

Acta Medica Okayama

Volume 42, Issue 1

1988

Article 9

FEBRUARY 1988

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Abstract

Type IV collagen-degrading enzyme activity was detected in human serum. Serum was preincubated with 4-aminophenylmercuric acetate and trypsin to activate the enzyme prior to assay. Type IV collagen, purified from human placentas and radiolabeled with [1-¹⁴C] acetic anhydride, was used as the substrate. The enzyme activity was measured at pH 7.5 and inhibited by treatment with ethylenediaminetetraacetic acid or heat. The assay of type IV collagen-degrading enzyme in human serum might be useful for estimating the degradation of type IV collagen.

KEYWORDS: type IV collagen-degrading enzyme, collagen degradation, collagenase, type IV collagen, metalloproteinase

Type IV Collagen-Degrading Enzyme Activity in Human Serum

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Type IV collagen-degrading enzyme activity was detected in human serum. Serum was preincubated with 4-aminophenylmercuric acetate and trypsin to activate the enzyme prior to assay. Type IV collagen, purified from human placentas and radiolabeled with [$^1\text{-}^{14}\text{C}$] acetic anhydride, was used as the substrate. The enzyme activity was measured at pH 7.5 and inhibited by treatment with ethylenediaminetetraacetic acid or heat. The assay of type IV collagen-degrading enzyme in human serum might be useful for estimating the degradation of type IV collagen.

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Collagen is a major extracellular protein characterized by a unique amino acid composition. Type I, II and III collagens are interstitial collagens which are degraded by vertebrate collagenase (EC 3. 4. 24. 7). Type IV collagen is a major component of basement membranes. The type IV collagen molecule is cleaved specifically one quarter from the N terminus by type IV collagen-degrading enzyme (1), and is not cleaved by vertebrate collagenase. Type IV collagen-degrading enzyme is a neutral metalloproteinase, and its activity is inhibited by ethylenediaminetetraacetic acid (EDTA) (2). The enzyme activity has been measured in the culture medium of murine melanoma and sarcoma cells (3) and in human liver homogenate (4), but it has not been detected in serum. In the present study, type IV collagen-degrading

enzyme activity was detected in human serum.

Materials and Methods

Preparation of substrate. Type IV collagen was purified from human placentas according to the method of Sage *et al.* (5), and radiolabeled with [$^1\text{-}^{14}\text{C}$] acetic anhydride (New England Nuclear, Boston, MA) by a method modified from that of Cawston *et al.* (6).

Enzyme activation. Venous blood was obtained from a healthy subject in the early morning. Serum was separated by centrifugation and stored at -20°C until assay. Sixty μl of serum were preincubated with 1 mM 4-aminophenylmercuric acetate (APMA; Wako Pure Chemical Industries Ltd., Osaka) at 37°C for 2 h, and then with 435 μg trypsin (Sigma Chemical Co., St. Louis, MO) at 37°C for 5 min followed by the addition of 6.5 mg soybean trypsin inhibitor (Sigma).

Assay for type IV collagen-degrading enzyme activity. Type IV collagen-degrading enzyme ac-

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tivity was measured by a modification of the method of Liotta *et al.* (3). Sixty-five μg of [^{14}C]-labeled type IV collagen and the serum preincubated with APMA and trypsin prior to assay were incubated at 37°C for 5 h in 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM CaCl_2 , 200 mM NaCl and 1 mM diisopropyl fluorophosphate (DFP; Sigma). The final reaction volume was 650 μl . The reaction was terminated by adding trichloroacetic acid (final 2%) and tannic acid (0.1%). The mixture was further kept at 4°C for 30 min and centrifuged at 3,000 rpm for 30 min. Radioactivity in the supernatant was counted with a liquid scintillation counter (LSC-703; Aloka Co. Ltd., Tokyo). Each assay was done in duplicate. The reaction mixture with 10 mM EDTA was simultaneously run as the control according to the method of Liotta *et al.* (3). One unit of type IV collagen-degrading enzyme activity was defined as 1 μg of collagen degraded at 37°C per min.

Results

Type IV collagen-degrading enzyme was present in an inactive form in human serum (Table 1). To detect this enzyme activity

Table 1 Activation of type IV collagen-degrading enzyme in human serum

Treatment ^a	Activity of type IV collagen-degrading enzyme (mU/ml serum) ^b
None	N. D.
APMA: 1 mM, 2 h	N. D.
APMA: 1 mM, 6 h	N. D.
Trypsin: 0.7 mg/ml, 5 min	N. D.
Trypsin: 7.3 mg/ml, 5 min	38
APMA: 1 mM, 2 h, and Trypsin: 7.3 mg/ml, 5 min	192
APMA: 1 mM, 2 h; Trypsin: 7.3 mg/ml, 5 min, and 10 mM EDTA ^c	N. D.

a: Treatments were done at 37°C.

b: 1 U = 1 μg of collagen degraded at 37°C per min.

c: EDTA was added after activation treatment.

Abbreviations: N. D., Not detected; APMA, 4-aminophenylmercuric acetate; EDTA, ethylenediaminetetraacetic acid.

in serum, enzyme activation prior to assay was essential. APMA alone did not activate the enzyme, but trypsin alone did slightly. The maximal activity was obtained after activation was carried out with 1 mM APMA for 2 h followed by 435 μg trypsin for 5 min.

The enzyme activity was linear to 75 μl of serum and to 6-h incubation (Fig. 1). Addition of EDTA inhibited the enzyme activity (Table 1). The activity was lost after incubation at 90°C for 5 min.

Discussion

Type IV collagen-degrading enzyme activity was detected in human serum. The enzyme acted at neutral pH and the activity was lost after heat treatment. Addition of EDTA to the reaction mixture at a final concentration of 10 mM inhibited the enzyme activity. The products of the enzyme reaction were examined using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) at neutral pH and PAGE at acidic pH (7). EDTA inhibited the appearance of degradation products of type IV collagen. The enzyme activated under the present conditions did not degrade [^{14}C]-labeled type I collagen (data not shown). The data indicate that this enzyme is a neutral metalloproteinase, which degrades type IV collagen, but not type I collagen.

Simultaneous treatment with APMA and trypsin activated type IV collagen-degrading enzyme in serum, where many types of enzyme inhibitors such as α_2 -macroglobulin and β_1 -anticollagenase exist. However, the same treatment did not activate vertebrate collagenase in serum. Vertebrate collagenase is thought to also be present in serum in an inactive form, supposedly as an enzyme-inhibitor complex. This complex was not dissociated by the present treatment with APMA and trypsin. Therefore, vertebrate collagenase may bind to its inhibitor(s) in a

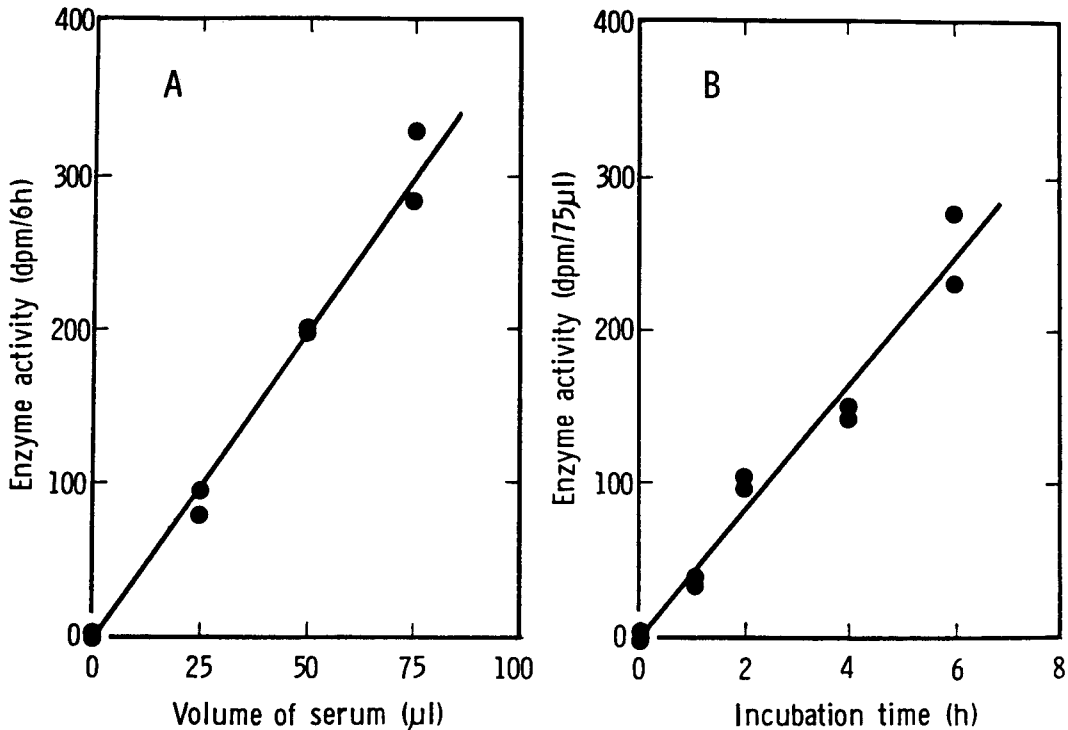


Fig. 1 The effects of serum volume and incubation time on type IV collagen-degrading enzyme activity. A: Zero, 25, 50 and 75 μ l serum were incubated with [14 C]-labeled type IV collagen for 6 h. B: Seventy-five μ l of serum were incubated for 0, 1, 2, 4 and 6 h. Each assay was run in duplicate. The assay procedure is described under Materials and Methods.

different manner, from the way type IV collagen-degrading enzyme binds to its inhibitor. It is not clear at the present whether or not the inhibitors of two types of collagen-degrading enzymes are the same protein.

Gel filtration of serum showed several peaks of type IV collagen-degrading enzyme activity (data not shown). Since all peaks demonstrated the activity after enzyme activation, it is suggested that this enzyme is present not only as an enzyme-inhibitor complex but also as a fragment of the type IV collagen-enzyme-inhibitor complex. The activation system of the present study appears to work on this fragment of the type IV collagen-enzyme-inhibitor complex.

Recently, serum markers reflecting collagen metabolism (both synthesis and degradation) in fibrotic liver have been reported, such as type III procollagen N peptide and

7S fragment of type IV collagen (8). At the present, however, there is no serum marker which reflects collagen degradation alone. Although further study is necessary to elucidate whether or not serum type IV collagen-degrading enzyme activity reflects type IV collagen degradation alone, the assay of the enzyme activity in human serum may be useful as an indication of type IV collagen degradation.

Acknowledgments. We wish to thank Professor Emeritus Hideo Nagashima, M.D. for his kind instruction and critical review of this manuscript. We also wish to thank Yoshihiro Kimura, M.D. for his experimental assistance.

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Received October 12, 1987; accepted November 24, 1987