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Purification and characterization of human 92-kDa type IV collagenase (gelatinase B)

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Abbreviation: MMP, matrix metalloproteinase

Abstract

Human 92-kDa type IV collagenase (gelatinase B), a family of matrix metalloproteinases (MMP), play important roles in the degradation of the basement membrane and the migration of leukocytes and metastatic tumor cells during inflammation and invasion. To investigate the biochemical and enzymatic characteristics of human neutrophil type IV collagenase, the enzyme was extracted from human leukocytes and purified by a combination of Ultrogel AcA 54 and Bio-Rex 70 chromatographies. The purified enzyme showed a single band of molecular weight of 92 kDa on SDS-PAGE. Human neutrophil type IV collagenase degraded gelatin by cleaving the specific sites, but did not affect intact type I collagen. Human 92-kDa type IV collagenase activity was inhibited by EGTA, EDTA and tetracycline. Tetracycline showed the strongest inhibitory effect on the gelatinolytic activity of the 92-kDa type IV collagenase. These inhibitory effects may be due to the chelation effect of these agents since 92-kDa type IV collagenase is a metallo-enzyme.

Keywords: human neutrophil, type IV collagenase, tetracycline

Introduction

The matrix metalloproteinases (MMP) are a family of enzymes that are believed to play a leading role in both the normal turnover and pathological destruction of the extracellular matrix (Woessner, 1991). Currently, the human MMP family can be grouped into four categories that consist of interstitial collagenases, gelatinases or type IV collagenases, stromelysins and putative metalloproteinases (PUMP) on the basis of the similarities in their protein substrate specificities and the domain organizations of their polypeptide chains (Netzel-Arnett *et al*., 1993).

In 1972 Harris and Krane described a metalloendopeptidase from human synovium explants that could degrade denatured interstitial collagens. Since its description by Sopata and Dancewicz (Sopata and Dancewicz,1974), human neutrophil gelatinase has been examined by several investigators (Murphy *et al*., 1980, 1982), and Hibbs *et al*. (1985) described 92-kDa type IV collagenase from human neutrophils in 1985.

Because of difficulties in isolating the enzyme in sufficient quantity as pure form, very little knowledge has been accumulated on their detailed biochemistry (Docherty *et al*., 1990).

In 1985 Hibbs *et al*. isolated and purified neutrophil gelatinase by inducing neutrophils to secrete this enzyme in short-term culture. However, the yield was extremely low: From the starting material of 1.5 g proteins, they obtained only 2.85 mg of purified enzyme (0.19% yield).

Tetracycline inhibition of neutrophil-associated collagenolysis has been the focus of a number of investigations (Gabler *et al*., 1991). Gloub and associates (Gloub *et al*., 1983, 1984, 1985, 1987) have provided evidence that tetracyclines can inhibit collagenase activity *in vitro* and *in vivo*, by binding to Ca^{2+} and Zn^{2+}. Zucker *et al*. (1989) described that tetracycline inhibits human 72-kDa type IV collagenase isolated from human small cell lung cancer.

In this report we described a simplified method of isolation of human 92-kDa type IV collagenase from human leukocytes and characterization of this enzyme.

Materials and Methods

Materials

Materials used in this work were obtained from the following sources: sodium acetate, aminophenylmercuric acetate (APMA), bovine serum albumin, EDTA, EGTA, tetracycline, Brij 35, N-succinyl-ala-ala-ala-p-nitroanilide (SANA), N-succinyl-ala-ala-pro-phe-p-nitroanilide (SAPNA), Tris base, and calcium chloride from Sigma Chemical Co., St. Louis, MO. U.S.A.; high molecular weight and low molecular weight electrophoresis calibration kits from Pharmacia, Brussels, Belgium; Ultrogel AcA 54 from LKB,Uppsala, Sweden; Bio-Rex 70, and all reagents for SDS-PAGE from BioRad, Hercules, CA, U.S.A.; Spectrapor dialysis membane from Spectramedical Co, Houston, TX. U.S.A.; PM 10 concentration membrane from Amicon

Co., Beverly, MA, U.S.A.

Preparation of leukocytes

Human leukocytes were prepared from fresh blood or from donor blood stored overnight at 4-10˚C (Kosin Medical Center, Pusan, Korea). Human leukocytes were purified by the method of Kang *et al*. (1987). Whole blood was centrifuged at 200 *g* for 30 min at 22˚C. Single buffy coat fractions were carefully collected with Pasteur pipette and collected fraction was centrifuged at 300 *g* for 25 min at 22˚C. The leukocytes were isolated from the cell pellets and suspended in a solution of 0.05 M Tris-HCl, pH 7.4, containing 0.2 M NaCl, 5 mM $CaCl_2$ and washed three times with the same buffer. Leukocytes were homogenized in 0.5 M Tris-HCl, pH 7.4, containing 2 M NaCl, 0.05 M $CaCl_2$ by Polytron (Kinematica CH-6010, Brinkman Co., Kriens-Luzern, Switzerland) and centrifuged at 32,000 *g* for 60 min at 4˚C. Supernatant was collected and used for further purification.

Gel filtration

Aliquots of the crude extract were chromatographed at 4˚C through a 2.5 × 100 cm column of Ultrogel AcA 54 equilibrated with 50 mM Tris-HCl buffer, pH 7.3, containing 0.15 M NaCl, 5 mM $CaCl_2$ and 0.1% Brij 35. Fractions were assayed for protein and gelatinase. The fractions demonstrating gelatinolytic activity were pooled and diluted 2 fold with 50 mM sodium acetate containing 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and 0.1% Brij 35 for ion exchange chromatography.

Ion exchange chromatography

Aliquots of sample purified by gel filtration were applied to a column(3.0×27 cm) of Bio-Rex 70 which had been previously equilibrated in the 50 mM sodium acetate containing 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and 0.1% Brij 35. The column was washed with the starting buffer until the absorbance at 280 nm reached base-line, and the bound fraction was eluted with a linear gradient of sodium acetate from 0.05 M to 0.7 M. The fractions were assayed for gelatinolytic activity. The fractions containing gelatinolytic activity were pooled and concentrated to one-fourteenth of the original volume by ultrafiltration system with PM 10 membrane. The concentrate was dialyzed against 50 mM sodium acetate containing 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and 0.1% Brij 35.

Gel electrophoresis

SDS-PAGE was performed using a slab gel apparatus (Hoefer) according to the method of Laemmli (Laemmli, 1970). The gels were stained in 0.1% Coomassie Brilliant Blue in 25% methanol, 10% acetic acid and destained in 25% methanol, 10% acetic acid.

Enzyme assay

The enzyme was activated by incubation with 3 mM of p-aminophenylmercuric acetate(APMA) at 37˚C for 1 h. Denatured type I collagen was incubated with the activated enzyme for 17 h at 37˚C. Enzyme activity was identified by SDS-PAGE.

Inhibition of 92-kDa gelatinase activity

The activated enzyme was preincubated with 20 mM EGTA, 20 mM EDTA and 20 mM tetracycline, respectively, for 30 min at 37˚C. Denatured type I collagen were incubated with the preincubated enzyme in the presence of 20 mM EGTA, 20 mM EDTA and 20 mM tetracycline, respectively, for 17 h at 37˚C. Reaction products were separated on 10% poly-acrylamide gel and enzyme activity was identified.

Results

Purification of 92-kDa type IV collagenase by Ultrogel AcA 54 chromatography

Ultrogel AcA 54 gel filtration provided a satisfactory method for the initial separation of the gelatinase from majority of other proteins (Figure 1). The proteins appeared as several peaks and the fractions 42-48 contained gelatinolytic activity (Figure 2). The fraction 51-60 contained a trace of gelatinolytic activity but it also contained elastinolytic activity. The fractions 27-39 contained hemoglobin and other macromolecules which were contained in leukocyte extract. The fractions 60-63 contained elastolytic activity and the fractions 66-69 contained cathepsin G.

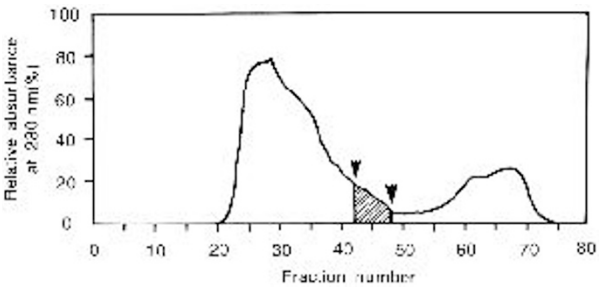

Figure 1. Purification of 92-kDa type IV collagenase by Ultrogel AcA 54 chromatography. The column (2.5 x 100 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.3, containing 0.15 M of NaCl, 5 mM $CaCl_2$ and0.1% Brij 35. The sample from leukocyte extract was applied and 8 ml of fractions were collected at a flow rate of 8 ml/15 min. All chromatographic procedures were performed at 4˚C.— : protein profile.

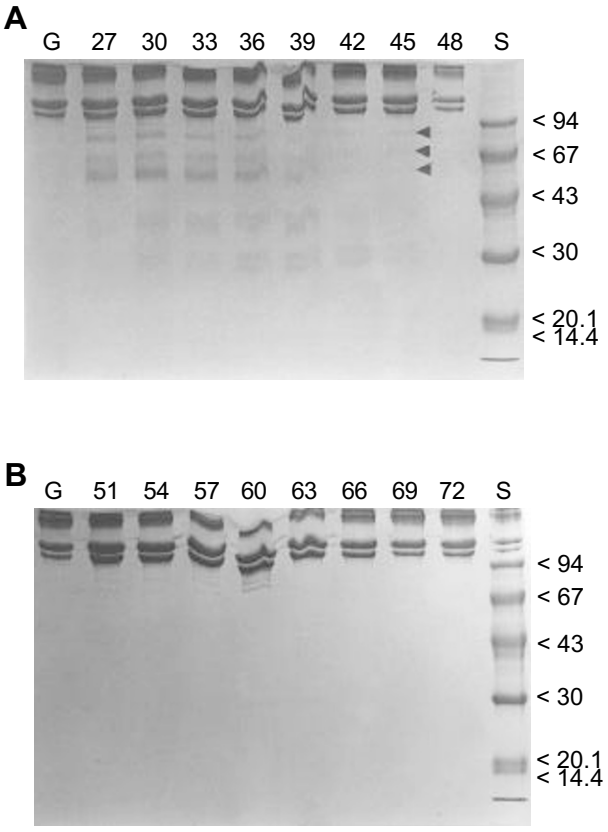

Figure 2. SDS-PAGE of fractions isolated by Ultrogel AcA 54 chromatography. Lane G: denatured type I collagen; lanes of the fraction 27-72: 45 μg of denatured type I collagen were incubated with 70 μl of aliquots of sample isolated by Ultrogel AcA 54 chromatography for 17 h at 25˚C. Reaction products were separated on 10% polyacrylamide gel (panels A and B). The positions of degradation products of collagen are marked. Lane S, molecular mass markers were run on a separate tract of the same gel and are indicated.

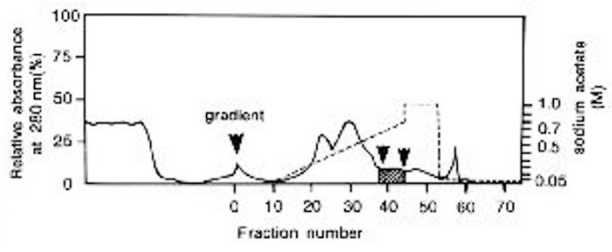

Figure 3. Purification of 92-kDa type IV collagenase by Bio-Rex 70 ion exchange chromatography. The column (2.5 x 100 cm) was equilibrated with 50 mM sodium acetate containing 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and0.1 % Brij 35. After sample (the gelatinolytic enzyme partially purified by gel chromatography on Ultrogel AcA 54) was applied, the column was washed with the same buffer until the absorbance at 280 nm reached base-line. Elution was performed at pH 7.4 with linear gradient of sodium acetate from 0.05 M to 0.7 M. 7 ml fractions were collected at a flow rate of 7 ml/ 5 min.

Purification of 92-kDa type IV collagenase by Bio-Rex 70 ion exchange chromatography

Gelatinolytic activity was further purified by Bio-Rex 70 ion exchange chromatography. From this chromatography, several different proteins were separated (Figure 3). The gelatinolytic activity was seen in the fraction number 32-44 (Figure 4), at sodium acetate concentration between 0.5 M and 0.7 M. The fractions 38-44 were pooled, concentrated and dialyzed against 50 mM sodium acetate containing 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and 0.1% Brij 35. The volume and protein concentration of the dialysate was 3.5 ml and 1.1 mg/ml, respectively.

Molecular weight determination and gelatinolytic activity of purified enzyme on the SDS-PAGE

SDS-PAGE of the purified enzyme and degradation

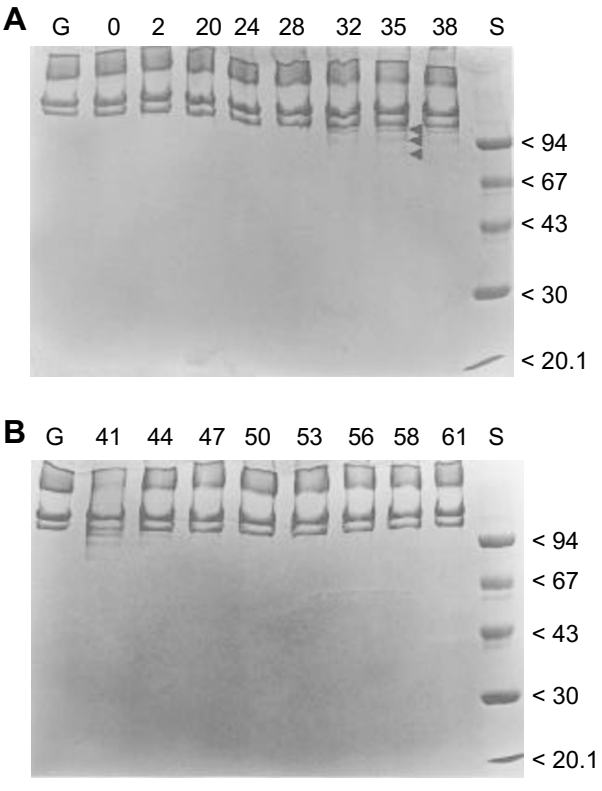

Figure 4. Enzyme activity of fractions isolated by Bio-Rex 70 ion exchange chromatography. Lane G: denatured type I collagen; lanes of the fraction 0-61: 73.5 μg of denatured type I collagen were incubated with 70 μl of aliquots of sample isolated by Bio-Rex 70 ion exchange chromatography for 17 h at 37˚C. Reaction products were separated on 10% SDS-polyacrylamide gel (panels A and B). The positions of degradation products were marked. Lane S, molecular mass markers were run on a separate tract of the same gel and are indicated.

product was carried out and the results are shown in Figure 5. The purified gelatinase showed a single band with an apparent molecular weight of 92-kDa (lane 2). Lane 3 showed that denatured type I collagens (gelatins) with molecular weight of 124,000 and 112,000 and the fragments after hydrolysis by gelatinase with molecular weight of 100,000, 94,000, 92,000 and 60,000.

Inhibition of 92-kDa gelatinase activity

As shown in Figure 6, lanes 3 and 4 showed that denatured type I collagens (gelatins) with molecular weight of 124,000 and 112,000 and the fragments after hydrolysis by gelatinase with molecular weight of 100,000, 94,000, 92,000 and 75,000. Lanes 5 and 6 showed that denatured type I collagens with molecular weights of 124,000 and 112,000 and the fragments after hydrolysis by gelatinase with molecular weight of 92,000. But there was no fragments after hydrolysis by gelatinase in the lane 7. 92-kDa gelatinolytic activity was inhibited markedly by the metal chelators, EGTA, EDTA, and tetracycline. Among these inhibitors, tetracycline was the strongest inhibitor of the 92-kDa gelatinase.

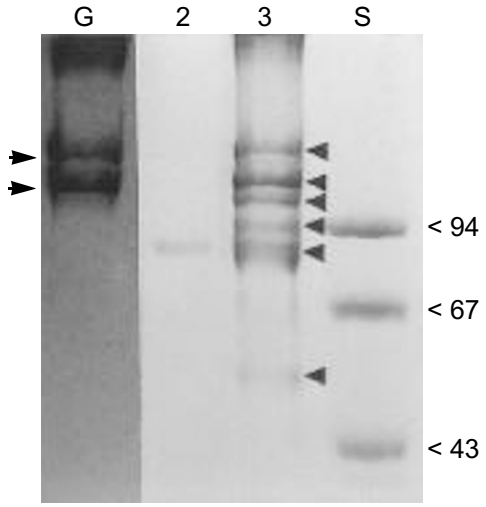

Figure 5. Analysis of purified enzyme and its gelatinolytic activity on the SDS-PAGE. Lane G: denatured type I collagen; lane 2: purified enzyme; lane 3: 73.8 μg of denatured type I collagen was incubated with 200 μg of purified enzyme for 4 h at 37˚C. Reaction products were separated on a 10% SDS-polyacrylamide gel. Molecular mass markers were run on a separate tract of the same gel and are indicated.

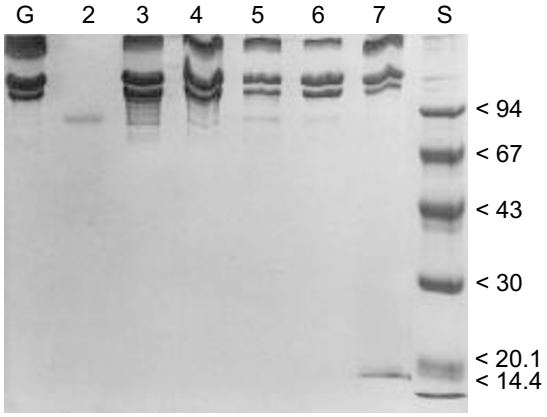

Figure 6. Inhibition of 92-kDa type IV collagenase by EGTA, EDTA and tetracycline. Lane G, 14.7 μg of denatured type I collagen, lane 2: purified enzyme; lane 3~4, 14.7 μg of denatured type I collagen were incubated with 2.2 μg of purified enzyme for 17 h at 37˚C; lane 5~7: 14.7 μg of denatured type I collagen were incubated with 2.2 μg of purified enzyme in the presence of 20 mM EGTA (lane 5), 20 mM EDTA (lane 6) and 20 mM tetracycline (lane 7), respectively, for 17 h at 37˚C. Reaction products were separated on a 10% SDS-polyacrylamide gel. Molecular mass markers were run on a separate tract of the same gel and are indicated.

Discussion

In this study we have isolated 3.85 mg of 92-kDa type IV collagenase from 132 mg of leukocyte extract (2.9% yield), by a combination of Ultrogel AcA 54 gel filtration and Bio-Rex 70 ion exchange chromatographies. Purified enzyme showed a single band on SDS-PAGE with a molecular mass of 92-kDa. Considering previous data on two step chromatography techniques for the same enzyme (Hibbs *et al*. 1985), the presently described two step purification method is much simpler and more convenient. The purification of metallo-proteinases from human polymorphonuclear neutrophil leukocytes is technically difficult for two main reasons: (a) the metalloproteinase contents are relatively low, and (b) both enzymes are degraded by the serine proteinases, which are present in large amounts in the azurophil granules (Murphy *et al*., 1980). It has been calculated that 1×10^9 neutrphils contain approx. 0.2 mg of collagenase and 0.1 mg of gelatinase, in contrast to 4 mg of elastase and 4 mg of cathepsin G (Murphy *et al*., 1982). We simplified the purification procedure of 92-kDa type IV collagenase to minimize the loss of enzyme during the multistep preparation. Therefore this method of preparation is very rapid and suitable for purification of enzyme from large quantities of starting material.

To investgate the biological and enzymatic characteristics of the purified enzyme, inhibition study of gelatinolytic activity of the enzyme by metal chelators was performed. As can be seen in Figure 6, gelatinolytic activity of the purified enzyme was inhibited markedly by 20 mM EGTA, 20 mM EDTA and 20 mM tetracycline, respectively. The reason for these inhibition could be that the active site zinc atom had been chelated and released from the catalytic domain

of the enzyme, resulting in the loss of hydrolytic activity. In good agreement with the report by Zucker *et al*., (1989), the gelatinolytic activity of type IV collagenase was more strongly inhibited by tetracycline than EGTA and EDTA.

Acknowledgement

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References

Docherty, A. J. P. and Murphy, G. (1990) The tissue metalloproteinase family and the inhibitor TIMP: a study using cDNAs and recombinant proteins. *Ann. Rheu. Dis.* 49: 469-479

Gabler, W. L. and Creamer, H. R. (1991) Suppression of human neutrophil function by tetracyclines. *J. Periodont. Res.* 26: 52-58

Gloub, L. M., Lee, H. M., Lehrer, G., Nemiroff, A., McNamara, T. F., Kaplan, R. and Ramamurthy, N. S. (1983) Minocycline reduces gingival collagenolytic activity during diabetes: preliminary observation and a proposed new mechanism of action. *J. Periodont. Res.* 18: 516-526

Gloub, L. M., Ramamurthy, N. and McNamara, T. F. (1984) Tetra-cyclines inhibit tissue collagenase activity. *J. Periodont. Res.* 19: 651-655

Gloub, L. M., Wolff, M. and Lee, H. M. (1985) Further evidence that tetracyclines inhibit collagenase activity in humn crevicular fluid and from other mammalian source. *J. Periodont. Res.* 20: 12-23

Golub, L. M., McNamara, T. F., Angelo, G. D., Greenwald, R. A. and Ramamurthy, N. S. (1987) A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. *J. Dent. Res.* 66: 1310-1314

Harris, E. D. and Krane, S. M. (1972) An endopeptidase from rheumatoid synovial tissue culture. *Biochim. Biophys. Acta* 258: 566-576

Hibbs, M. S., Hasty, K. A., Seyer, J. M., Kang, A. H. and Mainardi, C. L. (1985) Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J. Biol.Chem.* 260: 2493-2500

Kang, K., Jeong, H. Y. and Ghim, S.-Y. (1987) Simultaneous separation of pure neutrophils and lymphocytes from normal human blood by step gradient centrifugation. *Korean J. Immunol.* 9: 27-31

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685

Murphy, G., Bretz, U., Baggiolini, M. and Reynolds, J. J. (1980) The latent collagenase and gelatinase of human polymorphonuclear neutrophil leukocytes. *Biochem. J.* 192: 517-525

Murphy, G., Reynolds, J. J., Bretz, U. and Baggiolini, M. (1982) Partial purification of collagenase and gelatinase from human polymorpho-nuclear leukocytes. *Biochem. J.* 203: 209-221

Netzel-Arnett, S., Sang, Q., More, W. G. I., Navre, M., Birkedal Hansen, H. and Van-Wart H. E. (1993) Comparative sequence specificities of human 72- and 92-kDa gelatinases (type IV collagenases) and pump (matrilysin). *Biochemistry* 32: 6427-6432

Sopata, I. and Dancewicz, A. M. (1974) Presence of gelatin specific proteinase and its latent form in human leukocytes. *Biochim. Biophys. Acta* 370: 510-523

Woessner, J. F. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* 5: 2145-2154

Zucker, S., Wieman, J., Lysik, R. M., Imhof, B., Nagase, H., Ramamurthy, N., Liotta, L. A. and Golub, L. M. (1989) Gelatin-degrading type IV collagenase isolated from human small cell lung cancer. *Invasion Metastasis* 9: 167-181