°C. After the preincubation, hyaluronate substrate was added and the incubation was resumed for an additional 16 h, at which time the samples were assayed for terminal *N*-acetylhexosamine.

In some instances the serum-free medium was removed from the cell cultures and incubated directly for 3 h at 37° or at 4°C without prior ammonium sulfate precipitation. After this preincubation, hyaluronidase was isolated from the medium by ammonium sulfate precipitation, as described above, and the hyaluronidase activity remaining in the two samples (37° vs. 4°C preincubation) was compared.

In some experiments, protease inhibitors were added to the samples before preincubation at neutral pH. These included (a) 1 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in 95% ethanol, (b) 1 mM parachloromercuribenzoic acid (PCMB) dissolved in 50 mM NaOH, (c) a mixture containing 100 mM ξ -NH₂-caproic acid, 5 mM benzamidine-HCl, and 10 mM EDTA (20), (d) 0.1% soybean trypsin inhibitor (SBTI), 0.1% ξ -NH₂-caproic acid, and 2 mM *N*-ethylmaleimide (NEM), alone and in combination.

For mixing experiments, known aliquots of hyaluronidase-containing samples from cell extracts and from medium samples were preincubated at neutral pH, either alone or after mixing together. After the preincubation at neutral pH and 37°C for 3 h, samples were corrected to the appropriate ionic strength and pH with formate buffer and incubated with hyaluronate substrate as indicated above. Control samples were preincubated at neutral pH at 4°C.

Ion-Exchange Chromatography on Carboxymethyl Cellulose

Samples of cell extracts and resolubilized ammonium sulfate precipitates of serum-free culture medium were adjusted to 0.02 M sodium formate, 0.03 M NaCl, pH 3.7 (0.2 \times formate buffer) by either dilution or dialysis and applied to columns of carboxymethyl cellulose (CMC) (17 \times 80 mm) (CM-52, Whatman), previously equilibrated with 0.2 \times formate buffer, pH 3.7. Routinely, two successive step elutions of 50–75 ml each were applied, one at 0.1 M NaCl, 0.02 M sodium formate, pH 3.7, and the second at 0.2 M NaCl, 0.02 M sodium formate, pH 3.7. 5-ml fractions were collected and assayed individually for hyaluronidase activity as indicated above. Hyaluronidase-containing frac-

tions were then pooled and reassayed for hyaluronidase activity and protein concentration, according to the method of Lowry et al. (17). In some instances, samples were first fractionated on Sephadex G-150 (see reference 23) and then applied to CMC. For linear gradients, samples were applied to CMC as usual and then a gradient from 0.03 to 0.28 M NaCl, in a total volume of 200 ml of 0.02 M sodium formate, pH 3.7, was applied. 5-ml fractions were collected and assayed individually for hyaluronidase activity.

In some instances, medium-derived hyaluronidase that eluted from CMC at 0.1 M NaCl was pooled, digested with neuraminidase, and rechromatographed on CMC, or tested for thermal stability at neutral pH. Two sources of neuraminidase were used. One (type IX, Sigma Chemical Co., St. Louis, Mo.) was affinity chromatographically purified. The other (No. 8300, Bethesda Research Laboratories, Rockville, Md.) was a highly purified preparation shown to be free of six different glycosidase, protease, and phosphatase activities. Neuraminidase digestions were done in 1× formate buffer at pH 3.7, containing 0.3% bovine serum albumin (BSA) (8) at a concentration of 0.08 U neuraminidase/ml sample, at 37°C for 3 h. In the presence or absence of neuraminidase, no hyaluronidase activity was lost under these incubation conditions because the enzyme is stable at pH 3.7. Neuraminidase activity in each batch of enzyme was verified by incubation at 37°C for 3 h in the same formate buffer, containing BSA, with sialyllactose (Sigma Chemical Co.) as substrate, and the released sialic acid was determined colorimetrically by the alkali-Ehrlich method (8). To demonstrate further that the neuraminidase was active in the actual digests of hyaluronidase-containing samples, aliquots of the latter were removed, incubated with sialyllactose substrate, and released sialic acid was determined colorimetrically. Routinely, reaction mixtures contained 0.02 U of neuraminidase and 50–100 µg of sialyllactose substrate/250 µl of reaction mixture.

In other experiments, samples of medium-derived hyaluronidase that eluted from CMC at 0.1 M NaCl were digested with alkaline phosphatase (bacterial type III or IIR, Sigma Chemical Co.) (11, 16) and then rechromatographed on CMC. Hyaluronidase-containing samples were dialyzed into Tris buffer (0.1 M Tris, 0.15 M NaCl) at pH 8, and digested with alkaline phosphatase (2 U/ml sample) at room temperature for 2 h. No hyaluronidase activity was lost under these incubation conditions. Samples were then dialyzed back into 0.2× formate buffer at pH 3.7, and rechromatographed on CMC. To demonstrate that the alkaline phosphatase was active under these incubation conditions, aliquots of each preparation of alkaline phosphatase were incubated in the Tris buffer, pH 8, at 2 U/ml, with *p*-nitrophenol phosphate (Sigma Chemical Co.) as substrate. Released, free *p*-nitrophenol was assayed colorimetrically (9). Under these conditions, complete digestion of the substrate was obtained.

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RESULTS

Thermal Stability at Neutral pH of the Cell- and Medium-derived Forms of Hyaluronidase

Comparison of the activities of the two forms of the enzyme after incubation over a varying temperature range from 4° to 37°C (Table I) or at fixed temperatures for increasing incubation times (Fig. 1) demonstrated that, at neutral pH, the secreted, medium-associated form of hyaluronidase is significantly more thermally stable at 30°–37°C than the cell layer-associated form of the enzyme. Similar results were obtained with hyaluronidase prepared from confluent cultures of chick embryo skin or muscle fibroblasts. Table I shows that, although both cell- and medium-derived samples retained complete activity when neutralized and maintained at 4°C, incubation for 3 h at 30°C and neutral pH led to an ~30% loss in cell-associated hyaluronidase activity, whereas the secreted medium form of the enzyme lost only ~5% of its activity under these same conditions. Likewise, a similar incubation at 33°C resulted in

TABLE I
Thermal Stability at Neutral pH of Cell- and Medium-derived Hyaluronidase

Preincubation temperature °C	Muscle fibroblasts		Skin fibroblasts	
	Cells	Medium	Cells	Medium
	% Initial enzyme activity			
4°	100 ± 0 (6)	100 ± 0 (6)	100 ± 0 (4)	100 ± 0 (4)
22°	89 ± 1 (3)	98 ± 3 (4)	92 ± 0 (2)	94 ± 3 (3)
30°	70 ± 10 (4)	96 ± 2 (5)	68 ± 6 (3)	93 ± 2 (3)
33°	43 ± 5 (5)	82 ± 4 (6)	41 ± 13 (3)	76 ± 6 (4)
35°	0 ± 0 (1)	ND	0 ± 0 (1)	ND
37°	0 ± 0 (5)	41 ± 6 (6)	0 ± 0 (4)	38 ± 9 (4)

Values are expressed as the percentage of hyaluronidase activity remaining after a 3-h incubation at neutral pH at the indicated temperatures. After this preincubation at neutral pH, samples were acidified with formate buffer to pH 3.7, incubated for 8 or 16 h with hyaluronate substrate, and hyaluronidase activity was determined colorimetrically as indicated in Materials and Methods.

Each value represents the mean ± standard error. The number of experimental samples is indicated in parentheses. Each sample was assayed in duplicate.

ND, Not determined.

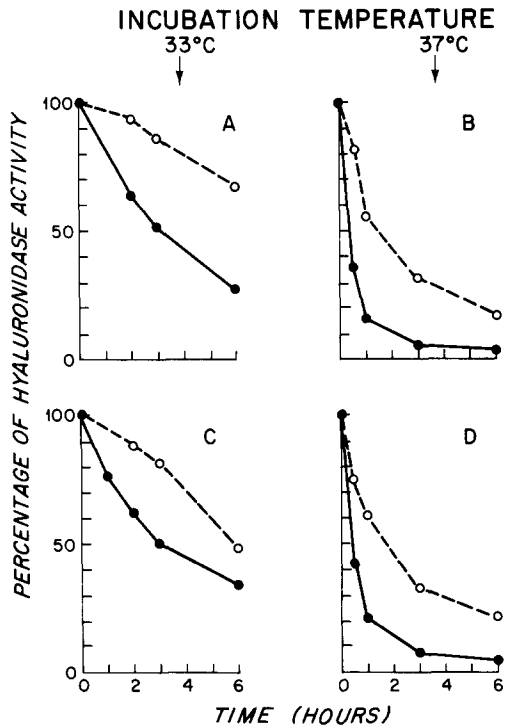


FIGURE 1 Thermal stability of the chick embryo fibroblast cell- (●) and medium-derived (○) forms of hyaluronidase at neutral pH. Samples were first preincubated under conditions of neutral pH, at either 33°C (A and C) or at 37°C (B and D) for the indicated time interval, then adjusted to pH 3.7 with formate buffer, incubated with hyaluronate substrate for an additional 16 h at 37°C, and assayed for hyaluronidase activity as indicated in Materials and Methods. Hyaluronidase activity remaining in each sample is indicated as the percentage of that present in the original sample. Each point represents the average of duplicate determinations. (A and B) Samples prepared from chick embryo muscle fibroblasts. (C and D) Samples prepared from chick embryo skin fibroblasts.

~60% loss of cell-derived hyaluronidase activity, whereas the medium form lost only ~20% activity under these conditions. Finally, incubation for 3 h at neutral pH and 37°C resulted in complete loss of cell-derived hyaluronidase activity, but the medium enzyme retained ~40% activity under these same incubation conditions. Similar data were obtained for the medium form of the enzyme whether the incubation at neutral pH and 37°C was done directly on samples of serum-free culture medium or on preparations of enzyme that had first been isolated from the culture medium by ammonium sulfate precipitation.

The greater thermal stability at neutral pH of the secreted, medium-derived hyaluronidase is further illustrated in Fig. 1. At a preincubation temperature of 33°C, 50% of the cell-associated enzyme activity was lost within 3 h, but a 50% loss in medium-derived hyaluronidase occurred only after 6–9 h, under these incubation conditions. At 37°C, a 50% loss of the cell-associated hyaluronidase activity occurred within 30 min, but an incubation time of ~90 min was required for a comparable decrease in medium-derived hyaluronidase activity to occur. In contrast to these results, preincubation of either cell- or medium-derived enzyme at pH 3.7 for up to 6 h at 37°C did not result in any loss of activity (Table II).

Inclusion of a battery of protease inhibitors (either alone or in combination) in cell-derived hyaluronidase samples failed to prevent loss of hyaluronidase activity at neutral pH and 37°C (Table III). Although inclusion of some protease inhibitors appeared to result in a partial reduction of hyaluronidase activity, preincubation for 2–3 h at 37°C and neutral pH of cell-associated hyaluronidase preparations in the presence or absence of protease inhibitors always resulted in complete loss of enzyme activity.

Mixing experiments also indicated that the higher thermal instability at neutral pH of the cellular form of hyaluronidase is not caused by proteases. These experiments also ruled out the presence of inhibitors in the cell-derived preparations. In these experiments, known aliquots of cell- and medium-derived hyaluronidase preparations were incubated at neutral pH and 37°C alone or together, and then assayed for hyaluronidase activity. Although under these conditions cell-de-

TABLE II
Stability of Cell-associated and Secreted Hyaluronidase at Acid pH and 37°C

Preincubation time	Muscle fibroblasts		Skin fibroblasts	
	Cells	Medium	Cells	Medium
<i>h</i>	<i>U/ml</i>			
0	3.7	4.8	3.0	6.4
1	3.7	5.2	3.2	6.8
3	3.7	4.8	3.1	7.0
6	3.8	4.6	3.4	6.8

Samples in formate buffer, pH 3.7, were preincubated at 37°C for the indicated time intervals. Hyaluronate substrate was then added and samples were incubated for an additional 16 h and then assayed colorimetrically as indicated in Materials and Methods.

rived hyaluronidase samples lost all activity, the mixed samples, containing both cell and medium hyaluronidase, had the same activity as the medium samples alone, at the end of the incubation at neutral pH (Table IV).

Chromatographic Behavior of Cell- and Medium-derived Hyaluronidase on CMC

Comparison of the chromatographic behavior of cell- and medium-derived hyaluronidase preparations on CMC demonstrated that the medium form of the enzyme is more acidic than the cellular form. With a step gradient, under conditions of acidic pH (3.7), >90% of the cellular form of hyaluronidase eluted from CMC at 0.2 M NaCl (Fig. 2A), whereas most of the medium-derived enzyme (60–90%) eluted at 0.1 M NaCl (Fig. 2B), with the remainder eluting at 0.2 M NaCl. When

TABLE III
Effect of Protease inhibitors on Cell-derived Hyaluronidase Activity At Neutral pH and 37°C

Inhibitor	Percentage of hyaluronidase activity	
	Controls (4°C)	Incubation at 37°C, 2 h
None	100	0
3 mM PMSF	76	3
1 mM PMSF + 1 mM PCMB	55	0
100 mM ϵ -NH ₂ -caproic acid, 5 mM benzamidine-HCl, + 10 mM EDTA	45	0
0.1% SBTI	97	0
0.1% ϵ -NH ₂ -caproic acid	96	0
2 mM NEM	85	0
0.1% SBTI + 0.1% ϵ -NH ₂ -caproic acid	ND	0
0.1% SBTI, 2 mM NEM	ND	0
0.1% SBTI, 2 mM NEM, + 0.1% ϵ -NH ₂ -caproic acid	ND	0
0.1% ϵ -NH ₂ -caproic acid + 2 mM NEM	ND	0

Cell-derived hyaluronidase preparations were neutralized by dialysis into F-12 culture medium and then incubated alone or with the indicated protease inhibitors for 2 h at 37°C. Control samples were left in F-12 at 4°C. After this preincubation at neutral pH, samples were acidified to pH 3.7 with formate buffer to a final concentration of 0.1 M Na formate-0.15 M NaCl, incubated with hyaluronate substrate for 16 h at 37°C, and hyaluronidase activity was determined colorimetrically as indicated in Materials and Methods. ND, Not determined.

TABLE IV
Effect of Mixing Cell- and Medium-derived Hyaluronidase Preparations at Neutral pH on Enzyme Activity

Sample	exp 1		exp 2	
	4°C	37°C	4°C	37°C
	$\mu\text{g GNAc}$			
Muscle fibroblasts				
Cells	2.1	0.2	3.3	0.7
Medium	5.7	2.7	7.2	4.7
Cells and medium	7.6	3.2	9.4	5.6
Skin fibroblasts				
Cells	2.5	0.4	2.4	0.7
Medium	2.9	1.7	7.3	4.3
Cells and medium	5.0	2.6	8.7	5.2

100- μl aliquots from cell extracts and media were preincubated alone or together for 3 h at 4° or 37°C, at neutral pH. After the preincubation period, samples were acidified to pH 3.7 with formate buffer at a final concentration of 0.1 M Na formate, 0.15 M NaCl, and then incubated with hyaluronate substrate for 16 h at 37°C, and the products of the digestion were measured colorimetrically as described in Materials and Methods. Hyaluronidase activity is expressed as μg of *N*-acetylglucosamine (GNAc) released per sample aliquot.

a linear gradient was used for elution, it was again found that the cell-derived hyaluronidase eluted at a higher ionic strength than the medium-derived enzyme (Fig. 3). The 0.1- and 0.2-M NaCl eluates from the step gradient of the medium enzyme (Fig. 2B) were also chromatographed in the linear gradient. The 0.2-M fraction gave an elution pattern almost identical to that of the cell preparation. The 0.1-M fraction, however, eluted in the same manner as the whole medium preparation, except that less overlap with the cell enzyme elution pattern was observed (Fig. 3). This suggests that the medium preparation contained a mixture of a small amount of the form of the enzyme obtained from the cells and another more acidic form that elutes at a lower ionic strength from the CMC columns and that comprises the bulk of the medium preparations.

Effects of Neuraminidase and Alkaline Phosphatase on Medium-derived Hyaluronidase

After neuraminidase digestion of the medium-derived hyaluronidase that elutes from CMC at 0.1 M NaCl, 70–100% of the enzyme eluted in 0.2 M NaCl (Fig. 4), the elution position of the cell-

associated hyaluronidase. Addition of 1 mg/ml sialyllactose, a substrate for neuraminidase, to these reaction mixtures largely protected the hyaluronidase from digestion by the neuraminidase, preventing the shift in elution position on CMC (84% of the enzyme eluted in 0.1 M NaCl, 16% in 0.2 M NaCl) (Fig. 4). Control samples, incubated in the absence of neuraminidase or sialyllactose, also eluted primarily in 0.1 M NaCl (89% of the enzyme eluted in 0.1 M NaCl, 11% in 0.2 M NaCl) (Fig. 4).

In some experiments, the neuraminidase was first inactivated by boiling before incubation with hyaluronidase-containing samples to eliminate the possibility that nonenzymatic contaminants of the neuraminidase preparations (see reference 33) might alter the hyaluronidase. Medium-derived hyaluronidase samples treated with heat-inactivated neuraminidase did not shift the position of elution on CMC, i.e., the enzyme continued to elute at 0.1 M NaCl.

In addition to shifting the elution profile on CMC of the medium-derived hyaluronidase toward that of the cell-associated enzyme, neuraminidase digestion of the medium form of hyaluronidase also decreased its thermal stability at neutral pH. Comparative temperature curves in which the temperature range was varied between 4° and 37°C demonstrated that the thermal stability of the neuraminidase-treated, medium-derived hyaluronidase preparations lies intermediate between that of cell-derived hyaluronidase and that of the untreated medium-derived hyaluronidase (Fig. 5).

In contrast to the effects of neuraminidase on the medium-derived form of hyaluronidase, treatment of the medium enzyme with alkaline phosphatase did not significantly affect its elution profile on CMC.

DISCUSSION

Cultures of chick embryo-derived fibroblasts, prepared from two different tissue sources, synthesize and secrete hyaluronidase (23). The cell-associated and secreted, medium-associated forms of this enzyme have specific characteristics in common, including acidic pH optima, substrate preference, and the products of digestion of hyaluronate substrate (23). We report here, however, that the hyaluronidase present within cells and that secreted into the medium represent two distinct forms of this enzyme.

The distinguishing feature of the secreted, me-

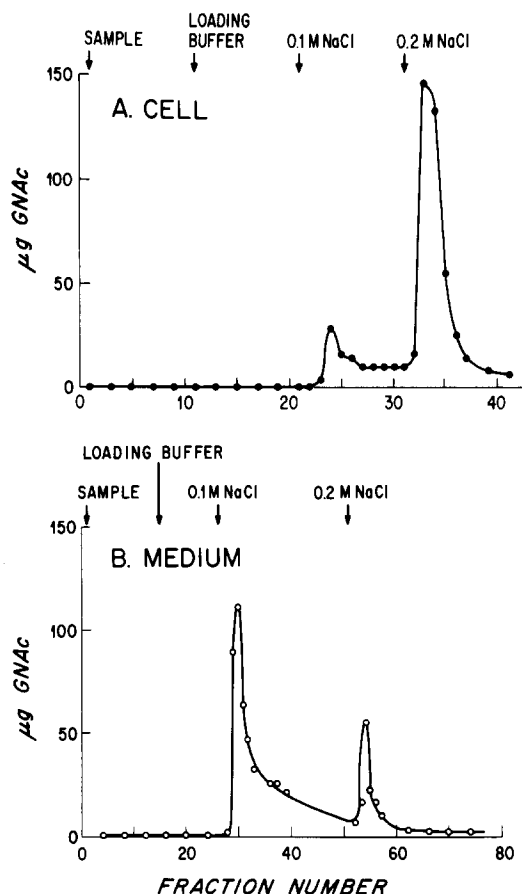


FIGURE 2 Ion exchange chromatography of chick embryo fibroblast cell- (A) and medium-derived (B) hyaluronidase preparations on CMC. Samples were applied to CMC in a buffer of 0.02 M sodium formate, 0.03 M NaCl, pH 3.7 (loading buffer). Loading buffer was then applied to the column. Samples were then eluted with two successive step gradients of 0.1 and 0.2 M NaCl in 0.02 M sodium formate, pH 3.7, as indicated. 4.5-ml fractions were collected and aliquots of each fraction were assayed individually for hyaluronidase activity as indicated in Materials and Methods. Hyaluronidase activity is expressed as μg of terminal *N*-acetylglucosamine (GNAC) released per fraction, after incubation at 37°C for 16 h.

diu form of hyaluronidase appears to be the presence of additional terminal sialic acid residues compared to the cell-associated enzyme. We do not yet know whether the two forms of enzyme are post-translational modifications of the same gene product. If so, it is also possible that the two forms differ with respect to other carbohydrate residues in addition to sialic acid. However, the

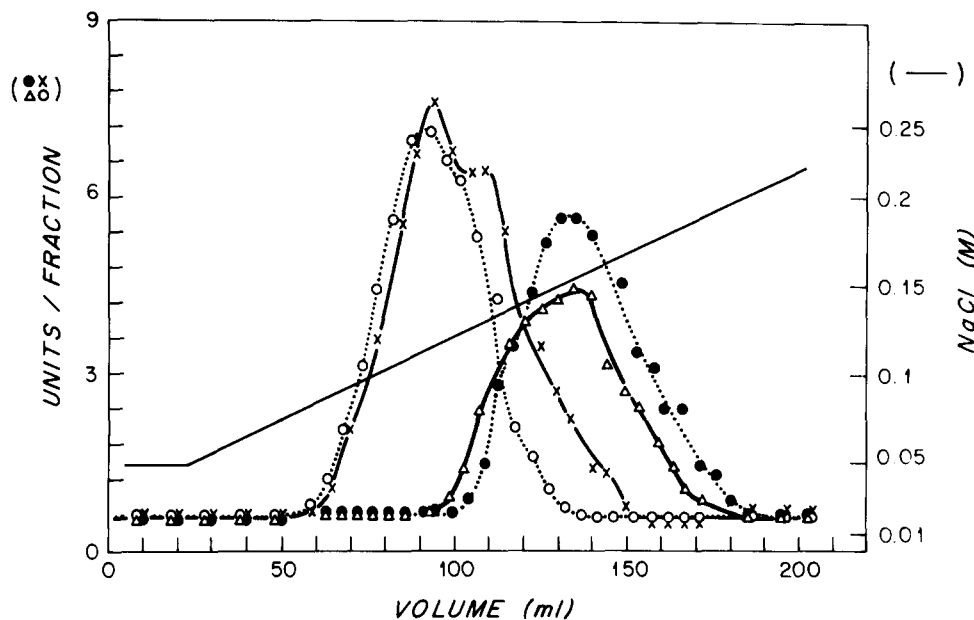


FIGURE 3 CMC chromatography of chick embryo fibroblast cell- and medium-derived forms of hyaluronidase in the presence of a linear gradient of NaCl. Samples were either directly applied to CMC or first isolated by step gradient elution from CMC (see Fig. 2), and then reapplied. Samples were loaded in 0.02 M sodium formate, 0.03 M NaCl, pH 3.7. A linear gradient of NaCl, from 0.03 to 0.28 M, in 0.02 M sodium formate, pH 3.7, was applied as indicated, and 4.5-ml fractions were collected. Aliquots of individual fractions were assayed for hyaluronidase activity, as indicated in the text. Hyaluronidase activity is expressed as units/fraction. (O) Medium-derived hyaluronidase, previously eluted at 0.1 M NaCl by step gradient CMC chromatography. (●) Medium-derived hyaluronidase, previously eluted at 0.2 M NaCl by step gradient CMC chromatography. (x) Medium-derived hyaluronidase applied directly to CMC. (Δ) Cell-derived hyaluronidase, previously eluted at 0.2 M NaCl by step gradient CMC chromatography.

presence of the additional sialic acid residues in the medium form appears to account for the two characteristics found to differ between the cell- and medium-derived forms, i.e., increased thermal stability at neutral pH and more acidic behavior on CMC chromatography. Treatment of the medium form of hyaluronidase with neuraminidase resulted in both a decrease in its thermal stability at neutral pH and a shift in its elution position on CMC toward that of the cellular form of the enzyme. That these changes resulted from neuraminidase action rather than contamination of the neuraminidase preparations by other enzymes or by nonenzymatic substances, e.g., merthiolates (see reference 33), was demonstrated in control experiments in which: (a) sialyllactose, a substrate for neuraminidase, prevented the change, and (b) boiled neuraminidase preparations were shown to have no effect on the hyaluronidase samples. In a study of the hyaluronidase present in extracts of

rat skin, Cashman et al. (7) also observed a shift from more acidic to more basic forms of hyaluronidase after treatment with neuraminidase.

Additional experiments confirmed that the decreased thermal stability of the cell-associated enzyme is intrinsic to this form of the enzyme rather than caused by degradation by proteases or inhibition by other factors in the preparations. Thus, protease inhibitors failed to prevent the loss of cell-associated hyaluronidase activity under conditions of neutral pH and 37°C, and mixing experiments demonstrated that inhibitors of hyaluronidase were not present intracellularly, as the addition of cell-associated enzyme preparations to medium enzyme preparations did not cause a decrease in activity of the medium-derived hyaluronidase. Although the medium enzyme is more stable than the cellular form, it still loses activity relatively rapidly at neutral pH and 37°C, the half time of loss of activity in the absence of cells being

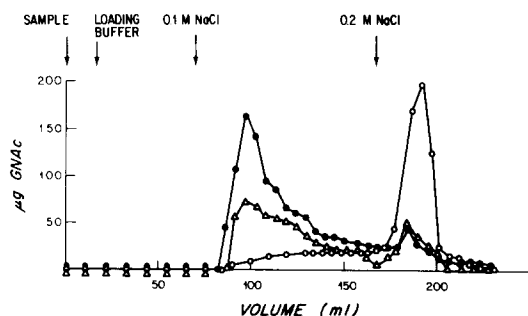


FIGURE 4 The effect of neuraminidase treatment on the chromatographic behavior of chick embryo fibroblast medium-derived hyaluronidase on CMC. Hyaluronidase-containing samples, previously eluted from CMC with 0.1 M NaCl, were separated into equal volumes and one-third of them were treated with neuraminidase, one-third with neuraminidase and sialyllactose, and one-third without additives, as indicated in Materials and Methods. Treated and untreated samples were then individually reapplied to CMC, and each was eluted with successive step gradients of 0.1 and 0.2 M NaCl. (○) + Neuraminidase; (△) + neuraminidase + sialyllactose; (●) no additives.

~1.5 h (Fig. 1). This and the fact that hyaluronidase accumulates linearly in the medium with time, reaching levels that are two to four times those present in the cells after a 48-h incubation period (23), indicate that these fibroblasts are likely to secrete hyaluronidase into the medium at levels considerably greater than those which we have actually been able to measure. Alternatively, the cells may protect the secreted enzyme in some manner, so that in culture the rate of loss of activity of the secreted hyaluronidase would be significantly decreased.

The secreted forms of a number of lysosomal exoglycosidases have been shown to contain specific recognition sites that bind to cell surface receptors and mediate active pinocytosis of these enzymes, followed by incorporation into secondary lysosomes (11, 12, 19, 25, 31). In the case of human fibroblasts, the recognition marker appears to be a phosphomannosyl moiety for β -glucuronidase (11, 13), α - and β -*N*-acetylglucosaminidase (12, 13), α -L-iduronidase (12, 19), α -mannosidase (13), and β -galactosidase (12). These observations have led to a hypothesis for lysosomal enzyme action in which these enzymes are secreted and reinternalized, via the specific binding sites, before function within secondary lysosomes (19). An alternative hypothesis has recently been proposed in which the phosphomannosyl recognition marker

is involved in segregating lysosomal enzymes from secretory products by retaining the former bound to the internal wall of exocytic vesicles during eversion, fusion with the plasma membrane, and secretion of unbound materials (26). According to this hypothesis, plasma membrane-bound lysosomal enzymes would then be reinternalized by endocytosis. Also, the presence of these enzymes with attached recognition markers in cell culture media would be caused by dissociation from the

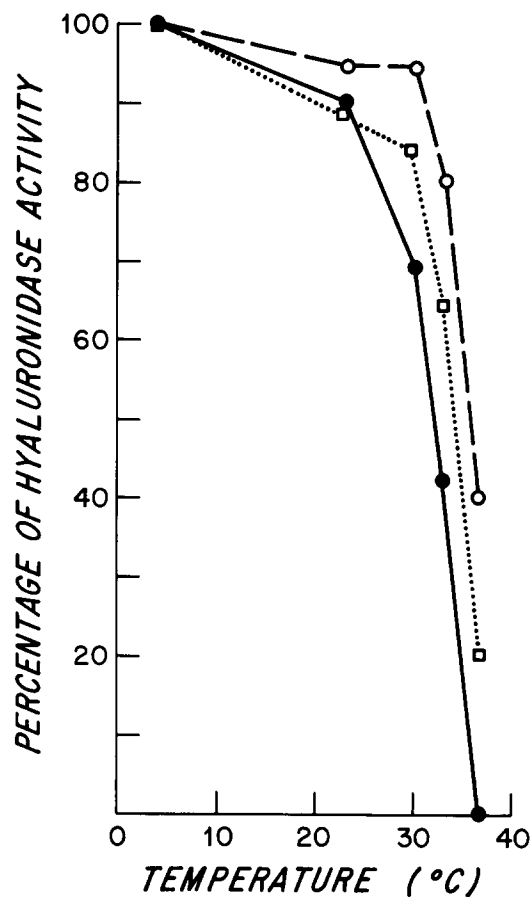


FIGURE 5 The effect of neuraminidase treatment on the thermal stability at neutral pH of the medium-derived form of hyaluronidase. Cell-derived hyaluronidase samples (●) and neuraminidase-treated (□) and untreated (○) medium-derived hyaluronidase samples were preincubated at neutral pH for 3 h at the indicated temperatures, then adjusted to pH 3.7 with formate buffer, incubated with hyaluronate substrate for 16 h at 37°C, and assayed for hyaluronidase activity. Hyaluronidase activity remaining in each sample is expressed as a percentage of that present in the original sample. Each point represents the average of duplicate determinations.

plasma membrane receptors under the culture conditions.

In the present study, we have not found evidence for phosphate groups attached to the secreted form of hyaluronidase as judged by the lack of change of behavior on ion exchange chromatography after treatment with alkaline phosphatase. Several other specific receptor-mediated recognition systems for glycoproteins, including lysosomal exoglycosidases, have been described in addition to that mediated by phosphomannosyl moieties. These involve galactose (5), *N*-acetylglucosamine (1, 14, 18, 27), and mannose (1, 2, 6, 34) moieties. To date, however, no demonstration of involvement of sialic acid as a specific recognition marker has been made. On the contrary, removal of terminal sialic acid groups is necessary for recognition of the penultimate galactose in glycoproteins cleared from blood by liver cells (5). Terminal sialic acid residues have also been reported present in a number of exoglycosidases, isolated from a soluble fraction prepared from rat kidney lysosomes (10). Treatment of these enzymes with neuraminidase was reported to convert them to more basic forms, resembling those found in the bound fraction of these lysosomal preparations (10). In studies on I-cell disease, in which lysosomal exoglycosidases accumulate extracellularly, Vladutiu (32) has proposed that inappropriate addition or failure to remove sialic acid residues, possibly resulting from a sialidase deficiency in these cells (28, 29), might block the phosphomannosyl recognition markers on these defective lysosomal enzymes. It has also been noted that enzymes secreted into culture media by I-cell fibroblasts are more thermally stable than their normal counterparts (see reference 26). Possibly, this difference is caused by the higher sialic acid content of the secreted I-cell exoglycosidases, as observed here for hyaluronidase.

The role of the additional sialic acid moieties present in the secreted form of hyaluronidase has yet to be resolved. It is unlikely that they are involved in modifying enzyme action, as both forms of enzyme are very similar in this respect. They both display activity only over a narrow acidic pH range with no activity at neutral pH, have the same substrate preference for hyaluronate, and give rise to a similar size range of oligosaccharide digestion products (23). These findings strongly suggest that this enzyme does not act extracellularly but that its action would be restricted to lysosomes. The possible role of the

sialic acid groups with respect to recognition and reuptake, as discussed above, will be clarified by experiments designed to test the relative efficiency of uptake of the two forms of hyaluronidase. A final consideration arises from the nature of the transport pathway of lysosomal enzymes from the Golgi apparatus to the lysosomes. These enzymes are believed to be transported from the Golgi apparatus to the plasma membrane, then either secreted and reinternalized (19) or briefly exposed to the extracellular milieu while bound to the plasma membrane and then endocytosed (26). Thus it would follow that these enzymes are exposed temporarily to a neutral pH environment before their incorporation into the acidic environment of the secondary lysosome. A possible function for the additional sialic acid residues may therefore be to protect lysosomal enzymes from loss of activity during this brief period.

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