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PURIFICATION OF GELATIN-SPECIFIC NEUTRAL PROTEASE FROM HUMAN SKIN BY CONVENTIONAL AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Human skin, maintained in serum-free organ culture, secretes a neutral metalloendopeptidase which is remarkably specific for gelatin. Because the product peptides from the action of collagenase on collagen become denatured into random coil polypeptides of 25000 and 75000 daltons at physiological temperature, it is thought that this "gelatinase" is the second, and possibly the only other enzyme in the pathway of extracellular collagen degradation.

New types of high-performance liquid chromatography (HPLC) columns have enabled us to improve the yields of active gelatinase from skin culture medium. Raw medium, which has been dialyzed and lyophilized, is fractionated with ammonium sulfate, and applied to Pharmacia Blue Sepharose in a batch step. The 0.4 *M* sodium chloride eluate is then subjected to gel filtration on Sephacryl S-200, followed by gradient elution from Amicon Green Sepharose. The fractions with gelatinolytic activity are applied to a Bio-Rad TSK-Phenyl-5PW HPLC column for mild hydrophobic chromatography with a gradient of decreasing ammonium sulfate concentration. In the final step, the enzyme is applied to a Pharmacia Mono-Q FPLC column and eluted with a gradient of sodium chloride. At this point, the enzyme appears as two bands, corresponding to enzymatic activity zymograms on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

INTRODUCTION

The complete degradation of collagen during connective tissue remodelling requires the action of at least two extracellular neutral metalloproteases. Collagenase catalyzes the initial cleavage of the helical collagen molecule at a locus 3/4 of the distance from the N-terminus[1]. The products of this cleavage spontaneously lose their helical conformation *in vitro* at physiological temperature (37°C) and are denatured to gelatin[2]. Gelatin is a poor substrate for collagenase[3,4] and must be broken down further by other proteases. We have reported the purification and some properties of a neutral metalloendopeptidase, secreted by human skin in serum-free organ

of the fact that this human skin gelatinase hydrolyzes gelatin into peptides of less than 5000 daltons, we have proposed that it is the major enzyme required for post-collagenase collagen degradation.

Since human skin gelatinase, purified by the methods published previously[5], loses proteolytic activity rapidly upon storage, we have revised the purification scheme in an effort to improve yields and maintain stability. The availability of high-performance liquid chromatography (HPLC) columns designed for purification of macromolecules which retain biological activity has enabled us to increase specific activity. The combined use of Pharmacia's Mono-Q ion-exchange column and Bio-Rad's TSK-Phenyl-5PW hydrophobic interaction column permit rapid and efficient means of semi-preparative separation of human skin gelatinase from organ culture medium.

EXPERIMENTAL

Culture methods

Normal human skin, obtained at surgery, was prepared for organ culture in serum-free medium, as previously reported[6]. All skin was treated for 20 min with 100 μg/ml of Amphotericin B, and cultured in the presence of 250 units of penicillin and 250 μg/ml of streptomycin. The medium was changed daily, and harvests of culture medium from days 3–7 were pooled, made 0.05 *M* in Tris (pH 7.5), and stored at −20°C.

Assay methods

Gelatinase was assayed as described previously[5]. Briefly, 25 μl native reconstituted [^{14}C]glycine-labeled collagen were denatured to gelatin by heating to 60°C for 20 min. Enzyme solution (75 μl) was added, and after incubation at 37°C, the reaction was terminated by adding 25 μl of 100% trichloroacetic acid. Extent of proteolysis was estimated by scintillation counting of the peptides soluble in 20% trichloroacetic acid.

Protein concentration was determined spectrophotometrically by the method of Groves *et al.*[7], using bovine serum albumin to establish a standard curve.

Electrophoretic techniques

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[8], using 10% gels. Proteins were visualized either by staining with Coomassie Blue, or by autoradiography after the protein mixture was iodinated with the Bolton–Hunter reagent[9].

Zymograms demonstrating gelatinolytic activity were obtained from SDS-polyacrylamide gels by Hori *et al.*'s[10] modification of the method of Heussen and Dowdle[11]. In this procedure, a 10% gel was cast, incorporating 1% gelatin. The enzyme solution was gently treated with SDS (room temperature for 30 min) before slow electrophoresis (6 mA) at 8°C. At the completion of the run, SDS was extracted from the gel with successive washes of 2.5% Triton X-100. The gel was then incubated at 37°C in 0.1 *M* glycine buffer, (pH 8.3), containing 10 m*M* calcium chloride and 1 m*M* zinc chloride, for 3–4 h. The reaction was stopped by incubating the gel for

15 min at room temperature in 50% trichloroacetic acid. After being thoroughly washed with water, the gel was stained with Coomassie Blue. Gelatinolytic activity was visualized as clear bands against the blue background.

Open column chromatography

Conventional chromatography was performed in jacketed columns, maintained at 4°C. Flow-rates were regulated with a Gilson peristaltic pump. The effluent was monitored with a Gilson MUVRP recording spectrophotometer and timed fractions were collected in an LKB Ultrorac collector. Sephacryl S-200 and Blue Sepharose are products of Pharmacia. Matrex Green A was obtained from Amicon.

HPLC

HPLC was performed on a Varian 5000 LC single-pump apparatus, with a central microprocessor. Column effluents were monitored with a Varian Vari-Chrom UV–VIS spectrophotometer, connected to a Fisher Recordall Series 5000 recorder. Columns used were a Mono-Q HR5/5 (50 × 5 mm) (Pharmacia) for ion exchange, and a TSK-Phenyl-5PW (75 × 7.5 mm) (Bio-Rad) for hydrophobic interaction chromatography. In lieu of guard columns, all solutions were passed through a 0.45-μm pre-column filter (Upchurch). All buffers were prepared with HPLC quality solvents and passed through 0.2-μm filters.

RESULTS

Initial preparation of medium

Serum-free medium from explant culture of human skin was concentrated by precipitation with ammonium sulfate to 80% of saturation. The precipitate was dialyzed against 5 m*M* Tris, (pH 7.5), and lyophilized. In the typical purification procedure described below, about 4 g of powder containing about 2100 mg protein, obtained from approximately 20 l of medium, was used as starting material. This powder was dissolved in 250 ml 0.05 *M* Tris (pH 7.5) containing 0.01 *M* calcium chloride, and further fractionated with ammonium sulfate. The material precipitating between 25 and 60% of saturation was redissolved in Buffer A (0.05 *M* Tris, pH 7.5, containing 0.005 *M* calcium chloride, 0.04 *M* sodium chloride, and 10% glycerol), and dialyzed against 1200 volumes of the same buffer. The enzyme solution was then applied to a 300 ml (20 × 5 cm) column of blue Sepharose which had been equilibrated with Buffer A. Virtually all of the enzyme activity and about 15% of the applied protein was eluted in a single step with buffer A containing 0.4 *M* sodium chloride. The eluate was dialyzed overnight *vs.* 16 l of 0.025 *M* Tris (pH 7.5), 0.005 *M* calcium chloride, and lyophilized.

Gel filtration

The Blue Sepharose eluate, containing 100-150 mg protein, was applied to a 95 × 2.6 cm column of Sephacryl S-200, equilibrated with Buffer A, containing 0.2 *M* sodium chloride. The sample was applied in a volume of 25 ml, and fractions were collected at a flow-rate of 17 ml/h into tubes containing 1/8 volume of 60% glycerol. Fractions were assayed for gelatinase activity, and those containing the highest activity were pooled, as shown in Fig. 1.

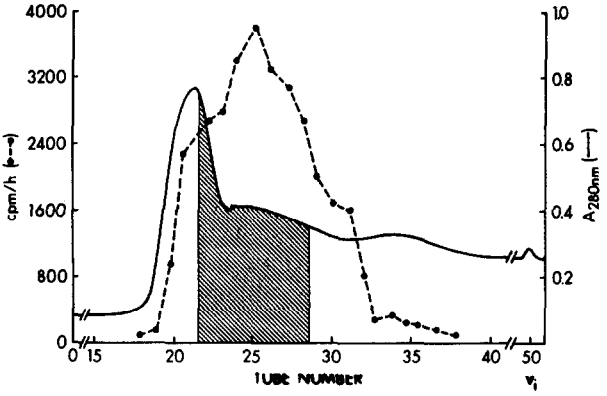

Fig. 1. Sephacryl S-200 chromatogram of Blue Sepharose eluate, containing human skin gelatinase. 75 μl of each 9.5 ml fraction was incubated with 25 μl of [^{14}C]gelatin (2000 cpm) for 30 min. The hatched area indicates the portion of the gel filtration eluate that was pooled for chromatography on Matrex Green A.

Green Sepharose chromatography

The gelatinase peak from S-200 was dialyzed *vs.* Buffer A and applied at a flow-rate of 50 ml/h to a 8 × 1.6 cm column of Matrex Green A, equilibrated with the same buffer. The column was washed with Buffer A, then developed with a 150-ml gradient of the same buffer with increasing sodium chloride concentration from 0.04 to 0.6 *M*. Fractions were assayed for gelatinase activity, and those containing the highest activity were pooled for HPLC, as shown in Fig. 2.

High-performance ion-exchange chromatography, first column

The gelatinase peak from Green Sepharose chromatography, which contained

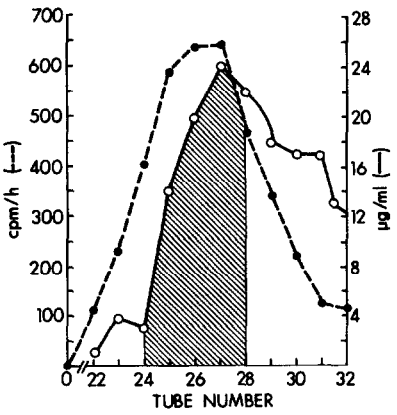

Fig. 2. A typical Matrex Green A chromatogram of the gelatinase peak from Sephacryl S-200. The peak from gel filtration was diluted to a final NaCl concentration of 0.04 *M* and applied to an 8-ml column of Matrex Green A at a flow-rate of 50 ml/h. The column was washed with 0.05 *M* Tris (pH 7.5), containing 0.005 *M* $CaCl_2$, 10% glycerol, and 0.04 *M* NaCl. A linear gradient, 150 ml total, was run from 0.04 to 0.6 *M* NaCl. 75 μl of each 6 ml fraction was assayed, and the tubes containing the highest gelatinase activity were pooled, as indicated by the hatch marks on the partial chromatogram shown above. O–O, A_{230}.

15 mg protein, was applied in two identical runs to a Mono-Q column using the following buffer system: Buffer 1 consisted of 0.01 *M* Tris (pH 8.4), 0.005 *M* calcium chloride, 20% glycerol, and 0.03% Brij 35. Buffer 2 was identical, but with the addition of 1 *M* sodium chloride.

The sample was diluted five-fold with Buffer 1 before pumping onto the column at 1 ml/min in the chromatogram shown in Fig. 3. (Usually for Mono-Q chromatography the sample was equilibrated with Buffer 1 by buffer exchange on Sephadex G-25.) The column was washed thoroughly with Buffer 1 before beginning the program shown in Fig. 3.

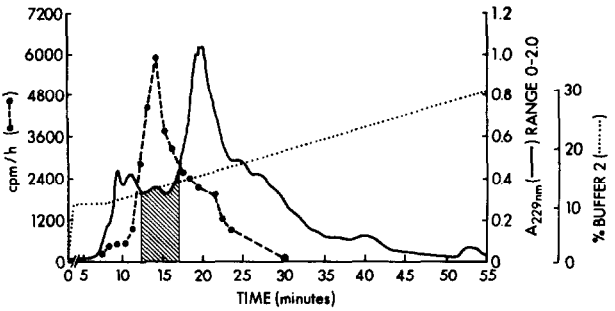

Fig. 3. High-performance ion-exchange chromatogram of human skin gelatinase on Mono-Q. The gelatinase pool from Matrex Green A chromatography was diluted five-fold in Buffer 1 and pumped through the column at 1 ml/min. After washing, a 67-min gradient program at a flow-rate of 1 ml/min, utilizing several steps, was initiated. Fractions (1 ml) were collected. The steps are indicated by the dotted line on the chromatogram. The enzyme peak was eluted at approximately 14% Buffer 2 (0.14 *M* NaCl), and the tubes in the hatched area were pooled for further purification.

As shown in Fig. 3, the main peak of gelatinase activity emerged before the main protein peak. Although there was a shoulder of enzymatic activity within the large protein peak, only the main peak, representing 32% of the applied activity, was pooled for the next purification step. Total recovered protein was 1.3 mg.

High-performance hydrophobic interaction chromatography

Ammonium sulfate, specially prepared for HPLC (Bio-Rad), was added to the gelatinase peak to a final concentration of 0.75 *M* and pumped at 1 ml/min through a TSK-Phenyl-5PW column which had been equilibrated with 0.1 *M* Tris (pH 7.5), 0.005 *M* calcium chloride, 0.75 *M* ammonium sulfate, 10% glycerol, and 0.007% Brij-35. Since serum albumin is a major contaminant of gelatinase preparations, the column was immersed in an ice bath, as described by Goheen and Engelhorn[12], who have shown that lowering the temperature decreases the width of the serum albumin elution peak. A linear gradient was run for 55 min, and 1-ml fractions were collected for enzyme assay. The peak of activity, shown in Fig. 4, contained 158 μg of protein, and 50% of the applied activity.

High-performance ion-exchange chromatography, second column

The gelatinase peak from the preceding column was reapplied to Mono-Q, using the same buffers 1 and 2, but with a slightly altered program, as shown in Fig.

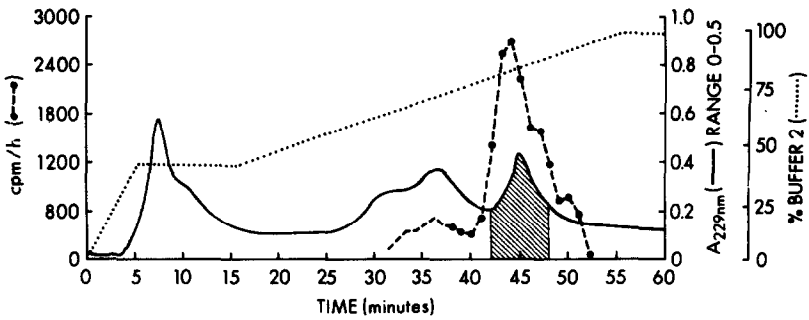

Fig. 4. High-performance hydrophobic-interaction chromatogram of gelatinase on TSK-Phenyl-5PW. The Mono-Q peak was made 0.75 *M* in $(NH_4)_2SO_4$ and diluted two-fold with Buffer 1, before being pumped through the column at a flow-rate of 1 ml/min. A 55-min program was used with several steps to attain 100% Buffer 2, as indicated by the dotted line on the above chromatogram. The column was kept immersed in an ice bath during the program. The enzyme peak was eluted at approximately 85% Buffer 2, and the tubes in the hatched area were pooled for further purification.

5. The gelatinase peak contained 31 μg of protein, and represented *ca.* 50% of the applied activity. The specific activity of the enzyme in this final peak was 2800 cpm/h/μg protein, representing a 2400-fold purification (Table I).

On PAGE, most of the protein in the gelatinase peak from the second Mono-Q column is present as two bands (Fig. 6). In Fig. 6, iodinated protein and untreated enzyme were run on the same gelatin gel, which was subsequently divided in half for processing. Both of the iodinated protein bands corresponded exactly to bands of gelatinolytic activity, shown on the gelatin zymogram half of the gel. When the gelatin impregnated gel was incubated in the presence of 50 m*M* EDTA, neither band of gelatinolytic activity appeared, indicating that the proteases in question are metalloenzymes.

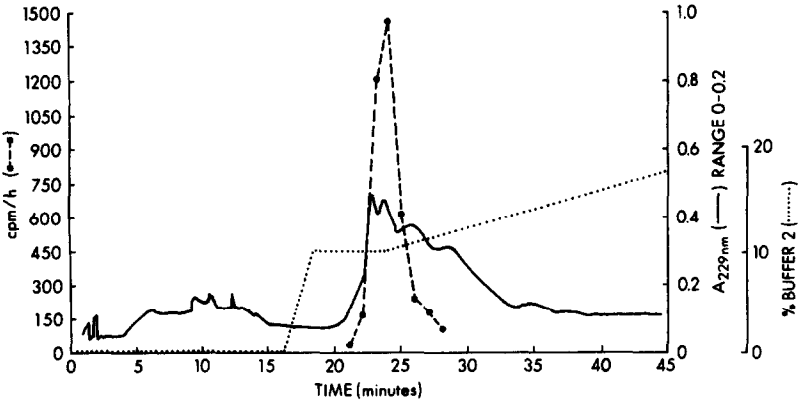

Fig. 5. Second high-performance ion-exchange chromatogram of gelatinase on Mono-Q. The peak of gelatinolytic activity from TSK-Phenyl-5PW was equilibrated with Buffer 1 on Sephadex G-25 and pumped onto the Mono-Q column at 1 ml/min. When the gradient, indicated by the dotted lines, was run at 1 ml/min, the enzyme was eluted at approximately 10% Buffer 2. One-minute fractions were collected. Buffers 1 and 2 were the same as described in Fig. 3.

TABLE I

PURIFICATION OF HUMAN SKIN GELATINASE

Source	*Total activity (cpm/h)*	*Protein (mg)*	*Specific activity (cpm/μg protein/h)*	*Purification (fold)*	*Recovery (%)*
(1) Organ culture medium which has been precipitated with ammonium sulfate (80% of saturation), dialyzed and lyophilized	2450000	2094	1.17	–	100
(2) Second ammonium sulfate fractionation	2768000	1030	2.7	2.3	≈100
(3) Blue Sepharose eluate	2643000	152	17.7	15	≈100
(4) Gelatinase Peak from S-200	1750000	43	40	34	71
(5) Gelatinase peak from Matrex Green A	1070000	15	71	61	44
(6) Gelatinase peak from first Mono-Q	362300	1.3	280	240	15
(7) Gelatinase peak from TSK-Phenyl-5PW	180000	0.158	1135	970	7.3
(8) Gelatinase peak from second Mono-Q	86800	0.031	2800	2400	3.5

DISCUSSION

We have found that there is a good deal of biological variability in organ cultures of human skin from different healthy individuals, both in terms of the quantity of gelatinase secreted and of contaminating proteins. Thus, semi-preparative HPLC must be preceded by analytical chromatograms in which only the minimum amount of protein necessary to detect enzymatic activity is used. Slight changes in the program have often been necessary because of the relative overabundance of a contaminating protein. Furthermore, because human skin gelatinase is labile in dilute solution, even in the presence of 20% glycerol and detergent, optimization must be limited in any one preparation. Therefore, with each enzyme preparation, new modifications are made in order to improve the yield and specific activity.

Conventional chromatographic procedures previously used to purify human skin gelatinase[5] showed a diffuse although apparently homogeneous protein band on both SDS and non-denaturing gel electrophoresis. The specific activity of these preparations obtained by HPLC is at least five fold higher than that obtained formerly, suggesting that further purification has been attained. Following HPLC, we now obtain two gelatinolytic protein bands, which differ in molecular weight by only about 7000 daltons. It is of interest that pure human skin fibroblast procollagenase[13] and rabbit synovial collagenase[14] are also secreted as two closely related species differing in size by only about 5000 daltons. To date, we have not been successful in separating the two species of gelatinase chromatographically, although efforts to accomplish this are in progress. In order to ascertain whether these two bands of gelatinase activity are closely related, we plan to elute the individual bands from PAGE for both chemical and immunological analysis.

Because human skin gelatinase and collagenase seem to be regulated coordi-

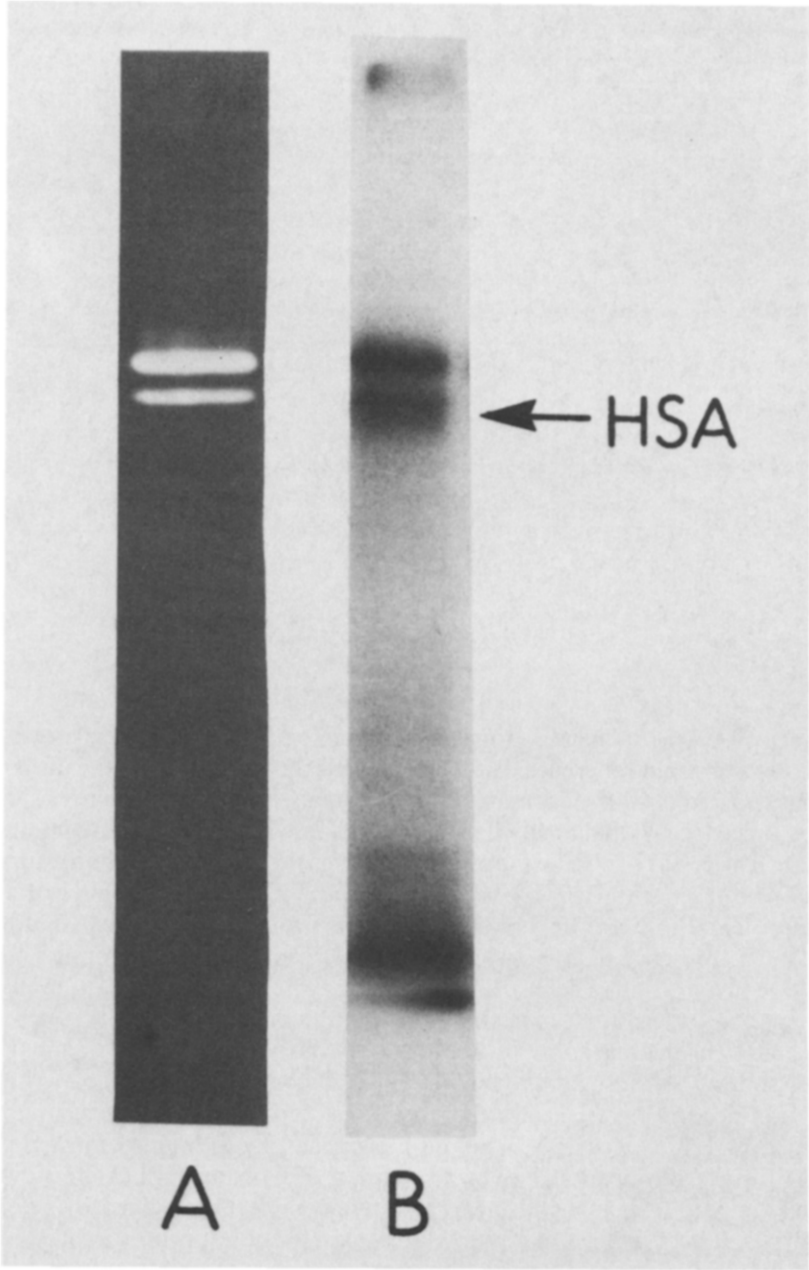

Fig. 6. SDS-PAGE, demonstrating that gelatinolytic activity coincides with major protein bands. An aliquot of the gelatinase peak from the second Mono-Q column was incubated with SDS for 30 min at room temperature before electrophoresis at 6 mA for 18 h at 8°C on a 10% acrylamide gel, which was copolymerized with gelatin. In a separate lane on the same gel, another aliquot was iodinated before electrophoresis. After electrophoresis was complete, the iodinated lane was processed for radioautography, while the corresponding lane was incubated to indicate enzyme activity, as described in the Experimental section. Clear areas indicate regions where gelatin has been hydrolyzed to small peptides by enzymatic activity which can be inhibited by EDTA. Lane A, zymogram indicating gelatinolytic activity. Lane B, autoradiogram of PAGE of final gelatinase peak. HSA = human serum albumin.

nately in cases thus far investigated[15,16] it will be most interesting to determine whether gelatinase, like collagenase[17], is produced as two closely related forms which differ only slightly in molecular weight.

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