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A Microtiter-Based Assay for Hyaluronidase Activity Not Requiring Specialized Reagents[1]

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A sensitive, rapid microtiter-based assay for hyaluronidase activity is described that does not require highly specialized biological reagents, as required heretofore. The free carboxyl groups of hyaluronan are biotinylated in a one-step reaction using biotin–hydrazide. This substrate is then covalently coupled to a 96-well microtiter plate. At the completion of the enzyme reaction, residual substrate is detected with an avidin–peroxidase reaction that can be read in a standard ELISA plate reader. Because the substrate is covalently bound to the microtiter plate, artifacts such as pH-dependent displacement of the biotinylated substrate do not occur. The sensitivity permits rapid measurement of hyaluronidase activity from cultured cells and biological samples with an interassay variation of less than 5%. Using this new assay, we measured the distribution profile of plasma hyaluronidase levels in normal human sera. A 1-μl sample of plasma was sufficient for assays in triplicate. Hyaluronidase activity in human foreskin primary keratinocyte cultures was also quantitated. A 25-fold increase in hyaluronidase activity was observed in keratinocyte cultures induced to differentiate in high calcium (1.5 mM), compared to levels in low calcium (0.05 mM) media. The microtiter-based assay may be used as a routine clinical laboratory procedure. © 1997 Academic Press

Hyaluronidases are a family of β-1–4-endoglucosaminidases that degrade hyaluronan (HA)[3] and, from vertebrate sources, can degrade, to a lesser extent, other glycosaminoglycans (for reviews, see 1, 2). These enzymes have been relatively neglected in comparison to other glycosidases, most likely due to the lack of simple and yet sensitive assays that measure degradation of substrate. Until recently, the most commonly used hyaluronidase assays were based upon the measurement of the generation of new reducing *N*-acetylamino groups (3), or loss of viscosity (4) or turbidity (5). These assays are either insensitive or lack specificity. More recently, a new generation of assays has been developed. A microtiter-based assay from this laboratory (6), requires the preparation of a highly specialized reagent, an HA-binding peptide derived from the proteoglycan, aggrecan. It is obtained from tryptic digests of bovine nasal cartilage and isolated by HA-affinity chromatography. This peptide must then be biotinylated (6). Residual substrate in the microtiter plate is then determined as a measure of enzymatic activity. The preparation of such a reagent is not feasible or practical in most laboratories. In addition, the HA substrate binds poorly to standard plastic microtiter plates. This noncovalent immobilization of HA on the microtiter plates is pH sensitive, making comparisons of enzymatic activity at different pH's difficult to interpret.

Hyaluronidases from vertebrate tissues can be separated into two classes: those with maximal activity near neutral pH, such as the enzyme from the plasma membrane of sperm, PH20 (7, 8), and the so-called lysosomal enzymes with maximal activity below pH 4.0 (9, 10). These two classes of enzymes are clearly distinct and appear to have very different biological functions. We have used the present assay to track enzyme activity in the purification and expression of the hyaluroni-

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[3] Abbreviations used: HA, hyaluronan, hyaluronic acid; bHA, biotinylated HA; sulfo-NHS, *N*-hydroxysulfosuccinimide; DMSO, dimethyl sulfoxide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; OPD, *o*-phenylenediamine; rTRU, relative turbidity reducing units; KGM, keratinocyte growth medium; Mes, morpholineethane sulfate.

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dase from human plasma (11). The assay is as sensitive as the one previously described from this laboratory (5), it can be performed using a much shorter incubation period of 15–60 min, and most importantly, it does not require preliminary preparation of a complex bioreagent. This assay will facilitate isolation of novel hyaluronidases from other tissues.

The HA substrate for hyaluronidases is becoming increasingly prominent in biology (12, 13). Its key role has been recognized in a number of basic biological processes, such as embryogenesis (12, 13), carcinogenesis (14, 15), wound healing (16), angiogenesis (17), and inflammation (18, 19). Clinically, aberrations of HA metabolism are associated with processes such as adult respiratory distress syndrome (20), organ transplant edema and rejection (21, 22), and as a marker for cancer remission and relapse (23). An inherited disorder involving serum hyaluronidase deficiency has been described recently (24). The hyaluronidase assay described herein may be used as a routine clinical laboratory procedure.

MATERIALS AND METHODS

Materials

Human umbilical cord HA from was purchased from ICN (Irvine, CA). COVALINK-NH microtiter plates were obtained from NUNC (Placerville, NJ), and sulfo-NHS and biotin hydrazide were from Pierce (Rockford, IL). Bovine testicular hyaluronidase, Wydase, was a gift of the Wyeth–Ayerst Co. (Philadelphia, PA). Human plasma samples were obtained from the UCSF blood donor facility. DMSO and guanidine hydrochloride were products of Fisher Scientific (Pittsburgh, PA). *O*-Phenylenediamine was purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Biotinylated HA

One hundred milligrams of human umbilical cord HA was dissolved in 0.1 M Mes, pH 5.0, to a final concentration of 1 mg/ml and allowed to dissolve for at least 24 h at 4°C prior to the coupling of biotin. Sulfo-NHS was added to the hyaluronate Mes solution to a final concentration of 0.184 mg/ml. Biotin hydrazide was dissolved in DMSO as a stock solution of 100 mM and added to the HA solution to a final concentration of 1 mM. A stock solution of 1-ethyl-3-(3-dimethylaminopropyl) carbidodiimide (EDAC) was prepared as a 100 mM stock solution in dH_2O and added to the HA–biotin solution at a final concentration of 30 μM. This solution was left stirred overnight at 4°C. Unlinked biotin and EDAC were removed after the addition of 4 M guanidine–HCl by dialysis against dH_2O with three changes of 1000× volumes of dH_2O. The dialyzed, biotinylated HA (bHA) was aliquoted and stored at −20°C for up to several months.

Immobilization of bHA onto ELISA Plates

Sulfo-NHS was diluted to 0.184 mg/ml in dH_2O with the bHA at a concentration of 0.2 mg/ml and pipetted into 96-well Covalink-NH plates at 50 μl per well. EDAC was diluted to 0.123 mg/ml in dH_2O and pipetted into the Covalink plates with the HA solution resulting in a final concentration of 10 μg/well hyaluronate and 6.15 μg/well EDAC. The plates were incubated overnight at 4°C or for 2 h at 23°C, which gave comparable results. After covalent immobilization of bHA on the microtiter plates, the coupling solution was removed by shaking and the plates were washed three times in PBS containing 2 M NaCl and 50 mM $MgSO_4$ (buffer A). The plates could be stored at 4°C for up to 1 week.

Assay for Hyaluronidase Activity

The Covalink plates with immobilized bHA were equilibrated with 100 μl/well assay buffer (0.1 M formate, pH 3.7, 0.1 M NaCl, 1% Triton X-100, 5 mM saccharolactone for lysosomal hyaluronidase; 0.1 M formate, pH 4.5, 0.15 M NaCl, 1% Triton X-100, 5 mM saccharolactone for neutral-active enzymes). A set of standards for the calibration of enzyme activity against relative turbidity reducing units (rTRU's) was generated by diluting Wydase in neutral enzyme buffer from 1.0 to 1×10^{-6} TRU/well and assaying 100 μl/well in triplicate. Samples of acid-active hyaluronidase were diluted in lysosomal assay buffer from 1:10 to 1:130,000 in immunoaffinity-purified preparations of recombinant human plasma hyaluronidase (11) and were pipetted in triplicate at 100 μl/well. For most assays of tissue extracts and human plasma, a 30-min incubation at 37°C was sufficient. Positive and negative control wells (no enzyme or no ABC, respectively) were also included in triplicate.

The reaction was terminated by the addition of 200 μl/well of 6 M guanidine–HCl followed by three washes of 300 μl/well with PBS, 2 M NaCl, 50 mM $MgSO_4$, 0.05% Tween 20 (buffer B). An avidin–biotin complex (ABC) kit (Vector Labs, Burlingame, CA) was prepared in 10 ml of PBS containing 0.1% Tween 20, which was preincubated for 30 min at room temperature during the hyaluronidase incubation.

The ABC solution was then added 100 μl/well, for 30 min at room temperature. For the negative control wells, ABC was not included. The plate was then washed five times with buffer B and an *o*-phenylenediamine (OPD) substrate was added at 100 μl/well by dissolving one 10-mg tablet of OPD in 10 ml of 0.1 M citrate–PO_4 buffer, pH 5.3, and adding 7.5 μl of 30% H_2O_2. The plate was incubated in the dark for 10–15 min and was then read using a 492-nm filter in an

ELISA plate reader (Titertek Multiskan PLUS, ICN) monitored by computer using the Delta Soft II plate reader software from Biometallics (Princeton, NJ). A standard curve using the bovine testicular hyaluronidase was generated by a four-parameter curve fit of the commercial hyaluronidase preparation and unknowns were interpolated through their absorbance at 492 nm.

Measurement of Kinetics of Different Hyaluronidases

Three different hyaluronidases were used for the analysis of time dependence of HA degradation. Using 0.01 rTRU of immunoaffinity-purified recombinant human plasma hyaluronidase (85,000 rTRU/mg), bovine testicular hyaluronidase (Sigma Type VI-s 3000 TRU/mg), or *Streptomyces* hyaluronidase (Calbiochem) samples were placed into a microtiter bHA plate at 0, 5, 10, 15, and 30 min in a 37°C water bath. Samples were then processed as usual and bHA degradation was measured at 492 nm.

Analysis of pH Dependence of Hyaluronidase Activity

To analyze the pH dependence of hyaluronidases, immunoaffinity-purified recombinant human plasma hyaluronidase and bovine testicular hyaluronidase were used. The pH dependence of enzyme activity was measured by diluting purified plasma hyaluronidase or partially purified bovine testicular hyaluronidase to 0.1 rTRU in the following buffers: 50 mM formate, pH 3–4.5, 50 mM acetate, pH 5–6, 50 mM Mes, pH 6–7, 50 mM Hepes, pH 7–8. Samples were assayed for 30 min at 37°C and activity expressed as a percentage of maximal activity. NaCl was not used in buffers because this has been reported to alter the pH optima of testicular hyaluronidase preparations (25, 26). We have verified this phenomenon. Physiological salt concentrations (0.15 M), decreased the apparent pH optimum. This was more pronounced in purified preparations of the testicular enzyme than in the original crude sample.

Establishment of Normal Distribution of Hyaluronidase Activity in Human Plasma

Levels of hyaluronidase in human plasma were established from 40 normal human plasma samples obtained from the UCSF Blood Bank. All plasma samples had been collected in EDTA. Residual cellular material was removed by centrifugation. Plasma samples were then assayed after a 30-min incubation using 1:200 dilutions in the formate assay buffer.

Preparation of Hyaluronidase Extracts from Keratinocyte Cultures

Hyaluronidase activity was characterized in normal keratinocytes. Primary foreskin keratinocyte cultures were generated from circumcision tissue from the newborn nursery at UCSF. Briefly, tissue was washed five times in PBS with penicillin, streptomycin, and fungizone followed by digestion overnight at 4°C in dispase. Epithelium was then stripped from mesenchymal tissue with forceps and digested in trypsin followed by plating on collagen-coated plates in KGM (keratinocyte growth medium) with 0.05 mM calcium (Clonetics, San Diego, CA). Cells were used between the first and fourth passage. To test the effects of induction of differentiation on hyaluronidase activity, cells were plated into six-well plates and at confluence the medium was changed to either KGM with 1.5 mM calcium or left at 0.05 mM calcium. Cell layers and conditioned media were harvested after 72 h in culture. Cell layers were harvested with 60 mM octylglucoside with 50 U/ml DNase I (Boehringer Mannheim, Indianapolis, IN) and Complete protease inhibitor cocktail (Boehringer Mannheim) in PBS. Cells were extracted for 30 min on ice, followed by centrifugation at 10,000*g* for 10 min. Conditioned medium from each sample was treated with octylglucoside and protease inhibitors. Cell extracts were normalized on the basis of total cellular protein using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Extracts were then assayed at 1:10 dilutions in the described formate assay buffer at 37°C for 60 min. Activity was expressed as rTRU/mg cellular protein.

RESULTS

Covalent Biotinylation of HA

Hyaluronan from human umbilical cord was biotinylated in a one-step reaction using biotin–hydrazide and EDAC, resulting in a reaction product as depicted in Fig. 1. By limiting the EDAC, which couples the free carboxyl groups on HA with biotin–hydrazide, only a small fraction of the total glucuronic acid residues on HA are labeled. This amount of EDAC (3×10^{-5} M) added to HA (2.8×10^{-3} M) would result in a maximum of one molecule of biotin–hydrazide coupled per 93 disaccharide units of HA.

Incorporation of bHA into a Microtiter-Based Assay for Hyaluronidase

The bHA reagent was used to generate a microtiter-based hyaluronidase assay. The bHA was coupled to NH-bearing microtiter plates at a final concentration of 10 μg/well using EDAC. The amount of HA bound to the plates through this procedure was assayed with the HABP hyaluronan assay (27). Based upon the HABP hyaluronan assay, approximately 7.5 μg of the 10 μg added per well bound to the plate.

A four-parameter curve fit of bovine testicular hyaluronidase standard reactions measured at pH 3.7, and diluted from 1.0 to 1×10^{-6} TRU/well is presented in

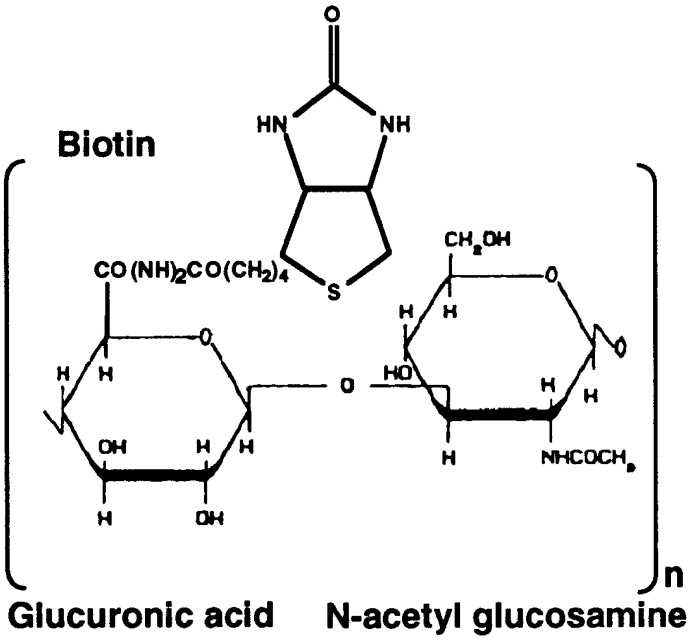


FIG. 1. Structure of the biotinylated disaccharide repeating subunits of HA resulting from a reaction between HA, biotin–hydrazide, and EDAC.

Fig. 2. Four-parameter curve fits were established from the equation $y = ((A - D)/(1 + (\text{conc}/C)^B)) + D$, where logit $y = \ln(y'/1 - y')$, $y' = (y - D)/(A - D)$, $B = -b/\ln 10$ and $C = \exp(a/B)$. The four parameters (A, B, C, D) were calculated with a software program that utilized the 2 + 2 algorithm with linear regression (28). This curve fit incorporates the sigmoidal aspects the standard curve seen in Fig. 2. Optimal accuracy for measurement of a sample occurs from 0.001 to 0.01 at TRU/well. During a 30-min incubation, 1/1000th of a TRU is clearly detectable. A standard logarithmic curve may also be utilized over a shorter range of values to establish a standard curve fit.

To establish linearity of the assay over time, samples of plasma, *Streptomyces* and testicular hyaluronidase

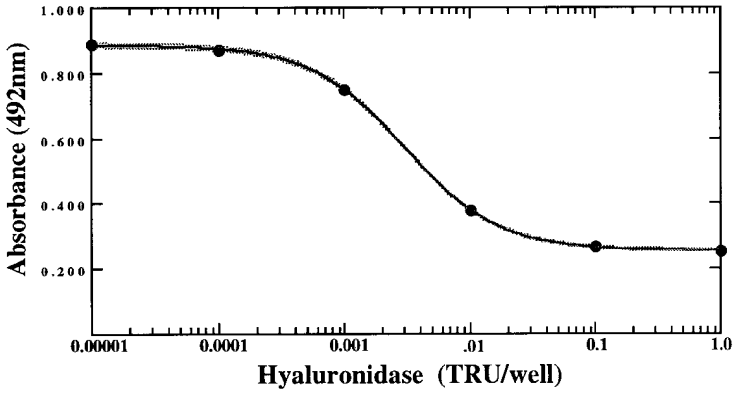


FIG. 2. A four-parameter curve fit of bovine testicular hyaluronidase assays as standard reactions performed at pH 3.7, diluted from 1.0 to 1×10^{-6} TRU/well.

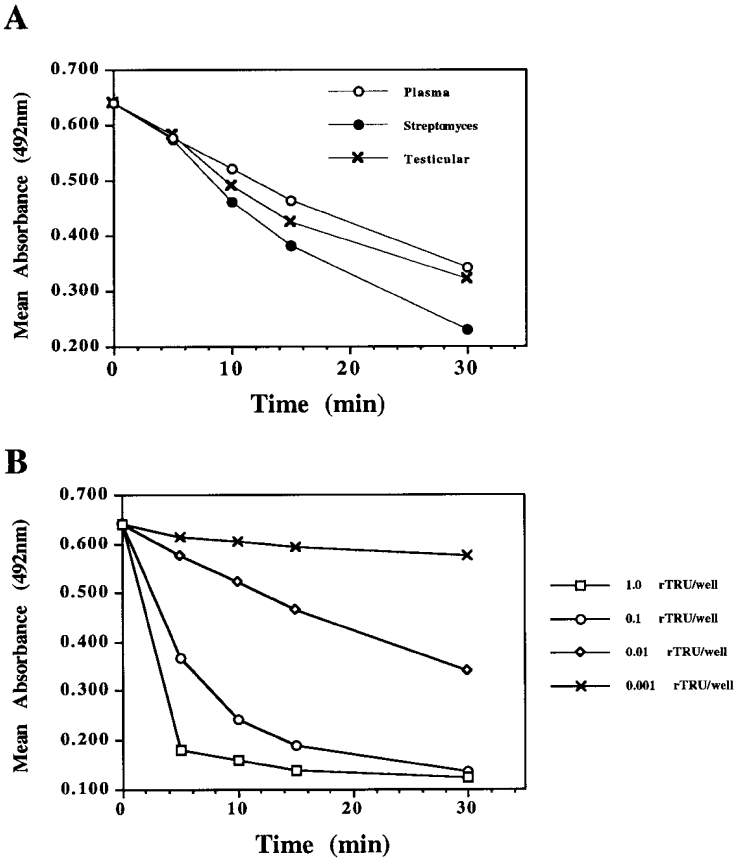


FIG. 3. (A) Linearity of the enzyme reaction over a 30-min incubation period, comparing three different hyaluronidases. 0.01 rTRU/well was utilized at each time, and assayed in triplicate. (B) Kinetic analysis of log dilutions of immunoaffinity-purified recombinant human plasma hyaluronidase. Human plasma hyaluronidase, from 1 to 0.001 rTRU/well, was assayed from 0 to 30 min.

were assayed as a function of time (Fig. 3A). Linearity over a 30-min incubation of enzyme was clearly present over this time period for recombinant human plasma hyaluronidase, whereas preparations of testicular hyaluronidase and *Streptomyces* hyaluronidase deviated slightly. Log dilutions of human plasma hyaluronidase (Fig. 3B) revealed that linearity was observed in more dilute preparations, presumably where substrate had not become limiting.

The pH dependence of a neutral- and an acid-active hyaluronidase were examined. In Fig. 4, recombinant human plasma hyaluronidase was compared to a commercial preparation of bovine testicular hyaluronidase (Sigma, type VI-S). The plasma enzyme had an acid optimum at pH 3.8, with no detectable activity above pH 4.5, whereas the bovine testicular hyaluronidase had a bimodal distribution of activity with optima at pH 4.5 and 7.5.

Analysis of Hyaluronidase Levels in Human Plasma

Hyaluronidase activity from the plasma of normal donors were assayed to establish the distribution of

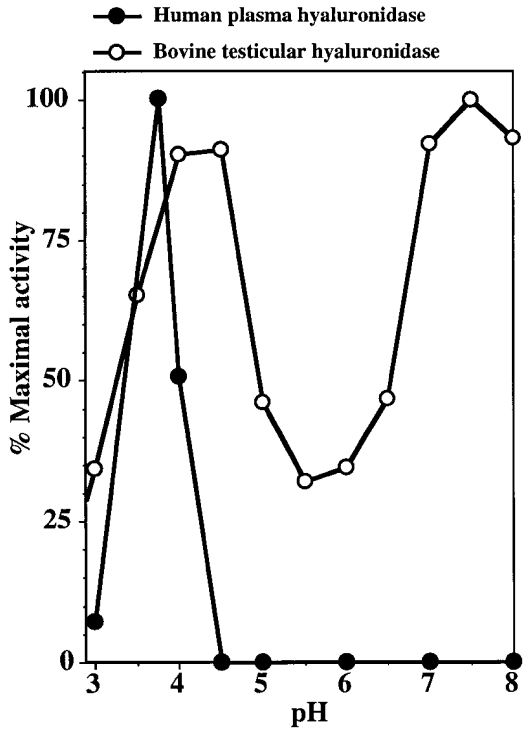


FIG. 4. Comparison of the pH profiles of recombinant human plasma hyaluronidase and a partially purified bovine testicular hyaluronidase preparation, Sigma, Type VI-S.

hyaluronidase levels in the human circulation. The histogram in Fig. 5 represents the distribution of hyaluronidase activity assayed at pH 3.7 from 40 healthy male and female subjects from ages 20 to 70 years. The mean plasma hyaluronidase level was 5.9 rTRU/ml. The standard deviation was 1.2 rTRU. The inter- and

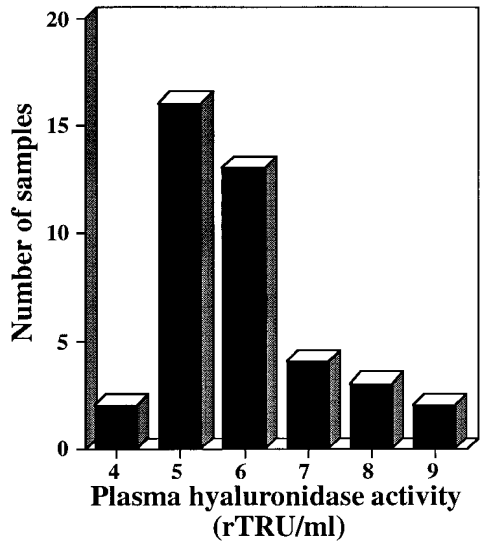


FIG. 5. Distribution of hyaluronidase levels in normal human plasma. Samples were from freshly collected specimens obtained from the UCSF Blood Bank. The 40 normal healthy donors ranged in age from 20 to 70 years. Samples were assayed at pH 3.7 as described under Materials and Methods.

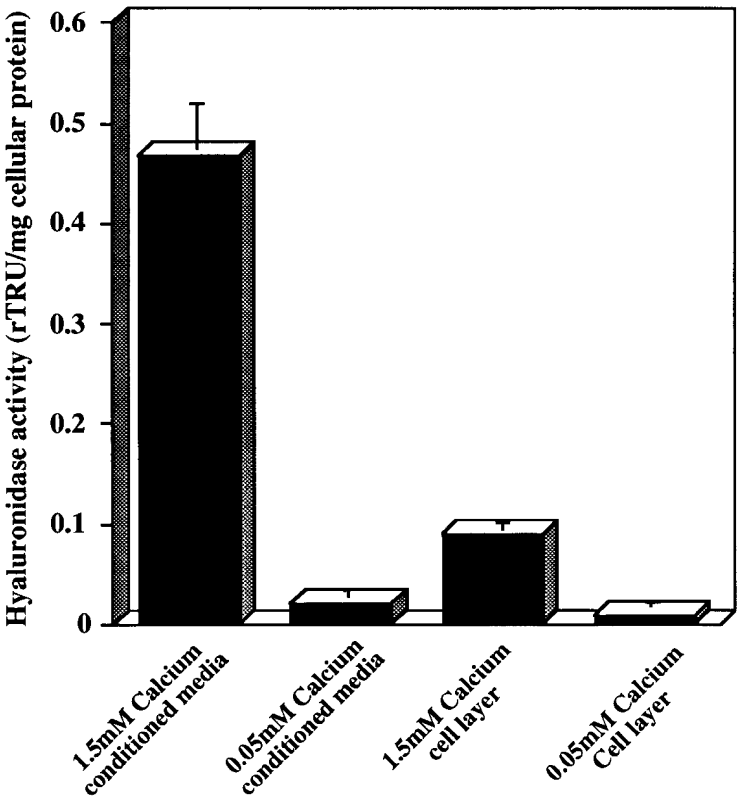


FIG. 6. Levels of hyaluronidase activity in human keratinocytes in primary culture, grown in low (0.05 mM) and high (1.5 mM) calcium. High calcium-containing medium induces differentiation and stratification in such cells, and enhances levels of hyaluronidase activity in both the medium and in the cell layer.

intraassay variations established from repeated sampling of a single plasma sample were less than 5 and 10%, respectively, thus establishing the reproducibility of this procedure. The interassay variation was calculated from comparing the standard deviation of the interpolated values of a serum sample assayed in 10 different wells in a single plate to the mean interpolated value of those 10 wells against a standard curve. The intraassay variation was obtained from comparing the standard deviation of interpolated values from the serum sample assayed in 6 different plates with different standard curves to the mean interpolated value of those 6 samples.

Effects of Calcium on Hyaluronidase Levels in Keratinocyte Cultures

Primary cultures of keratinocytes can be induced to stratify and express several markers for differentiation in a defined culture medium when calcium levels are elevated from 0.05 to 1.5 mM (29). The effects of calcium-induced differentiation on levels of hyaluronidase activity in keratinocyte cultures (Fig. 6) resulted in an approximately 25-fold increase in hyaluronidase activity in both conditioned media and cell layer, compared

to cultures in 0.05 mM calcium. EDTA was included with protease inhibitors in the extraction buffer, making it unlikely that calcium was directly effecting enzymatic activity. In purified preparations of human plasma hyaluronidase, EDTA had no inhibitory effect on enzyme activity, nor did added calcium have a stimulatory effect. All of the hyaluronidase activity secreted into the media of the keratinocyte cultures was immunoprecipitated in its entirety with monoclonal antibodies against the plasma enzyme (data not shown).

DISCUSSION

This new assay for hyaluronidase activity was developed to provide a faster and less labor-intensive procedure, compared to assays previously described. In addition, covalent coupling of the HA substrate to the microtiter plate made the assay more resistant to pH variations during the generation of pH optima curves and when different enzymes with different pH optima were being compared. This became particularly important after we realized that HA adherence to the standard plastic microtiter plates was pH-dependent, using the assay previously described (6).

Because HA does not contain free amines (Fig. 1), biotinylation of HA using NHS–biotin results in a very low level of biotinylation. This previously described method (30) using NHS–biotin may actually be a result of biotinylation of free amine groups from contaminating HA-binding proteins rather than the substrate itself. The use of EDAC as the limiting reagent to couple free carboxyl groups on HA, that are present at one site per disaccharide, with biotin hydrazide, can overcome this difficulty. This EDAC coupling procedure has been used successfully in our laboratory to link HA to Sepharose beads in the preparation of HA–Sepharose affinity resins.

We initially attempted to link biotin to HA with photoactivatable biotin, as has been described for DNA labeling. However, this resulted in a very low signal and, in addition, was very expensive in comparison to other biotin preparations. Biotin–hydrazide with EDAC as a coupling reagent was then tested as a method for linking biotin to HA. This procedure permitted the control of biotinylation by using the EDAC as the limiting reagent and was very easy to utilize. Through initial studies it was found that coupling the biotin at a ratio of 1 molecule of biotin per approximately 90 disaccharide units provided a strong signal in the avidin–peroxidase system and did not interfere with the ability of the enzyme to degrade substrate. Higher levels of biotinylation resulted in inhibition of enzyme activity and were beyond the range of sensitivity of the system. Both long- and short-chain biotin–hydrazide were analyzed, and short-chain biotin had the least interference with enzymatic activity.

This HA-biotinylation procedure was simple to perform, requiring only a one-step biotinylation procedure rather than the multistep modification of HA with pendant amine groups described previously (31). Excess biotin could easily be removed by dialysis. The guanidine–HCl was added to the preparation before dialysis to aid in the removal of any noncovalent interactions between biotin and HA.

Coupling the bHA to NH-microtiter plates was shown to be very efficient at a concentration of 10 μg/well. Approximately 75% of the added HA was bound to the microtiter plate, as determined by measuring the residual HA in solution using the HA-binding peptide competition assay for hyaluronate. Thus, approximately 7.5 μg of HA was coupled to each well of the microtiter plate. The amount of EDAC used to couple the HA to the plate was optimized as well, and it was demonstrated that a 10-fold increase in EDAC resulted in a significant loss of sensitivity to enzymatic degradation. This was presumably due to excessive crosslinking of the biotinylated substrate to the microtiter plate, because most endoglucosaminidases digest the substrate to the tetrasaccharide level as the smallest digestion product (32).

The present assay was very sensitive over short incubation periods and was approximately 1000 times more sensitive than the commonly used assays under identical incubation times. Increased sensitivity for detecting enzyme in cell cultures that produce very low levels of activity could be obtained by using a longer incubation time of 2–12 h. However, relative activities of these enzymes are difficult to evaluate over the longer incubations because of variations in enzyme stability and loss of the kinetic nature of the reaction. As seen in Fig. 2, the use of a four-parameter curve fit has a semilogarithmic relationship over a 3 log range between absorbance and activity, from 0.6 to to 0.006 relative TRU/ml during a 30-min incubation. If the incubation is extended to 2 h, this curve shifts from 0.06 to 0.00006 TRU/ml, resulting in a more sensitive measurement of activity. We routinely used the 1-h incubation for cell culture extracts and the 30-min assay for tissue extracts.

The utility of the assay for the measurement of pH optima of various enzymes was also evident. As seen in Fig. 3A, immunoaffinity-purified plasma hyaluronidase had the characteristic acid pH optimum of 3.8, whereas the commercial semipurified preparation of bovine testicular hyaluronidase, contained two distinct peaks of activity, one at neutrality and one at pH 4.0. The pH profile of immunoaffinity-purified recombinant human plasma hyaluronidase was identical to that of unprocessed human plasma (11), with no activity detected at neutral pH. This is the only enzyme detectable in plasma using this assay based upon immunological and amino acid sequencing criteria. The bimodal pH

optima for preparations of sperm hyaluronidase have been described using substrate gel zymography (33) and are not likely due to an artifact of the assay, as two disparate methods give the same result. It has been postulated that two hyaluronidase enzymes are present in these testicular preparations (33, 34).

REFERENCES

1. Frost, G. I., Csóka, T., and Stern, R. (1996) *Trends Glycosci. Glycotechnol.* **8,** 419–434.
2. Kreil, G. (1995) *Protein Sci.* **4,** 1666–1669.
3. Bonner, W. M., Jr., and Cantey, E. Y. (1966) *Clin. Chim. Acta* **13,** 746–752.
4. De Salegui, M., Plonska, H., and Pigman, W. (1967) *Arch. Biochem. Biophys.* **121,** 548–554.
5. Dorfman, A., and Ott, M. L. (1948) *J. Biol. Chem.* **172,** 367.
6. Stern, M., and Stern, R. (1992) *Matrix* **12,** 397–403.
7. Primakoff, P., Hyatt, H., and Myles, D. G. (1985) *J. Cell Biol.* **101,** 2239–2244.
8. Gmachl, M., Sagan, S., Ketter, S., and Kreil, G. (1993) *FEBS Lett.* **336,** 545–548.
9. Aronson, N. N., Jr., and Davidson, E. A. (1967) *J. Biol. Chem.* **242,** 437–440.
10. De Salegui, M., and Pigman, W. (1967) *Arch. Biochem. Biophys.* **120,** 60–67.
11. Frost, G. I., Csóka, T. B., Wong, T., and Stern, R. (1997) *Biochem. Biophys. Res. Commun.* **236,** 10–15.
12. Brown, J. J., and Papaioannou, V. E. (1993) *Development* **117,** 483–492.
13. Meyer, M. F., and Kreil, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4543–4547.
14. Knudson, W. (1996) *Am. J. Pathol.* **148,** 1721–1726.
15. Sherman, L., Sleeman, J., Herrlich, P., and Ponta, H. (1994) *Curr. Opin. Cell Biol.* **6,** 726–733.
16. Estes, J. M., Adzick, N. S., Harrison, M. R., Longaker, M. T., and Stern, R. (1993) *J. Pediatr. Surg.* **28,** 1227–1231.
17. West, D. C., and Kumar, S. (1989) *Ciba Found. Symp.* **143,** 187–201, 201–207, 281–285.
18. Edelstam, G. A., Laurent, U. B., Lundkvist, O. E., Fraser, J. R., and Laurent, T. C. (1992) *Inflammation* **16,** 459–469.
19. Noble, P. W., McKee, C. M., Cowman, M., and Shin, H. S. (1996) *J. Exp. Med.* **183,** 2373–2378.
20. Kropf, J., Grobe, E., Knoch, M., Lammers, M., Gressner, A. M., and Lennartz, H. (1991) *Eur. J. Clin. Chem. Clin. Biochem.* **29,** 805–812.
21. Rao, P. N., Bronsther, O. L., Pinna, A. D., Demetris, A., Snyder, J., Fung, J., and Starzl, T. E. (1993) *Transplant. Proc.* **25,** 2141–2142.
22. Itasaka, H., Suehiro, T., Wakiyama, S., Yanaga, K., Shimada, M., and Sugimachi, K. (1995) *J. Surg. Res.* **59,** 589–595.
23. Hernandez Hernandez, J. R., Garcia Garcia, J. M., Martinez Muniz, M. A., Allende Monclus, M. T., and Ruibal Morell, A. (1995) *Int. J. Biol. Markers* **10,** 149–155.
24. Natowicz, M. R., Short, M. P., Wang, Y., Dickersin, G. R., Gebhardt, M. C., Rosenthal, D. I., Sims, K. B., and Rosenberg, A. E. (1996) *N. Engl. J. Med.* **335,** 1029–1033.
25. Gold, E. W. (1982) *Biochem. J.* **205,** 69–74.
26. Gacesa, P., Savitsky, M. J., Dodgson, K. S., and Olavesen, A. H. (1979) *Biochem. Soc. Trans.* **7,** 1287–1289.
27. Fosang, A. J., Hey, N. J., Carney, S. L., and Hardingham, T. E. (1990) *Matrix* **10,** 306–313.
28. Rodbard, D., Lenox, R. H., Wray, H. L., and Ramseth, D. (1976) *Clin. Chem.* **22,** 350.
29. Hennings, H., and Holbrook, K. A. (1983) *Exp. Cell Res.* **143,** 127–142.
30. Hoare, K., Savani, R. C., Wang, C., Yang, B., and Turley, E. A. (1993) *Connect. Tissue Res.* **30,** 117–126.
31. Pouyani, T., and Prestwich, G. D. (1994) *Bioconjugate Chem.* **5,** 370–372.
32. Aronson, N. N., Jr., and Davidson, E. A. (1967) *J. Biol. Chem.* **242,** 441–444.
33. Cherr, G. N., Meyers, S. A., Yudin, A. I., VandeVoort, C. A., Myles, D. G., Primakoff, P., and Overstreet, J. W. (1996) *Dev. Biol.* **175,** 142–153.
34. Hunnicutt, G. R., Mahan, K., Lathrop, W. F., Ramarao, C. S., Myles, D. G., and Primakoff, P. (1996) *Biol. Reprod.* **54,** 1343–1349.