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Isolation and characterization of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) from human rheumatoid synovial fluid

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The tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 were purified to apparent homogeneity from human rheumatoid synovial fluid (HRSF). The inhibitors were isolated by dissociation of non-covalent gelatinase/TIMP complexes. TIMP-1 migrated as a single polypeptide with M_r 28 500 on SDS-PAGE, while the M_r of TIMP-2 was 21 000. The inhibitory activity was stable under heat and acid pH. N-terminal sequence data were obtained for the first 15 residues of both inhibitors and showed identity to the human fibroblast inhibitors TIMP-1 and TIMP-2. This is the first demonstration that TIMP-1 and TIMP-2 can be directly purified from human rheumatoid synovial fluid. The complex formation between the metalloproteinase inhibitors and leucocyte metalloproteinases was shown by immunoblotting.

Tissue inhibitor of metalloproteinase; Gelatinase; Rheumatoid synovial fluid

1. INTRODUCTION

The tissue inhibitor of metalloproteinases, TIMP-1, is a glycoprotein with an apparent molecular mass of 28 500 and was isolated from different tissues and body fluids [1–4]. TIMP-2 is a non-glycosylated protein with a molecular mass of 21 000, in most cases isolated as a complex with the proenzyme of gelatinase, i.e latent M_r 72 000 gelatinase from different culture media [5–7]. Both inhibitors specifically inhibit the matrix metalloproteinases, collagenase, gelatinase, stromelysin and PUMP-1, which degrade extracellular matrix components such as collagens, proteoglycan, fibronectin and others. It has been found that TIMP-1 forms a complex with latent M_r 92 000 gelatinase, while TIMP-2 is able to form a complex with M_r 72 000 gelatinase. All TIMP complexes can be activated e.g. by APMA and then generate gelatinolytic activity, which can be inhibited by the addition of more TIMP-1 or TIMP-2 [6,8,9]. These results indicate that regulation of the extracellular matrix degradation is more complex than assumed before and that both inhibitors TIMP-1 and TIMP-2 play important roles in controlling matrix metalloproteinase activity in vivo. A decrease in TIMP-1 and TIMP-2 levels can be expected to initiate uncontrolled degradation of the extracellular matrix as observed in pathological conditions, such as rheumatoid arthritis.

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2. MATERIALS AND METHODS

2.1. *Purification of human TIMP-1 and TIMP-2 from rheumatoid synovial fluid*

600 ml rheumatoid synovial fluid was dialyzed against 50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 300 mM NaCl and 0.02% NaN_3 (buffer A). The sample was supplemented with hyaluronidase and stirred overnight at 20°C. This solution was centrifuged for 20 min at 48 000 × *g* to remove insoluble material and was then applied to an anion-exchange column (DEAE-A50; 3.2 × 10 cm) equilibrated with buffer A. The break-through fractions containing the gelatinase/TIMP complexes free of hyaluronic acids were adjusted to 0.5 M NaCl and stirred with 30 ml gelatin Sepharose for 1 h at 4°C. The loaded gelatin sepharose was washed with 50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 500 mM NaCl, 0.02% NaN_3 (buffer B) and filled into a column (1.6 × 30 cm). The gelatinase/TIMP complexes were eluted by adding 500 mM NaCl and 2.5% (v/v) Me_2SO to buffer B. The eluate was concentrated by ultrafiltration using an Amicon PM10 membrane. The dissociation of the gelatinase/TIMP complexes was performed by gelfiltration chromatography using Ultrogel AcA 44 (2.6 × 77 cm) in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% $NaDodSO_4$ (buffer C). The inhibitor containing fractions were dialyzed against 20 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$, 10 mM NaCl, 0.005% (w/v) Triton X-100, 0.02% NaN_3 (buffer D).

This solution was subjected to a Q-Sepharose fast-flow column (1.6 × 3 cm). TIMP-1 was retarded by this column, while adsorbed TIMP-2 could be eluted with a linear gradient of 0.01 to 0.2 M NaCl (80 ml). TIMP-1 or TIMP-2 fractions were concentrated by ultrafiltration using an Amicon PM5 membrane, dialyzed against 20 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 0.005% Triton X-100, 0.02% NaN_3 and used for further characterization.

2.2. *SDS-PAGE*

SDS-PAGE was performed according to the method of Laemmli [10]. Proteins were stained with silver as described by Heukeshoeven and Dernick [11].

2.3. *Protein determination*

Colum eluates were monitored for protein by measuring $A_{280\ nm}$.

2.4. *Sequence determination*

Amino-terminal sequence determinations were performed as recent-

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ly published [12] using a microsequencer (Model 810, Knauer, Berlin, Germany).

2.5. *Detection of matrix metalloproteinase/TIMP complexes by immunoblotting*

Purified TIMP-1 or TIMP-2 was blotted from unstained SDS-PAGE onto PVDF membranes by using the Biometra fast blot system. The membrane was incubated for 30 min with 3% BSA (w/v) in TBST-buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl, 0.05% Tween 20), for 10 min in TBST-buffer and 90 min with 50 μg latent or activated matrix metalloproteinase (PMNL collagenase or gelatinase) in TBST-buffer. The control remained in TBST-buffer. Membranes were washed with TBST-buffer for 5 min. Inhibitor/matrix metalloproteinase complexes were incubated with rabbit polyclonal antibodies specific for PMNL-gelatinase or collagenase. After 90 min reaction time the blots were washed with TBST-buffer for 10 min. The antigen/antibody complexes were detected using anti-rabbit alkaline phosphatase conjugated second antibodies as recently published [13].

2.6. *Assay of metalloproteinase inhibitor activity*

Inhibitory activity was determined measuring the cleavage of the synthetic octapeptide (dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) [14] by active PMNL gelatinase or collagenase in the presence or absence of metalloproteinase inhibitor.

2.7. *Purification of PMNL gelatinase and collagenase*

PMNL gelatinase and collagenase were purified according to the methods described in [15,16].

2.8. *Characterization of rabbit polyclonal anti-PMNL gelatinase and collagenase antisera*

The specificity of the antibodies was recently published in [13].

2.9. *Preparation and characterization of rabbit polyclonal TIMP-1 antisera*

The method for purification and characterization was recently described [13].

2.10. *Detection of TIMP-1 by immunoblotting*

This was carried out as for the detection of matrix metalloproteinase/TIMP complexes, but without incubation with enzyme solutions.

3. RESULTS AND DISCUSSION

The metalloproteinase inhibitors TIMP-1 and TIMP-2 were purified to homogeneity from human rheumatoid synovial fluid by a series of chromatographic steps (Table 1). Purification could be carried out, as TIMP complexes of latent and active gelatinase were bound to gelatin sepharose column, allowing easy separation of the complexes from most contaminating proteins. The isolated gelatinase/TIMP complexes were dissociated by gelfiltration in the presence of 0.1% SDS (Fig. 1). This demonstrates that gelatinase/TIMP complexes exist in a non-covalent form. In contrast to other reports [6,7] the isolated gelatinase was active, which could be due to using human rheumatoid synovial fluid or activation by SDS. The separation of TIMP-1 from TIMP-2 was achieved by chromatography on Q-Sepharose fast-flow. While TIMP-1 was not or only weakly bound to this column, TIMP-2 was eluted between 0.08 and 0.15 M NaCl. Further contaminating protein eluted with 0.5 M NaCl or 1.0 M NaCl (Fig. 2). This chromatographic procedure yielded pure TIMP-1 and TIMP-2 free of any other proteins. This is documented by SDS-PAGE (Fig. 3) showing a single protein band for each inhibitor with a molecular mass of 28 500 (TIMP-1) or 21 000 (TIMP-2). N-terminal sequence determination

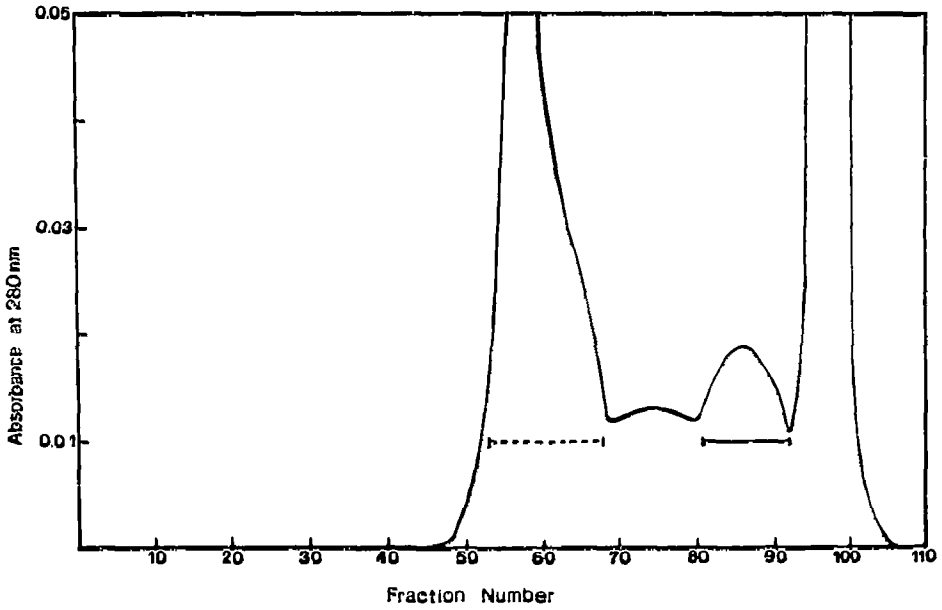


Fig. 1. Gelatinase/TIMP complex dissociation by SDS-gelfiltration on Ultrogel AcA 44 column. Concentrated fractions from gelatinsepharose (2 ml) were applied to an Ultrogel AcA 44 column (2.6 × 77 cm) equilibrated with buffer C. Fractions (1.5 ml) were collected at a flow rate of 10 ml/h and analyzed for protein by silver stained SDS-PAGE. Gelatinase (- - - -) or inhibitor (—) containing fractions were combined and dialyzed against buffer D.

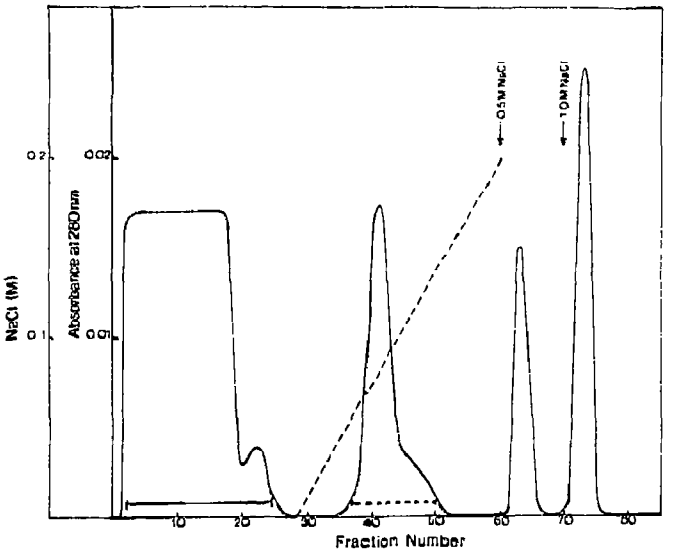


Fig. 2. Separation of TIMP-1 from TIMP-2 by Q-Sepharose. Dialyzed inhibitor containing fractions were applied to a Q-Sepharose column (1.6 × 3 cm) equilibrated with buffer D. Fractions (2 ml) were collected at a flow rate of 30 ml/h. Bound protein was eluted using a linear gradient (0.01–0.2 M NaCl) and analyzed for TIMP-1 and TIMP-2 by silver stained SDS-PAGE and inhibitor assay. TIMP-1 (- - - -) or TIMP-2 (—) containing fractions were combined and used for further characterization.

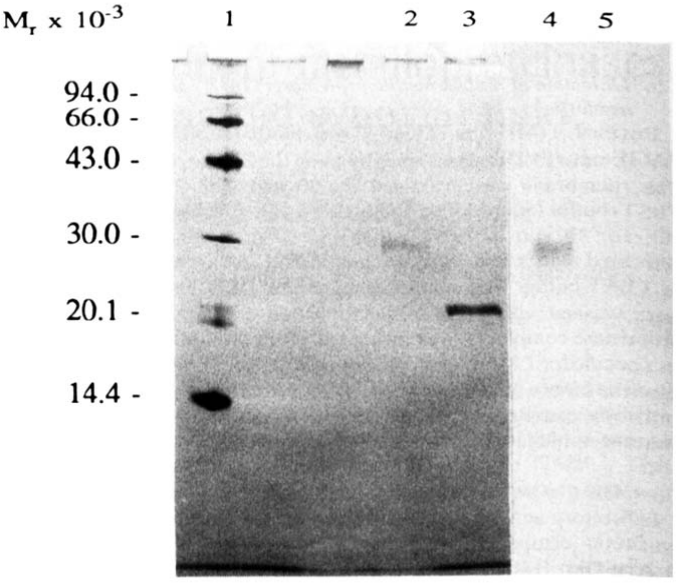


Fig. 3. Isolated human rheumatoid synovial fluid inhibitors and immunological detection by TIMP-1 antibodies. Lane 1, molecular mass standard; lane 2, HRSF-TIMP-1 (10% SDS-PAGE, silver stained); lane 3, HRSF-TIMP-2 (10% SDS-PAGE, silver stained); lane 4, HRSF-TIMP-1, immunologically detected by TIMP-1 antibody; lane 5, HRSF-TIMP-2, no cross reaction with TIMP-1 antibody.

revealed total identity of the isolated rheumatoid synovial fluid inhibitor TIMP-1 with human fibroblast inhibitor TIMP-1 in the first 15 residues (Table 2). Furthermore, the first 15 amino acid residues of the M_r 21 000 inhibitor shared total homology with TIMP-2 isolated from human melanoma cells [7] or recombinant TIMP-2 [17]. Both inhibitors were stable up to pH 2 and temperatures up to 100°C for 30 min, and were inactivated by reduction and carboxamidomethylation. A specific antibody raised against the isolated TIMP-1 only detected this inhibitor but not TIMP-2 (Fig. 3). Rheumatoid synovial fluid TIMP-1 and TIMP-2 are both able to inhibit active leucocyte gelatinase and collagenase. Autocatalytic degradation of these enzymes did not take place in the presence of either of these inhibitors.

Complex formation between the active leucocyte ma-

Table 1

Summary of purification of TIMP-1 and TIMP-2 from human rheumatoid synovial fluid

Purification step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purity
Crude extract after zentrifugation	2561.000	8707.4	3.4	-
DEAE-Sepharose	1455.000	7420.5	5.1	1.5
Gelatin-Sepharose	8.400	1764.0	210.0	61.8
SDS-Gelfiltration	0.420	1499.2	3612.5	1062.5
Q-Sepharose TIMP-1	0.110	1300.2	11820.0	3476.5
Q-Sepharose TIMP-2	0.022	249.9	11360.0	3341.2

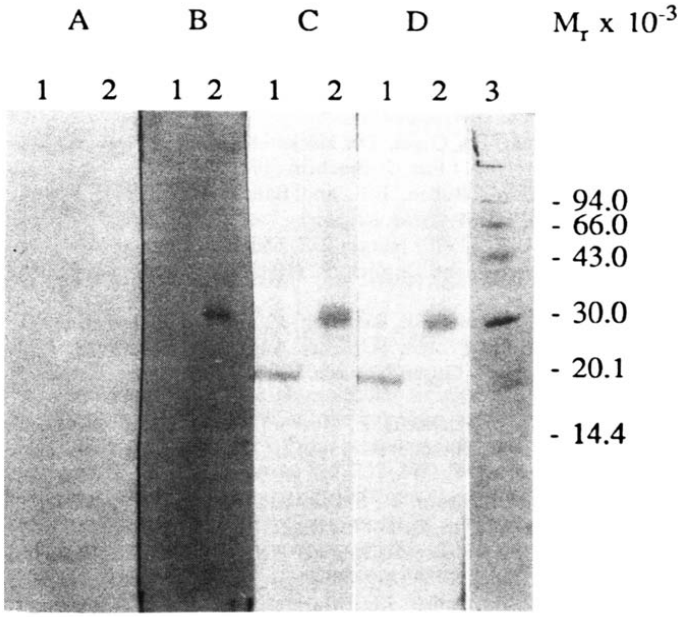


Fig. 4. Detection of human leucocyte metalloproteinase/TIMP complexes by immunoblotting. Lane 1, HRSF-TIMP-1; lane 2, HRSF-TIMP-1. A, blotted inhibitors incubated for 2 h with latent leucocyte collagenase and analyzed with collagenase antibody; B, blotted inhibitors incubated for 2 h with latent leucocyte gelatinase and analyzed with gelatinase IgG; C, as A, but incubation with activated leucocyte collagenase; D, as B, but incubation with activated leucocyte gelatinase.

trix metalloproteinases and these inhibitors was also demonstrated by immunoblotting (Fig. 4). Blotted inhibitors were incubated with activated gelatinase or collagenase. Only a single stained band was detected for each inhibitor by the specific antibodies, which recognized the complex with the enzyme. Similar results were obtained for TIMP-1 using PMNL progelatinase, while such a complex was not detected for TIMP-2. This is in agreement with the results of Goldberg [16], who found that M_r 92 000 progelatinase isolated from SV 40 transformated fibroblasts is able to form a complex with TIMP-1. In contrast PMNL procollagenase remained unbound to TIMP-1 or TIMP-2 and no complex formation was observed. In summary TIMP-1 and TIMP-2 are able to form stable complexes with activated PMNL collagenase and gelatinase, but only TIMP-1 can form a complex with PMNL progelatinase. This demonstrates that there must be a specific binding site for TIMP-1 in latent M_r 92 000 gelatinase. Howard et al. have shown that M_r 72 000 progelatinase forms complexes with TIMP-2 and perhaps TIMP-2 is a stabilizer of this enzyme [9]. It would seem that TIMP-1 has the same function for M_r 92 000 progelatinase and that the matrix metalloproteinase inhibitors have more assignments than just blocking the activated enzymes.

Furthermore, it was shown that TIMP-1 and TIMP-2 suppressed collagen degradation by activated leucocyte collagenase (results not shown). This underlines that these inhibitors play an important role in the control of matrix metalloproteinases in tissue remodelling. These enzymes are able to degrade many extracellular matrix components. Therefore, decreased inhibitor levels could

Table 2

The first 15 N-terminal amino acids of human rheumatoid synovial fluid inhibitors (TIMP-1 and TIMP-2) and comparision with sequences of inhibitors from other sources.

Sources[a]	Amino acid residues			
	1	5	10	15
HRSF-TIMP-1	X-T-X-V-P-P-H-P-Q-T-A-F-X-N-S-			
H-TIMP-1	C-T-C-V-P-P-H-P-Q-T-A-F-C-N-S-			
HRSF-TIMP-2	X-S-X-S-P-V-H-P-Q-Q-A-F-X-N-A-			
H-TIMP-2	C-S-C-S-P-V-H-P-Q-Q-A-F-C-N-A-			

a HRSF-TIMP-1, human rheumatoid synovial fluid TIMP-1

H-TIMP-1, human TIMP-1 reported by Docherty et al. [18]

HRSF-TIMP-2, human rheumatoid synovial fluid TIMP-2

H-TIMP-2, human TIMP-2 reported by Stetler-Stevenson et al. [7] and Boone et al. [17]

X = unidentified amino acid

lead to unbalanced matrix degradation as observed in pathological conditions such as rheumatoid arthritis.

This is the first report that TIMP-1 as well as TIMP-2 are present in human rheumatoid synovial fluid and that both inhibitors can be isolated from this body fluid by a simple chromatographic procedure. Future investigations need to clarify the roles of TIMP-1 and TIMP-2 in diseases such as rheumatoid arthritis. This could perhaps help to develop therapeutic approaches to inhibit matrix degradation.

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