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Purification, Cloning, and Expression of Human Plasma Hyaluronidase[1]

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Hyaluronidase was purified from human plasma using Triton X-114 phase extractions and ion-exchange chromatography. Monoclonal antibodies generated against the purified protein by a novel screening assay were utilized to isolate homogeneous enzyme for microsequencing. The amino acid sequences obtained matched a cDNA in the Expressed Sequence Tag database which, with 5′-RACE-PCR, was used to clone the plasma hyaluronidase gene, termed Hyal-1. Hyal-1 codes for a protein of 435 amino acids that is over 40% identical to PH-20, a sperm-specific hyaluronidase. Unlike PH-20, which is only expressed in testis, transcripts of Hyal-1 were found in multiple tissues. Hyal-1 stably expressed in human embryonic kidney cells resulted in a 3,000 fold increase of secreted immunoreactive hyaluronidase activity that was biochemically indistinguishable from human plasma hyaluronidase. By immunological, molecular and biochemical criteria, we conclude that Hyal-1 is the predominant hyaluronidase found in human plasma. © 1997 Academic Press

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan (GAG), and a major component of the extracellular matrix. It is composed of repeating disaccharide units and is the only non-sulfated GAG. It is prominent in connective tissue, skin, cartilage, synovial fluid (1) and whenever rapid tissue turnover is occurring, such as during embryonic development (2) wound healing (3) and malignancy (4,5). The enzymes that catabolize HA, the hyaluronidases (E.C 3.1.25), are a family of β,1-4 endoglucosaminidases that depolymerize HA and chondroitin sulfate (for review, see 6,7). In vertebrates, these enzymes are grouped into two classes, the neutral-active hyaluronidases such as sperm-associated PH-20 (8,9), and those with an acid pH optimum such as those found in human liver (10) and plasma (11). A genetic deficiency of human plasma hyaluronidase was recently described (12). Attempts to isolate hyaluronidase from human plasma have met with limited success because of it's high specific activity and very low abundance. In order to purify human plasma hyaluronidase to homogeneity in sufficient quantity for amino acid sequencing, we generated monoclonal antibodies specific to the purified enzyme using a novel enzyme-capture assay. This enabled us to clone the gene that codes for human plasma hyaluronidase, which we term Hyal-1.

MATERIALS AND METHODS

Assays for hyaluronidase activity and protein concentration. Hyaluronidase assays (13, 14) and HA substrate gel zymograms (15) were performed as previously described. Bovine testicular hyaluronidase (Sigma VI-S, 3,000 TRU/mg, St. Louis, MO) was used for comparisons. Protein concentrations were measured using the Lowry (Pierce, Rockford IL) assay.

Purification of human plasma hyaluronidase. To 2 l of human plasma, 0.02% Na Azide, 50 mM NaCl, 5% sucrose and 7.5% Triton X-114 (Boehringer Mannheim, Indianapolis, IN) were dissolved at 4°C with stirring for 90 min followed by centrifugation at 10,000 × g for 30 min. The plasma was then subjected to phase extraction at 37°C and centrifuged at 10,000 × g for 30 min at 37°C. The detergent-rich phase was removed and diluted to 2 l with ice cold 50 mM Hepes, pH 7.5, 0.15 M NaCl, followed by repartitioning at 37C° with centrifugation. This procedure was repeated three times. The final detergent phase was diluted six-fold with 25 mM Mes, pH 6.0, and 20 ml of equilibrated SP-Sepharose (Pharmacia, Piscataway NJ) resin was added and stirred overnight at 4°C. The beads were collected by centrifugation and washed with 25 mM Mes, pH 6.0, containing 46 mM octylglucoside (Boehringer Mannheim). Hyaluroni-

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Abbreviations: HA, hyaluronan; rTRU, relative turbidity reducing units; HEK-293, human embryonic kidney-293 cells; PCR, polymerase chain reaction; cDNA, complementary DNA; RACE, rapid amplification of cDNA ends.

Data Deposition: Human plasma hyaluronidase (Hyal-1) is available under GenBank Accession No. U96078.

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dase was eluted from the beads with 0.3 M NaCl in Mes buffer pH 6.0 with several washes, concentrated on a YM3 membrane (Amicon, Beverly, MA) and desalted into 10 mM PO_4 pH 7.4 with 25 mM NaCl, 46 mM octylglucoside on an F.P.L.C. Fast-Desalting column (Pharmacia). The preparation was then combined with 10 ml of hydroxylapatite resin (Biorad, Richmond, CA) equilibrated in the same buffer, and left on a rocker at 4°C overnight. Hyaluronidase did not adsorb to the resin and was recovered in the supernatant. The supernatant was concentrated to 0.5 ml on a Centriplus YM3 concentrator (Amicon), applied to a 12.5% polyacrylamide gel on a Phast Gel System (Pharmacia), and silver stained.

Generation of anti-hyaluronidase monoclonal antibodies. BALB/c mice were immunized using purified antigen from the post-hydroxyapatite step using established procedures (16). Hybridomas secreting anti-hyaluronidase antibodies were screened with a novel enzyme capture assay. HA was coated onto microtiter plates under the same conditions as those described for the hyaluronidase assay (10) except that 1.25 μg/well of goat anti-mouse IgG (Jackson Immunolabs, West Grove, PA) was included with the HA so that both HA and goat anti-mouse IgG were bound to the plates. Hybridoma supernatants were incubated with diluted human plasma for 60 min at 37°C followed by incubation in the HA/anti-mouse-IgG plates for 60 min at 37°C. Plates were washed 5 times with PBS containing 0.1% Triton X-100, and 10 mg/ml BSA followed by the addition of formate assay buffer and incubation at 37°C for 60 min. Digested HA as a result of immunoprecipitated hyaluronidase was detected as in the standard assay. One of the single cell cloned hybridoma lines was grown as ascites in BALB/c mice. IgG_{2a} was purified through Protein-A affinity chromatography.

Immunoprecipitation and immunoaffinity purifications. Purified IgG_{2a} from the 17E9 anti-hyaluronidase hybridoma clone was used for immunoprecipitations and purifications unless otherwise specified. For the immunoprecipitation of hyaluronidase from human plasma, serial dilutions of purified 17E9 IgG or control mouse IgG2a were mixed with plasma diluted in RIPA buffer (1% NP-40, 1% deoxycholate, 1% Triton X-100, 5 mM EDTA in PBS) followed by immunoprecipitation with protein-A beads. Residual activity in the supernatant was then measured in the hyaluronidase assay. For the immunoaffinity purification of hyaluronidase, 3 mg of purified IgG from the 17E9 hybridoma clone was coupled to a 1 ml Hi-Trap-NHS activated column (Pharmacia). Plasma or HEK-293 recombinant hyaluronidase conditioned media was diluted 1:2 with RIPA buffer, and passed through the anti-hyaluronidase IgG column. The column was first washed with PBS containing 2 M NaCl, 100 mM octylglucoside followed by washing with 100 mM citrate pH 4.0, 0.15 M NaCl and octylglucoside, and then eluted with the same buffer adjusted to pH 3.0.

Microsequencing of hyaluronidase. For N-terminal amino acid sequencing, the immunoaffinity purified protein was electroblotted from an SDS gel to a PVDF membrane (ABI, Foster City, CA) and sequenced by Edman degradation. Internal peptides of immunoaffinity purified plasma hyaluronidase were obtained through digestion with cyanogen bromide, separated on a Vydac C-18 column, and sequenced as above.

Isolation of the plasma hyaluronidase cDNA. A TBLASTN (17) homology search of the EST database (18) revealed a single I.M.A.G.E. Consortium clone (19) (GenBank accession no. AA223264) with 100% identity to the N-terminal and an internal amino acid sequence of plasma hyaluronidase. We sequenced this EST with the *Taq* dye deoxy terminator cycle sequencing kit and an ABI Prism automated sequencer (Applied Biosystems, Foster City, CA). To obtain the 5′ end of the cDNA, nested 5′ RACE (20) was performed on a Marathon Ready human heart cDNA library (Clontech, Palo Alto, CA). For the first PCR reaction HPHRACE1 (5- ATCGAAGACACTGACATCCACGTCCACACC-3′) was used with the AP2 primer from Clontech. For the nested reaction HPHRACE2 (5′-TGCCTCTCCAGGCACCACTGGGTGTTTGC-3′) was used with the AP2 primer. Annealing/extension was at 72°C for 40 cycles in the first PCR reaction and for 15 cycles in the nested reaction. A single sharp band of 800 bp was observed on agarose gel electrophoresis and was ligated into the pCR2.1 vector (Invitrogen, San Diego, CA) which was used to transform TOP10F' competent cells. Positive colonies were sequenced as above. As expected, the 800bp product overlaped 100% with the 5′ end of the EST by 300 bp.

Expression of recombinant plasma hyaluronidase in HEK-293 cells. For generation of the hyaluronidase cDNA coding sequence, PCR was performed with the EST as template with the following primers: HPHF1 5′-GTGCCATGGCAGCCCACC-3′ and HPHR1 5′-ATCACCACATGCTCTTCCGC-3′ with annealing at 58°C for 10 cycles. The product was cloned into the expression vector pCR3.1-Uni (Invitrogen) to produce the vector Hyal-1-p3.1 and used to transform One Shot TOP10F' competent cells. Hyal-1-p3.1 was purified from positive colonies, and transfected into HEK-293 cells using 9 μg of purified plasmid with 60 μl of Lipofectin (Gibco BRL). After 48 h. cells were plated out using the limiting dilution method into 24 well plates with 500 μg/ml G418. After 14 days, the conditioned media of resistant colonies was assayed for hyaluronidase activity using the described protocol. Colonies with high level expression were expanded for further characterization. For the analysis of the recombinant hyaluronidase, an HEK-293-line overexpressing Hyal-1-p3.1 was grown for 48 h. serum free and the conditioned media passed through a 17E9 anti-plasma hyaluronidase immunoaffinity column. Recombinant enzyme was eluted using the same protocol as for human plasma. Purified recombinant hyaluronidase was then blotted to PVDF and sequenced.

Organ survey of hyaluronidase transcripts. Nested PCR primers amplifying the 1.3 kb coding region of the plasma hyaluronidase cDNA were used to analyze the tissue distribution of transcripts in λgt10 cDNA libraries. For the first round of PCR the following primers were used: HPHF2 (5′-AGGTTGTCCTCGACCAGTC-3′) and HPHR2 (5′-ATGTGCAACTCAGTGTGTGGC-3′) at an annealing temp. of 58°C. The second PCR reaction consisted of 15 cycles at an annealing temp. of 58°C with primers HPHF1 and HPHR1 (see above).

RESULTS

Purification of hyaluronidase. The enzyme partitioned into the temperature-induced Triton X-114 detergent phase and gave a 60-fold enrichment. The post-hydroxylapatite preparation of hyaluronidase was purified to apparent homogeneity as determined by silver staining (Fig. 1), resulting in an overall purification of 1.5-million fold (Table 1). The specific activity of the enzyme was approximately 85,000 rTRU/mg protein. The protein migrated on 12.5% SDS-PAGE with a relative molecular mass of 57 kDa (Fig. 1).

Generation of monoclonal antibodies against plasma hyaluronidase. The post-hydroxylapatite preparation was used to generate monoclonal antibodies. An enzyme capture assay was used for screening hybridomas that exploited the lack of enzymatic activity or HA-binding affinity of plasma hyaluronidase at neutral pH. The hybridoma supernatants were incubated with crude plasma at neutral pH in the HA/anti-mouse IgG microtiter plates to immunoprecipitate the antibody-antigen complex. This screening procedure yielded eight clones from twenty 96-well plates. One clone of the IgG_{2a} class, 17E9, was used to generate ascites.

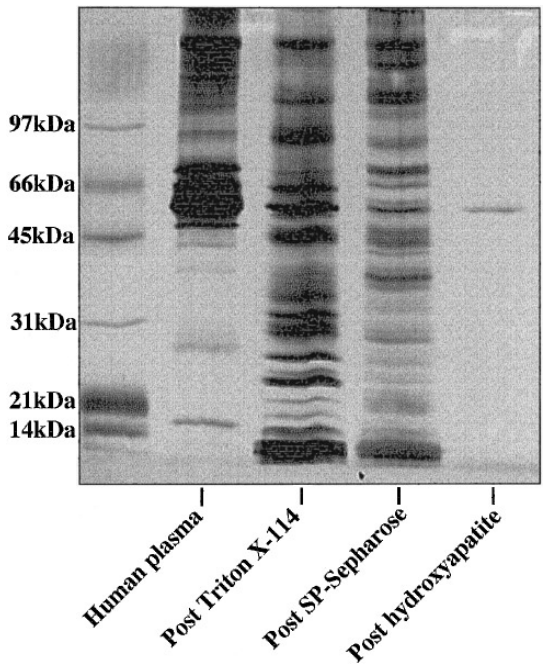

FIG. 1. Analyses of hyaluronidase throughout the purification procedure. 4 μl samples at each step were electrophoresed on 12.5% acrylamide gels and silver stained. Hyaluronidase migrated with an apparent molecular mass of 57 kDa in the post hydroxylapatite concentrate step.

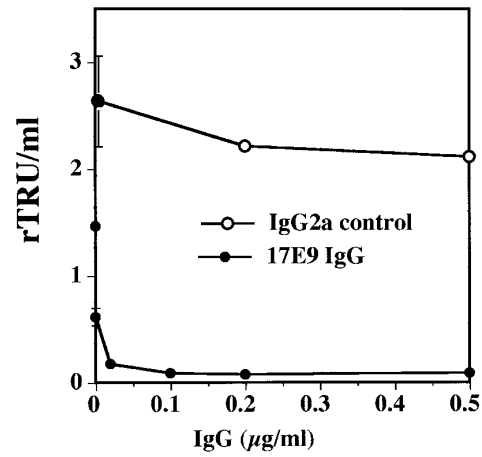

FIG. 2. Immunoprecipitation of hyaluronidase from human plasma with monoclonal antibodies. Serial dilutions of purified 17E9 anti plasma hyaluronidase IgG_{2a} or corresponding amounts of mouse control IgG_{2a} were combined with human plasma diluted with RIPA buffer, followed by immunoprecipitation with Protein-A Sepharose beads. Supernates were then assayed using the hyaluronidase assay as described.

Addition of serial dilutions of the 17E9 antibody to human plasma followed by immunoprecipitation with Protein-A resulted in precipitation of all detectable hyaluronidase activity (Fig. 2). Hybridoma supernatants from the seven other clones were also capable of immunoprecipitating all detectable hyaluronidase activity from human plasma (not shown).

Immunoaffinity purification and microsequencing. Hyaluronidase could be purified to homogeneity in a single step from human plasma by immunoaffinity chromatography using the 17E9 antibodies. After washing the column under stringent conditions, the enzyme eluted at pH 3.0, and was purified to homogeneity as determined by SDS-PAGE and N-terminal amino acid sequencing. Three additional internal sequences were obtained from CnBr digests of the immunopurified protein.

Isolating the cDNA of plasma hyaluronidase. The Hyal-1 cDNA was 2507 kb in length and contained an open reading frame of 1308 bp that coded for a protein of 435 amino acids. The N-terminal and internal amino acid sequences (overerlined in Fig. 3) of plasma hyaluronidase were 100% identical to the conceptual translation of the Hyal-1 cDNA. This strongly suggests that Hyal-1 is the gene that codes for plasma hyaluronidase. Alignment of the predicted translation of Hyal-1 and human PH-20 indicates 40% amino acid sequence identity and 60% homology (Fig. 3).

Expression of recombinant plasma hyaluronidase. The parental HEK-293 cell line produced almost undetectable levels of hyaluronidase in the conditioned media (0.005 rTRU/ml) and cell layer whereas the stably transfected cells secreted approximately 15 rTRU/ml, a 3,000 fold increase (Fig. 4). This increase of enzymatic activity was confirmed using a well characterized color-

TABLE 1

Purification Scheme for Human Plasma Hyaluronidase

Purification step	Volume (ml)	Activity (rTRU/ml)	Protein (mg/ml)	Specific activity (rTRU/mg)	X-Fold purification
Starting material	2,100	5	86	0.058	1.0
Final detergent phase	650	4.71	1.3	3.62	63
SP-sepharose	60	42.5	0.85	50	875
Hydroxyapatite	1.0	1943	0.0225	86,355	1.5×10^6

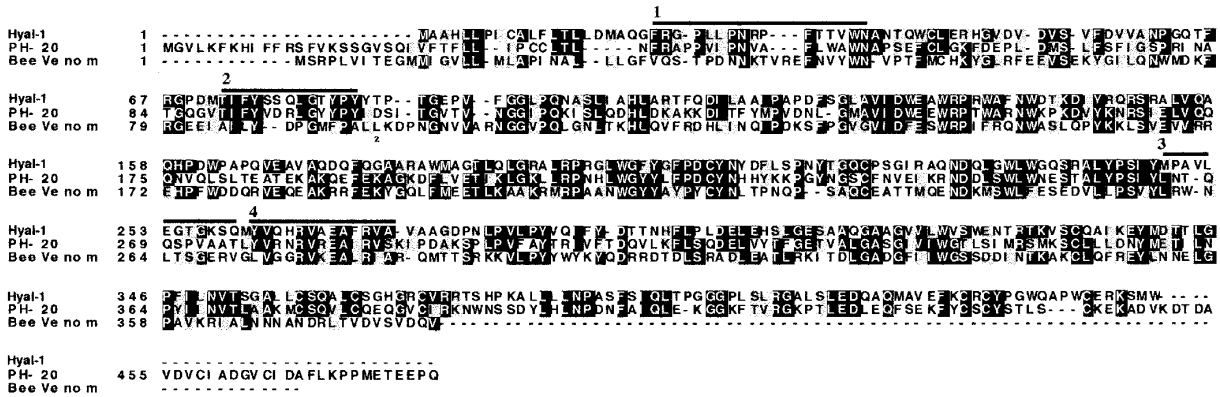

FIG. 3. Alignment of plasma hyaluronidase-Hyal-1, human PH-20 and bee venom hyaluronidase. Identical amino acids are boxed, similar amino acids are shaded. Peptide sequences obtained from the immunoaffinity purified protein are overlined. Plasma hyaluronidase has 40% amino acid identity with human PH-20, a sperm-specific hyaluronidase.

imetric assay for hyaluronidase activity (14), which also revealed that the enzyme cleaved the β,1-4 linkages as opposed to β,1-3 linkages in HA. To ensure that the hyaluronidase activity found in the recombinant HEK-293 cell clones was the product of the transfected cDNA, we immunoaffinity purified the hyaluronidase from serum free conditioned medium of the HEK-293 overexpressing clone and sequenced the eluent from the 17E9 column. This yielded the same processed N-terminus (FRGPLLVP) found in human plasma and a migrated as a single band on SDS-PAGE (Fig. 5A). This band aligned with the purified plasma using both silver stain and substrate gel zymography (Fig. 5B). A commercial preparation of testicular hyaluronidase was run for comparison of the specific activity. The pH activity curve of recombinant plasma hyaluronidase (Fig. 6) had the same profile as the immunoaffinity-purified plasma enzyme, with no activity above pH 4.5, in contrast to bovine testicular hyaluronidase, which displayed a bimodal curve of activity with maxima at pH's 4.5 and 7.5.

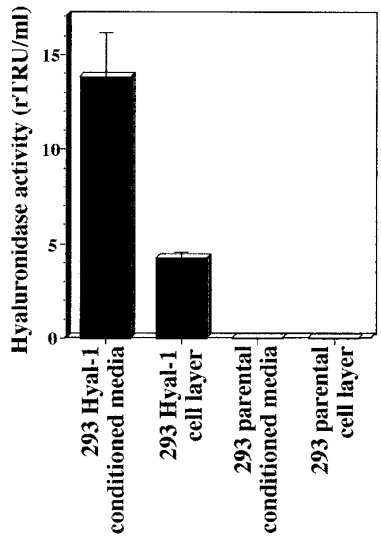

FIG. 4. HEK-293 cells stably transfected with coding cDNA secrete hyaluronidase activity not present in the parental line. Cells were transfected with a CMV driven pCR3.1-Uni vector containing the Hyal-1 gene from nucleotides 606–1942. Cells from a hyaluronidase-secreting G418-resistant HEK-293 clone and the parental cell line control were grown for 48 h. in the absence of serum. Conditioned media and cell layer were harvested and assayed for hyaluronidase activity using the described microtiter assay. Error bars indicate the standard error of three samples.

Organ survey of hyaluronidase transcripts. Nested amplimers were used to analyze the tissue distribution of Hyal-1 transcripts in λgt10 cDNA libraries. As illustrated in Fig. 7, PCR products were found in heart, kidney, liver, lung, placenta, and skeletal muscle, but were not detected in brain. Whether human plasma hyaluronidase is the predominant acid-active hyaluronidase described in other tissues awaits further studies. However, Hyal-1 is the only hyaluronidase present in human plasma.

DISCUSSION

An HA-degrading activity was first described in acidified human plasma in 1966 (14). This enzyme was characterized as a single species by substrate gel zymography (21). There have been several attempts to purifiy such an activity (11, 22, 23), but isolation of this enzyme protein to homogeneity has been extraordinarily difficult. We now report the isolation of this hyaluronidase. The enzyme is present at an extremely low concentration, 60 ng/ml, has a high specific activity of 85,000 rTRU/mg and is highly unstable in the absence of detergents. A 1.5 million-fold purification was necessary to achieve homogeneity. Generation of monoclonal antibodies was essential

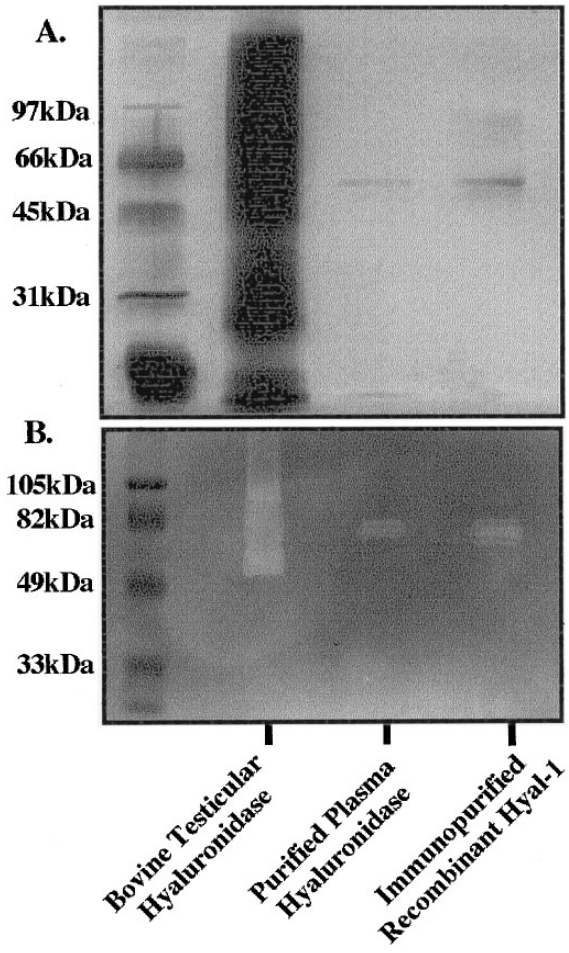

FIG. 5. A. Immunoaffinity-purified recombinant hyaluronidase migrated with a relative molecular mass indistinguishable from biochemically purified plasma hyaluronidase. Bovine testicular hyaluronidase, corresponding to an equal level of activity, was electrophoresed with plasma hyaluronidase and recombinant Hyal-1 for comparison. B. Substrate gel zymography of testicular, plasma and recombinant hyaluronidase. Samples were electrophoresed on a 10% SDS PAGE gel impregnated with 50μg/ml HA. After substituting SDS with Triton X-100, the gel was incubated in formate buffer, pH 3.7, overnight. The gel was then stained for HA with Alcian blue and counterstained with Coomassie blue R-250.

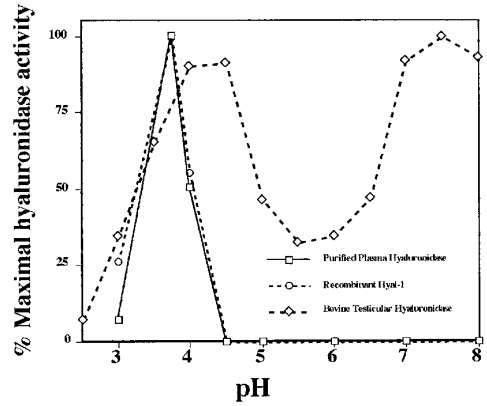

FIG. 6. Secreted recombinant hyaluronidase in HEK-293 cells has the same pH activity profile as native plasma hyaluronidase. Immunopurified plasma hyaluronidase, immunopurified recombinant hyaluronidase and bovine testicular hyaluronidase were assayed in the microtiter assay from pH 3.0–8.0. Activities at given pH intervals were plotted as a percent of maximum activity.

to purify enough enzyme for sequence analysis. The homology between plasma hyaluronidase and PH-20, a sperm-specific hyaluronidase, suggests that all mammalian β,1-4 hyaluronidases may be members of a conserved family (6). All detectable hyaluronidase activity in normal human plasma could be identified as a single protein by both immunoreactivity and amino acid sequencing. It is likely that this enzyme is the hyaluronidase absent in the recently described patient with serum hyaluronidase defiency (12).

Few serum proteins partition into the detergent rich phase as did hyaluronidase. This property is usually reserved to lipid-modified or integral membrane proteins (24). Phospholipase treatments did not expose a glycosyl phosphatidylinositol-anchor, as has been described for some forms of PH-20 (25). Analysis of the Hyal-1 mature amino acid sequence with Kyte-Doolitle hydropathy plots (not shown), failed to reveal any potential transmembrane domain that could explain such phase partitioning properties. Nevertheless, recombinant Hyal-1 from the HEK-293 overexpressing clones possessed identical phospholipase-resistant detergent-phase partitioning characteristics as the biochemically purfied plasma hyaluronidase. We must assume that either the lipid modification is resistant to cleavage, as has been demonstrated for other GPI-linked proteins

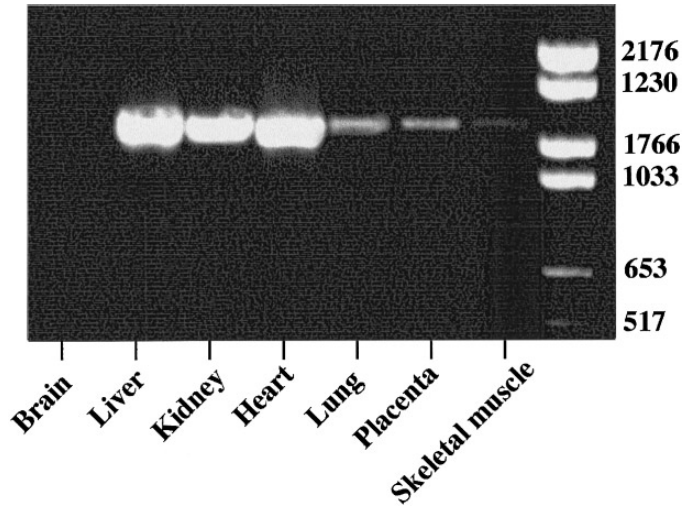

FIG. 7. A PCR organ survey of plasma hyaluronidase reveals transcripts in heart, kidney, liver, lung, placenta, and skeletal muscle, but not in brain. λgt10 human cDNA organ libraries were analyzed for plasma hyaluronidase transcripts using a nested PCR reaction that amplified the same 1.3 kb open reading frame that was used for expression.

(26), or that some other form of lipid association is responsible for the hydrophobic characteristics of this enzyme.

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