Purification and microsequencing of hyaluronidase isozymes from human urine

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Abstract **We recently cloned the major hyaluronidase of human plasma, which we termed HYAL1. All hyaluronidase activity could be purified from human urine on an anti-HYAL1 monoclonal antibody column. However, urine contains two hyaluronidases of 57 kDa and 45 kDa, whereas plasma only contains the 57 kDa activity. Microsequencing confirmed that both urinary isozymes have N-termini identical to plasma hyaluronidase, but a second N-terminus was found in the smaller isozyme, apparently derived from the terminal 25 amino acids of HYAL1, at the C-terminus. The two polypeptides of the 45 kDa isozyme resulting from endoproteolytic cleavage of the 57 kDa isoform are presumably linked by disulfide bonds. Sperm contains two isozymes of the testicular hyaluronidase, PH-20, and the lower molecular weight isozyme is believed to be an endoproteolytically processed form of the larger protein. Analogously to PH-20, the smaller isozyme of HYAL1 is likely to be a proteolytically processed product of the larger isozyme.**

Key words: Hyaluronidase; Microsequencing; Isozyme; Human urine; Monoclonal antibody

1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan that has a key role in the structure and organization of the extracellular matrix (ECM) [1]. It is ubiquitous in higher eukaryotes and is elevated whenever rapid tissue proliferation and regeneration are occurring, such as during embryogenesis, wound healing, and malignancy. Until recently, the nature of the enzymes that depolymerize HA, the hyaluronidases [2], has been virtually unknown. The first eukaryotic hyaluronidase to be cloned was from bee venom [3]. It was found that bee venom hyaluronidase has significant homology to a sperm membrane protein, PH-20, that had previously been isolated using a monoclonal antibody [4]. It was demonstrated subsequently that PH-20 has hyaluronidase activity [5]. PH-20 mRNA expression is restricted to the testis, and it was assumed that PH-20 homologs did not exist in other tissues. Recently we purified and cloned the major hyaluronidase activity of human plasma, which we termed HYAL1, and observed that the protein is homologous to PH-20, with 40% amino acid identity [6]. Highest levels of expression of the HYAL1 mRNA were found in liver and kidney. The production of monoclonal antibodies was essential for purification of sufficient enzyme for microsequencing. Hyaluronidase is present at high specific activity in human urine, approximately 100-fold that of human plasma [7,8]. It has been hypothesized that hyaluronidase plays a role in the action of antidiuretic hormone by increasing the permeability of the nephron walls, which have an ECM rich in HA [8,9]. However, this has remained controversial [10–12]. As an initial step in exploring this hypothesis in greater detail, we wished to determine if urinary hyaluronidase is identical to the plasma enzyme. To answer this question, we purified hyaluronidase from human urine using an anti-HYAL1 monoclonal affinity column and sequenced the proteins that bound strongly to the column.

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Abbreviations: ECM, extracellular matrix; HA, hyaluronic acid, hyaluronan; IgG, immunoglobulin gamma; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TRU, turbidity reducing unit

2. Materials and methods

2.1. Preparation of the anti-plasma hyaluronidase monoclonal affinity column

For the immunoaffinity purification of hyaluronidase, 3 mg of purified IgG from a single cell hybridoma clone that immunoprecipitated all hyaluronidase activity from human plasma (clone 17E9) was coupled to a 1 ml Hi-Trap-NHS-activated column (Pharmacia, Uppsala, Sweden) to produce an anti-HYAL1 affinity column as previously described [6].

2.2. Assays for hyaluronidase activity and protein

Hyaluronidase activity was measured using a classic colorimetric assay [13], a new microtiter-based assay [14], and HA substrate gel zymography [15]. All of the assays were standardized to the turbidity reducing unit (TRU) with commercial hyaluronidase (Sigma type VI hyaluronidase) as a standard. One TRU of hyaluronidase is defined as the amount of enzyme that will decrease the turbidity producing capacity of 0.2 mg HA to that of 0.1 mg HA in 30 min at 37°C [16]. Protein concentrations were measured with the detergent-compatible Micro BCA assay (Pierce, Rockford, IL, USA). Proteins were visualized with the Pharmacia Phast system on 12.5% SDS-PAGE gels according to the manufacturer's instructions.

2.3. Immunoaffinity purification of human urinary hyaluronidase

Twenty liters of human urine were collected from laboratory volunteers, concentrated to 1 l and desalted into PBS on a CH2PRS cartridge concentrator fitted with a S3Y10 spiral cartridge (Amicon, Beverly, MA, USA). To the concentrated urine, 1% Triton X-100, 1% sodium deoxycholate, 1% NP-40, and 0.02% sodium azide were added with stirring, followed by centrifugation at $10000 \times g$ for 1 h to remove insoluble material. The urine was filtered through a 0.22 μm bottletop filter (Corning Costar Corp., Cambridge, MA, USA) and applied to the anti-HYAL1 column on an FPLC system (Pharmacia) at 1 ml/min. The column was then washed with 5 ml of 2 M NaCl in 1% Triton X-100, followed by 0.1 M Na acetate pH 5.0, 0.1 M NaCl, 50 mM octylglucoside, and finally with 0.1 M Na citrate, pH 2.7, 0.1 M NaCl, 50 mM octylglucoside. Fractions of 1.5 ml were collected during the washing procedure and assayed for hyaluronidase activity as described above.

2.4. Effect of protease inhibitors on the urinary hyaluronidase

Because of the large volume involved in the above purification, protease inhibitors could not be used. To determine the effects of

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urinary proteases on hyaluronidase in the urine, 50 ml samples of urine were collected directly onto protease inhibitors (Complete protease inhibitor tablets, Boehringer Mannheim, Germany), and concentrated to 2.5 ml on a 8050 Stirred Ultrafiltration Cell (Amicon) fitted with a YM10 membrane. Assays were then performed as described above.

2.5. Sequence analysis of human urinary hyaluronidase

Fractions with hyaluronidase activity were concentrated by precipitation in 100% acetone at −20°C overnight and resuspended in 40 μl Laemmli buffer [17]. The fractions were then separated in a 12% SDS-PAGE gel on a Mighty Small II system (Hoefer Scientific Instruments, San Francisco, CA, USA). After electrophoresis, the bands of protein were excised with a scalpel, extracted, and sequenced by Edman degradation.

2.6. Characterization of urinary hyaluronidase

Affinity-purified hyaluronidase was diluted 1:500 in 0.1 M citrate-phosphate buffer, and activity was measured by the colorimetric and microtiter assays described above between pH 2.7 and 7.0. Activity was measured in the presence and absence of 0.1 M NaCl.

3. Results

3.1. Immunoaffinity purification of human urinary hyaluronidase

Levels of hyaluronidase activity in the pooled unconcentrated urine were approximately 4 TRU/ml with a protein concentration of 70 μg/ml. This equates to a total amount of activity of 8×10^4 TRU in 20 000 ml at a specific activity of 57 TRU/mg protein. This is approximately 100-fold greater than that of human plasma, which has a specific activity of 0.6 TRU/mg. All of the hyaluronidase present in urine was bound to the anti-HYAL1 column. No activity was detected in the 2 M NaCl (fractions 12–15, Fig. 1) or the pH 4.5 wash (fractions 19–22, Fig. 1). All of the activity eluted as a very sharp peak during the subsequent pH 2.3 wash (fraction 28, Fig. 1). 62 000 TRU were recovered in this fraction, which represents a yield of 78%. The SDS-PAGE electrophoresis of column

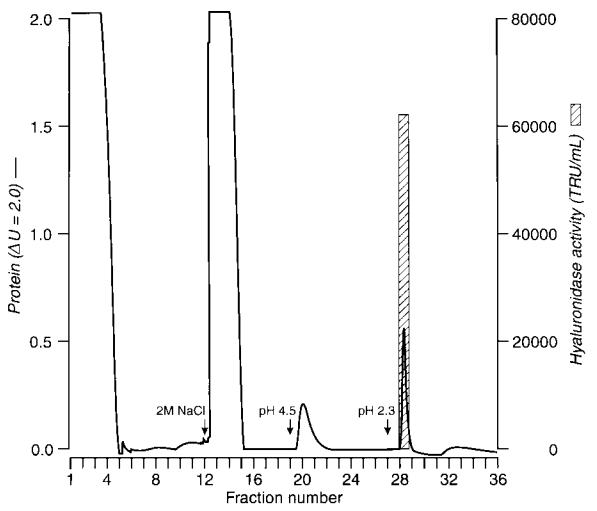

Fig. 1. Monoclonal antibody affinity chromatography of urinary hyaluronidase. Protein is represented by the solid line, scale on the left (Abs.280, ΔU = 2.0). Hyaluronidase activity is represented by the shaded bar, scale on the right. Fractions of 1.5 ml were collected. Fractions 1–4 contain the final 6 ml of urine passing through the column. Beginning at fraction 12 the column was washed with 2 M NaCl, and at fraction 19 with Na acetate pH 4.5. All of the hyaluronidase eluted during the Na citrate pH 2.3 wash in fraction 28.

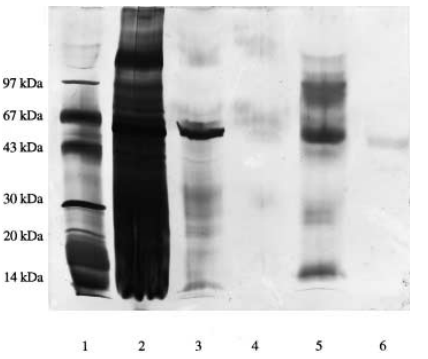

Fig. 2. Silver-stained gel of fractions from the monoclonal affinity chromatography. Lane 1: Bio-Rad low-range molecular weight markers (size on the left); lane 2: 20× concentrated urine; lane 3: 2 M NaCl wash; lane 4: pH 4.5 wash; lane 5: pH 2.3 wash (fraction 28); lane 6: recombinant HYAL1.

fraction 28 demonstrates that urine contains five proteins of 90, 57, 45, 28–30, and 14 kDa that bind to the monoclonal antibody (lane 5, Fig. 2). The 90 kDa protein disappeared at lower concentrations, or after reduction with 5% β-mercaptoethanol, and was probably a dimer of the 45 kDa activity (not shown). The size of the other proteins did not change after reduction. Only the 90, 57, and 45 kDa proteins possessed hyaluronidase activity on substrate gel zymography (Fig. 3). The pattern of activity closely resembled that previously reported for rat liver lysosomal hyaluronidase, which consists of a doublet of activity on HA zymograms [18]. The smaller proteins of 28–30 and 14 kDa did not have hyaluronidase activity on substrate gel zymography. These proteins are not found in human plasma and their function is unknown.

3.2. Sequence analysis of urinary hyaluronidase

The amino acid sequences obtained from the 57 kDa and 45 kDa isozymes can be seen in Table 1. Sequence A from both isozymes is identical to that obtained from human plasma hyaluronidase [6]. The sequences did not have significant homology to PH-20 because HYAL1 and PH-20 are not homologous at their N-termini [6]. The lower molecular weight isozyme contains a second sequence (sequence B) that is derived from the last 25 amino acids of HYAL1 before the C-terminus. This suggests that an internal cleavage had produced a smaller isozyme with two N-termini. A hypothetical diagrammatic representation of this process can be seen in Fig. 4. The 25 amino acid fragment may be linked to the rest of the protein by disulfide bonds between the cysteine residues of the fragment and other parts of the protein (Fig. 4).

Table 1
Amino acid sequences of the 57 kDa and 45 kDa isozymes

Isozyme (mol. wt.)	N-terminal amino acid sequence
57 kDa	Sequence A: FXGPLLPNRPFTTVWNXNTQW
45 kDa	Sequence A: FXGPLLPNXPFTTVWNA
	Sequence B: VEFKXRXYPGWQAPXXERK

Amino acid sequences were obtained by Edman degradation of gel-purified urinary hyaluronidase. Only one sequence was obtained from the 57 kDa isozyme, but two different sequences were obtained from the smaller 45 kDa isozyme. Sequence B from the smaller isozyme is derived from the last 25 amino acids of HYAL1 before the C-terminus.

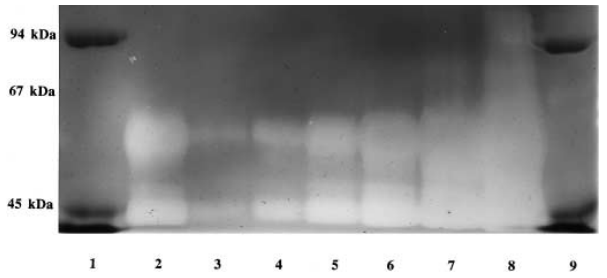

Fig. 3. Substrate gel zymography of immunoaffinity purified urine hyaluronidase. Molecular weight markers are in lanes 1 and 9 (Bio-Rad low range). 20× concentrated urine, collected over protease inhibitors, and diluted 5× in Laemmli buffer is in lane 2. Two clearings of hyaluronidase activity can be seen at 57 and 45 kDa. An aliquot of column fraction 28 was diluted in Laemmli buffer at the following concentrations: lane 3: 1/320; 4: 1/160; 5: 1/80; 6: 1/40; 7: 1/20; 8: 1/10. 20 μl was applied per lane. Two clearings of hyaluronidase activity can be seen at 57 and 45 kDa. At the very high concentration in lane 8, an additional band at 90 kDa can be seen.

3.3. *Characterization of human urinary hyaluronidase*

In the absence of NaCl, the urinary hyaluronidase was found to have a pH optimum of 3.4. The presence of 0.1 M NaCl caused an increase in the pH optimum to pH 3.8, and a decrease in overall activity. No activity was detected above pH 4.6 (Fig. 5).

3.4. *Effect of protease inhibitors on the urinary hyaluronidase*

Protease inhibitors did not change the characteristics of the urine hyaluronidase, including the appearance of the two isozymes of activity on substrate gel zymography (Fig. 3, lane 2). This indicates that the larger isoform was not cleaved by non-specific proteases in the urine, and was likely to have been processed prior to voiding, probably in the kidney.

4. Discussion

Human urine is known to contain significant levels of hyaluronidase at high specific activity, 100-fold that of plasma [7,8]. However, the nature of the protein bearing the activity has been unknown. As an initial step in investigating the hypothesis that hyaluronidase plays a role in antidiuresis [9–12], we wished to determine the relationship of urinary hyaluronidase to plasma hyaluronidase. Using a monoclonal antibody prepared against human plasma hyaluronidase [6], we were able to purify all hyaluronidase activity from human urine, indicating that all the hyaluronidase activity in urine is similar to the plasma enzyme. N-terminal microsequencing confirmed that human urinary hyaluronidase was identical to the plasma hyaluronidase, HYAL1. Unlike plasma, however, urine contains two isozymes of hyaluronidase, with molecular weights of 57 and 45 kDa. The lower molecular weight isozyme contains a second N-terminal sequence that was derived from the C-terminal 25 amino acids of the protein. This suggests that an internal cleavage had occurred that had created a second N-terminus. Because the lower molecular weight isozyme was 12 kDa smaller than the larger form, it would appear that approximately 100 internal amino acids had been removed by this cleavage reaction. However, the cleavage was not prevented if the urine was collected directly onto protease inhibitors (Fig. 3, lane 2), and we could detect no endopeptidase in human urine that was capable of reducing the plasma hyaluronidase from 57 to 45 kDa (data not shown). The processing, therefore, was likely to have occurred in the kidney prior to voiding. Rat liver lysosomes contain two isoforms of hyaluronidase [18], so the lower molecular weight isozyme of urinary hyaluronidase may be of lysosomal origin. Sperm contains two isozymes of the PH-20 hyaluronidase. The lower molecular weight isozyme of PH-20 is also believed to be a proteolytically processed form of the larger isozyme [4,19]. The two hyaluronidases PH-20 and HYAL1 may undergo similar proteolytic processing. This processing reaction may be similar to that described for a human chitotriosidase that has recently been identified [20]. The chitotriosidase is synthesized as a 50 kDa enzymatically active isozyme that is predominately secreted, whereas lysosomes contain a 39 kDa proteolytically processed isozyme [20]. There is structural similarity between chitin (poly β-*N*-acetylglucosamine) and HA (poly β-*N*-acetylglucosamine and β-glucuronic acid disaccharide) [21]. The similarity between the substrates catabolized by the two enzymes supports the hypothesis that the two enzymes may be analogously processed. However, the

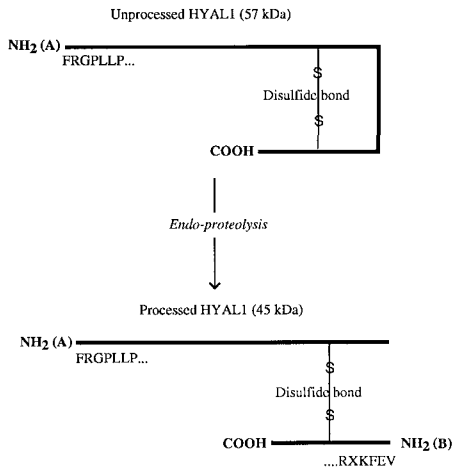

Fig. 4. Diagrammatic representation of the putative endoproteolytic processing of HYAL1. Unprocessed HYAL1 is shown in the upper figure. After endoproteolytic processing, two fragments are generated that produce two separate N-termini. The 25 amino acid fragment is presumably linked to the rest of the protein by disulfide bonds.

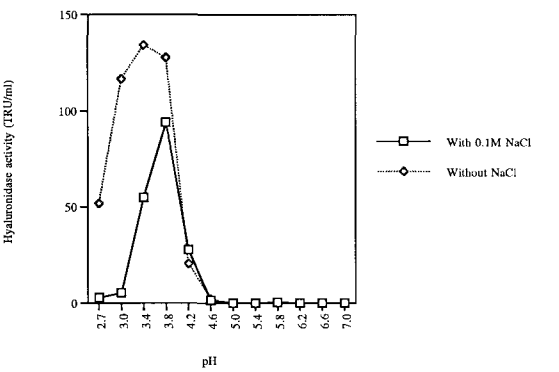

Fig. 5. pH activity curve of urinary hyaluronidase using the microtiter assay. Buffer was 0.1 M citrate-phosphate adjusted to the indicated pH, with 1% Triton X-100. Maximal activity is at pH 3.8 in the presence of 0.1 M NaCl, and pH 3.4 in the absence of NaCl. No activity was detected above pH 4.6. Similar results were obtained with the colorimetric assay.

identity of the protease(s) that carries out this reaction is currently unknown.

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