Purification of a serine-proteinase inhibitor from human articular cartilage

Identity with the acid-stable proteinase inhibitor of mucous secretions

Beate BÖHM,* Rainer DEUTZMANN† and Harald BURKHARDT*†

*Max-Planck-Society, Clinical Research Units for Rheumatology at the Medical Clinic III of the University Erlangen-Nürnberg, Erlangen, and †Department of Biochemistry, University of Regensburg, Regensburg, Federal Republic of Germany

An inhibitor of the serine proteinases human leucocyte elastase (EC 3.4.21.37), of cathepsin G (EC 3.4.21.20) and of trypsin (EC 3.4.21.4) has been purified from human articular cartilage. The apparent M_r of the cationic ($pI > 10$) protein was determined to 15000 by SDS/PAGE. It was shown to cross-react in Western blot with a specific antibody to a recombinant-derived serine-proteinase inhibitor of human mucous secretions. Identity of both inhibitors is indicated by the determination of the N -terminal amino acid sequence of the cartilage-derived serine-proteinase inhibitor. In all 24 residues the cartilage inhibitor was shown to be identical with the human secretory leucocyte proteinase inhibitor ('SLPI'). The inhibitor molecule may play a crucial role in the protection of cartilage matrix proteins against proteolytic attack.

INTRODUCTION

The integrity of the articular cartilage is maintained by a well-balanced metabolism of the matrix components. Under physiological conditions, the turnover of collagen and proteoglycans seems to be very low (Maroudas, 1975). In degenerative and inflammatory disease such as osteoarthritis or rheumatoid arthritis a disturbance of this metabolic balance occurs, resulting in a dramatic increase in proteolysis. The catabolism far exceeds the ability of the chondrocytes to increase the rate of matrix synthesis in order to compensate for the increased loss of matrix molecules. Among the various enzymes capable of degrading matrix components, neutral proteinases of the metallo- and serine type seem to be of major importance for the initiation of matrix breakdown in the extracellular compartment (Barrett, 1978). Polymorphonuclear leucocytes (PMN), which are abundant in effusions of inflamed joints, contain three of these proteinases: elastase, cathepsin G and collagenase (Baggiolini *et al.*, 1978). In the synovial fluid the destructive potential of these enzymes is normally controlled by the anti-proteinase potential of serum inhibitors, e.g. α_1 -proteinase inhibitor (α_1 -PI) or α_2 -macroglobulin (α_2 -M). However, a local disturbance of the enzyme-inhibitor balance may occur and promote tissue destruction (Burkhardt *et al.*, 1987). Therefore low- M_r proteinase inhibitors within the tissue itself may be even more important in the regulation of matrix turnover. The tissue inhibitor of metalloproteinases (TIMP), which was originally purified from rabbit bones (Cawston *et al.*, 1981), was shown to be synthesized by chondrocytes in culture (Duncan & Reynolds, 1983). Its synthesis is affected by growth factors such as transforming growth factor β (TGF β ; Edwards *et al.*, 1987).

TIMP has been cloned (Docherty *et al.*, 1985) and seems to exist in two different forms, namely TIMP 1 and TIMP 2 (Stetler-Stevenson *et al.*, 1989). The information concerning

serine-proteinase-inhibitory activity in articular cartilage is far less complete. Since Kuettner *et al.* (1974) and Sorgente *et al.* (1975) reported on a cationic protein from bovine cartilage with trypsin-inhibitory activity and an apparent M_r of 13000, several workers (Roughley *et al.*, 1978; Killackey *et al.*, 1983; Lesjak & Ghosh 1984) have confirmed the presence of serine-proteinase-inhibitory activity in cartilage of different origin, but failed to characterize the isolated molecules responsible for this activity. In a previous paper (Burkhardt *et al.*, 1987) we briefly summarized the conflicting data in more detail and reported on the partial purification and characterization of a serine-proteinase inhibitor from human articular cartilage. In the present paper we describe a modified purification scheme that permits us to separate the inhibitor from contaminating lysozyme and the identification of the inhibitor molecule as a homologue of the acid-stable proteinase inhibitor of human mucous secretions.

MATERIALS AND METHODS

Enzymes

Cathepsin G (EC 3.4.21.20) was purified from buffy coats of healthy blood donors according to the method of Engelbrecht *et al.* (1982). Trypsin (bovine pancreas) was purchased from Serva (Heidelberg, Germany) and elastase (EC 3.4.21.37) from Elastin Products (Owensville, MO, U.S.A.).

Assays

Elastase-inhibitory activity was measured using methoxy-succinyl (MeO-Suc)-Ala-Ala-Pro-Val-4-nitroanilide (4NA) (Najajima *et al.*, 1979) as chromogenic substrate. Trypsin inhibition was assayed by the method of Erlanger *et al.* (1961), employing benzoyl-DL-Arg-4NA. Suc-Phe-Pro-Phe-4NA was used to determine cathepsin G activity (Woodbury & Neurath,

Abbreviations used: PMN, polymorphonuclear leucocytes; α_1 -PI, α_1 -proteinase inhibitor; α_2 -M, α_2 -macroglobulin; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; MeO-Suc, methoxysuccinyl; 4NA, 4-nitroanilide; Tos-Lys-CH₂Cl, N - α -p-tosyl-lysylchloromethane ('TLCK'); TFA, trifluoroacetic acid; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/4.3 mM-Na₂HPO₄/1.4 mM-KH₂PO₄, pH 7.3); SLPI, human secretory leucocyte proteinase inhibitor; HUSI-I, human seminal plasma inhibitor.

† To whom correspondence and reprint requests should be sent, at the following address: Dr. H. Burkhardt, Max-Planck-Gesellschaft, Klinische Arbeitsgruppen für Rheumatologie, Schwabachanlage 10, D-8520 Erlangen, Federal Republic of Germany.

1980; Yoshida *et al.*, 1980). All assays were commenced after 15 min preincubation of the inhibitor probe with the enzyme.

Cartilage extraction

Human articular cartilage was taken for the knee joints of adolescent trauma victims within 24 h *post mortem*. From this starting material the inhibitor fractions were extracted by using a previously published protocol (Burkhardt *et al.*, 1987). Briefly, after extensive washing in normal saline and mechanical fractionation, the cartilage homogenate was stirred for 48 h at 4 °C in 200 mM-Tris/HCl (pH 7.4)/0.5 M-NaCl/50 mM-CaCl₂/2 M-guanidinium chloride/0.05 % (v/v) Brij 35. After centrifugation at 48000 g at 4 °C for 30 min the supernatant was filtered through an Amicon XM 100 membrane to remove large proteoglycans.

Ion-exchange chromatography

The cartilage extract was dialysed against 0.05 M-acetate buffer, pH 5.5, and applied to a CM-cellulose column (CM-52; Whatman; 2.8 cm × 45 cm) equilibrated with same buffer. The proteins were eluted with a linear increasing gradient of 0–1 M-NaCl and collected in 7 ml fractions.

A portion of the inhibitor fractions was re-equilibrated with the starting buffer (0.05 M-acetate, pH 5.5) and subjected to f.p.l.c. on a Mono S column (Pharmacia). The column was eluted under a pressure of 2 MPa using a linear gradient between 0 and 1 M-NaCl over 50 ml.

Affinity chromatography

Another portion of the inhibitor fractions eluted from the CM-52 column was equilibrated with 0.05 M-Tris/HCl (pH 8.2)/0.02 M-CaCl₂ and applied to 1 ml of trypsin-Sepharose (Sigma). After an incubation period of 1 h at room temperature, the Sepharose was washed with 50 ml of buffer. Elution of protein bound to trypsin-Sepharose was performed by the addition of 2 ml of buffer containing 10 mg of the specific irreversible trypsin inhibitor *N*- α -p-tosyl-L-lysylchloromethane hydrochloride (Tos-Lys-CH₂Cl) ('TLCK'; Bissendorf, Hannover, Germany).

Reverse-phase chromatography

In the last step of the purification procedure the inhibitor fractions were subjected to h.p.l.c. on a Vydac C₁₈ reverse-phase column. After concentration in a Speed-Vac centrifuge (Bachhofer, Reutlingen, Germany) the probes were dissolved in 500 μ l of 0.1 % trifluoroacetic acid (TFA) and loaded on to the column. For the mobile phase a gradient system of acetonitrile (0–100 %) in 0.08 % TFA was chosen.

Homogeneity of the peptides was controlled by SDS/PAGE in 18 % -(w/v)- acrylamide Minigels using the buffer system described by Laemmli (1970). Gels were stained with Coomassie Blue or silver as described by Oakley *et al.* (1980).

Western blot

After electrophoresis the cartilage-derived inhibitor and a sample of recombinant-derived acid-stable inhibitor of mucous secretions were blotted on to nitrocellulose filters (Towbin *et al.*, 1979). After blocking with low-fat milk protein, the blotted proteins were immunolabelled with a specific rabbit antibody to the recombinant-derived mucous inhibitor [antibody dilution 1:1000 in phosphate-buffered saline (PBS)], using peroxidase-labelled goat anti-rabbit IgG (Bio-Rad Laboratories) as second antibody, and chloronaphthol as colour substrate. The recombinant-derived inhibitor and the specific rabbit antibody were generously given by Dr. R. Heinzl-Wieland (Grünenthal G.m.b.H., Stolberg, Germany).

Affinity blotting

For the identification of protein bands with PMN elastase binding capacity in the beginning of the purification procedure, we applied a modified Western-blot technique. Using conventional blotting (Towbin *et al.*, 1979) and blocking with low-fat milk protein, the nitrocellulose filters were incubated with 15 μ g of PMN elastase in 5 ml of PBS for 2 h at room temperature. After thorough washing with PBS and a second blocking step, the blot was immunolabelled with a sheep anti-(PMN elastase) antibody (Serotec, Oxford, UK), followed by peroxidase-labelling rabbit anti-sheep IgG (Dianova, Hamburg, Germany). Chloronaphthol was employed as colour substrate.

Amino acid sequencing

The amino acid sequence was determined on a gas-phase sequencer (Applied Biosystems).

RESULTS

After guanidinium hydrochloride extraction and ultrafiltration, the inhibitor fractions were applied on CM-52 and eluted from the ion-exchange column at NaCl concentrations between 0.5 and 0.6 M. The chromatographic profile is shown in Fig. 1. The eluted fractions show one main inhibitory peak, with maximal inhibition values of 100 % for trypsin (60 munits/ml), PMN elastase (2 munits/ml) and cathepsin G (6 munits/ml).

SDS/PAGE revealed that this fraction was still heterogenous, containing lysozyme as the main impurity (Fig. 2).

In order to identify protein bands with inhibitory activity, we established a modified Western-blot technique. After conventional blotting (Towbin *et al.*, 1979) and blocking with low-fat milk protein, the nitrocellulose filters were incubated with PMN elastase. After thorough washing with PBS and a second blocking step, the blot was immunolabelled with an anti-(PMN elastase) sheep antibody, followed by peroxidase-labelled rabbit anti-sheep IgG. This technique revealed elastase-binding protein bands corresponding to an apparent molecular mass of 16 kDa after electrophoresis of the non-reduced fractions with inhibitory activity (Fig. 2).

Final purification was hampered by the difficulty in separating the inhibitor molecule from the lysozyme contamination. Two different strategies were followed. In a first attempt batch adsorption to trypsin-Sepharose was tested. After adsorption to

Fig. 1. Ion-exchange chromatography on CM-52

A guanidinium hydrochloride extract was fractionated by ultrafiltration, and the low-*M_r* (< 100 000) fraction equilibrated with 0.05 M-sodium acetate, pH 5.5. Column elution was performed with a linear 0–1 M-NaCl gradient. The Figure shows the elution profile and the inhibitory activity of 200 μ l of the fraction towards PMN elastase (◆), cathepsin G (▲) and trypsin (□).

Fig. 2. SDS/PAGE of the inhibitor after chromatography on CM-52

After chromatography on CM-52 the unreduced inhibitor fraction was applied to an SDS/18 %-(w/v)-polyacrylamide gel. The gel was stained with Coomassie Blue. Lane a, the inhibitor fraction. Marker proteins (lane b) and chick lysozyme (lane c); a' shows the blot of lanes a and b after incubation with PMN elastase and immuno-labelling with anti-elastase IgG (details of the method are described in the text).

Fig. 3. Reverse-phase h.p.l.c. of the inhibitor after affinity purification on trypsin-Sephacrose

After elution from the trypsin-Sephacrose column the inhibitor fractions were applied on a Vydac C₁₈ reverse-phase column. Elution was achieved by a linear gradient (straight diagonal line) of acetonitrile of 5–100% in 0.08% TFA (see the Materials and methods section). The inhibitor was eluted in the main fraction after 37 min.

Table 1. The four main sequences of the cartilage-derived proteinase inhibitor (CI) after separation on trypsin-Sephacrose: alignment with the SLPI sequence

*, Trypsin cleavage site in SLPI; **, chymotrypsin cleavage site in SLPI; aa, amino acid residues. Identical residues of the CI partial sequence and SLPI are boxed.

Position...	1	2	3	4	5	6	7	8	9	10	
	S	G	K	S	F	K	A	G	V	—	CI (sequence 1) SLPI (aa 1–10)
				S	F	K	A	G	V	C	
*	S	F	K	A	G	V	—	P	—	K	CI (sequence 2) SLPI (aa 4–13)
				A	G	V	C	P	P	K	
*	Y	K	K	P	E	—	E	S	D	W	CI (sequence 3) SLPI (aa 21–30)
				P	E	C	Q	S	D	W	
**	M	L	N	P	P	N	F	—	E	M	CI (sequence 4) SLPI (aa 73–82)
						N	F	C	E	M	

the gel, washing with starting buffer did not remove the inhibitor from the trypsin-Sephacrose; no elastase inhibitory activity was

Fig. 4. Cation-exchange f.p.l.c. of the inhibitor after CM-52 chromatography

After elution from the CM-52 column the inhibitor was equilibrated with 0.05 M-sodium acetate, pH 5, and loaded on to a Mono S column. The column was eluted with a linear gradient of 0–1 M-NaCl. The inhibitor fraction is marked with an asterisk.

found in the eluate. However, subsequent washing with buffer containing the trypsin inhibitor Tos-Lys-CH₂Cl eluted the elastase-inhibitory activity. In preliminary experiments a direct effect of Tos-Lys-CH₂Cl on PMN elastase activity had been excluded. Therefore a fraction was identified which is capable of binding to trypsin-Sephacrose in a reversible manner. Moreover, after removal from trypsin by a competitor which binds to the active centre of the enzyme irreversibly, this fraction still exhibits inhibitory activity towards PMN elastase. SDS/PAGE revealed that the inhibitor sample was still heterogeneous, though a progression in purification was achieved (results not shown). Therefore subsequent reverse-phase h.p.l.c. was performed. As shown in Fig. 3, at 37% acetonitrile a sharp peak was eluted from the Vydac C₁₈ column, which was subjected to amino acid sequencing. Four main sequences were identified: from the N-terminus, six residues were identified, starting at position 4. Comparison of these partial sequence data with known serine-proteinase inhibitors gave a hint to a relationship of the cartilage-derived inhibitor to human secretory leucocyte proteinase inhibitor (SLPI) (Table 1). Therefore we interpreted the four main sequences as a result of partial proteolysis of the inhibitor on the trypsin-Sephacrose column, while intramolecular disulphide bridges keep the molecule together. As attempts to obtain unequivocal sequence data after reduction and carboxymethylation failed, the purification strategy was changed.

After ion-exchange chromatography on CM52 we used a Mono S cation-exchange column for rechromatography of the inhibitor fractions. As shown in Fig. 4, two main peaks were eluted with a salt gradient. The minor peak exhibited elastase-inhibitory activity. This fraction was subjected to reverse-phase h.p.l.c. on a Vydac C₁₈ column. At 37% acetonitrile a fraction was eluted from the column that inhibited PMN elastase. This fraction proved to be homogeneous by silver staining after SDS/PAGE (Fig. 5). It migrated with the same electrophoretic mobility as the recombinant SLPI and cross-reacted in Western blot with a specific rabbit antibody to the recombinant-derived mucous inhibitor (Fig. 5).

The amino acid sequence analysis of the N-terminal 24 amino acid residues was determined by gas-phase sequencing (Table 2). Comparison with the SLPI molecule (Table 2) demonstrated identity with SLPI in 22 residues out of a total of 24 residues. For positions 10 and 18 the identification of the amino acids failed. In both positions there is a cysteine residue in the SLPI sequence.

The major peak from the Mono S column was subsequently

Fig. 5. Immunoblotting of the purified cartilage-derived serine proteinase inhibitor and recombinant inhibitor from human mucous secretions

After final purification on a reverse-phase C_{18} h.p.l.c. column the inhibitor (lane b) was applied to SDS/18%-(w/v)-polyacrylamide gels after reduction with dithiothreitol and silver-stained. Lane a, marker proteins; lane c, recombinant-derived inhibitor of mucous secretions; lane b', immunoblot of the cartilage-derived inhibitors; lane c', immunoblot of the recombinant-derived mucous-secretion inhibitor. The blots were immunolabelled with a rabbit antibody to the recombinant-derived mucous inhibitor (for details, see the Materials and methods section).

Table 2. *N*-Terminal sequence of the cartilage-derived proteinase inhibitor (CI) isolated by Mono S ion-exchange chromatography: comparison with the SLPI sequence

Position...	1	2	3	4	5	6	7	8	9	10	
	S	G	K	S	F	K	A	G	V	—	CI
	S	G	K	S	F	K	A	G	V	C	SLPI
Position...	11	12	13	14	15	16	17	18	19	20	
	P	P	K	K	S	A	Q	—	L	R	CI
	P	P	K	K	S	A	Q	C	L	R	SLPI
Position...	21	22	23	24							
	Y	K	K	P							CI
	Y	K	K	P							SLPI

subjected to reverse-phase h.p.l.c. The peak eluted at 45% acetonitrile was sequenced and identified as a homologue of chick lysozyme in the nine *N*-terminal residues analysed (sequence: KVFERELA).

DISCUSSION

Since the first reports of the presence of a serine-proteinase inhibitor in cartilage tissue (Kuettner *et al.*, 1974; Sorgente *et al.*, 1975), conflicting data have been published on the nature of the molecule responsible for this activity (Roughley *et al.*, 1978; Killakey *et al.*, 1983; Lesjak & Ghosh, 1984). In a previous paper (Burkhardt *et al.*, 1987) we reported on the partial purification and characterization of the inhibitor and briefly reviewed the discrepancies found in the literature. More recently DiMuzio *et al.* (1987) described the separation of an elastase inhibitor with a molecular mass of 15 kDa. By blotting from a two-dimensional gel and microsequencing, an *N*-terminal sequence of 12 residues

was obtained, but not published. Whether this inhibitor also inhibited trypsin or cathepsin G was not stated.

Our data indicate identity of the cartilage-derived serine-proteinase inhibitor with the acid-stable proteinase inhibitor of mucous secretions [human seminal plasma inhibitor (HUSI-I); Seemüller *et al.*, 1986] and SLPI (Thompson & Ohlsson, 1986). During the purification procedure it proved to be very difficult to separate the inhibitor molecule from contaminating lysozyme. The strategy of using affinity chromatography on trypsin-Sepharose led to partial sequence data which indicated internal cleavage of the molecule at three sites at least. The sequences suggested sequence identity with the inhibitor from mucous secretions as shown in Table 1. Also in agreement with this hypothesis, limited cleavage of native SLPI at residues 20–22 and 72–74 has been reported (Thompson & Ohlsson, 1986), corresponding to the localization of active sites for trypsin and chymotrypsin by X-ray-crystal-structure analysis of the enzyme-inhibitor complex (Grütter *et al.*, 1988). Therefore we hypothesize that partial proteolysis of the cartilage inhibitor is responsible for the generation of heterogeneous *N*-termini. Together with the disulphide bridges stabilizing the partially degraded molecule, this assumption could explain the four sequences obtained after purification on trypsin-Sepharose. In all, 18 residues could be aligned with the SLPI sequence, with the exception of one glutamic acid residue which corresponds to a glutamine residue in position 27 of the SLPI sequence.

Unequivocal support for identity of the serine-proteinase inhibitor from cartilage with SLPI was obtained after *N*-terminal sequencing of the intact molecule purified by ion-exchange chromatography: 22 residues were identical with the SLPI sequence. Positions 10 and 18 remained unidentified; these positions correspond to cysteine residues in the SLPI.

The identity of the cartilage-derived inhibitor with SLPI is also supported by the following criteria: (1) the inhibition spectrum of both proteins include elastase, cathepsin G and trypsin (Fig. 2; Thompson & Ohlsson, 1986); (2) for both inhibitors the basic nature (Burkhardt *et al.*, 1987; Thompson & Ohlsson, 1986) has been described ($pI > 10$); (3) the acid-resistance of the cartilage-derived inhibitor is proven by the fact that elastase inhibition is still detectable after reverse-phase chromatography in 0.1% TFA; (4) on SDS/PAGE the reduced cartilage inhibitor migrated together with the recombinant-derived mucous inhibitor in a very short distance above the lysozyme marker band (Fig. 5). The aberrant electrophoretic mobility of SLPI on SDS/PAGE compared with M_r calculation on the basis of the known sequence (11 726) was related to its cationic nature (Thompson & Ohlsson, 1986); (5) a specific antibody to the recombinant-derived SLPI cross-reacts with the cartilage inhibitor in the immunoblot (Fig. 5).

The existence of only one inhibitor of this type is supported by the literature, as there is no evidence for a genetic divergence of SLPI-related proteinase inhibitors. Some closely related inhibitors from different mucous secretions, which had been originally regarded as distinct proteins, were in fact proven to be identical with SLPI: cervix uteri secretion inhibitor ('CUSH') (Heinzel *et al.*, 1986), HUSI-I (Seemüller *et al.*, 1986) and bronchial leukocyte proteinase inhibitor ('BLPI') (Boudier *et al.*, 1988). Moreover, the SLPI gene was shown to be a single-copy gene (Stetler *et al.*, 1986).

In contrast with the reported exclusive detection in mucous secretions, our data are the first evidence for an additional role of the SLPI inhibitor in cartilage. Whether it is synthesized locally by the chondrocytes or the synovial fibroblasts remains to be elucidated. Another possibility would be the diffusion of the inhibitor from the circulation into the matrix. In this case the negatively charged proteoglycans could function as a kind of

cation-exchanger to concentrate the inhibitor molecule in the matrix. Regardless of the site of synthesis, the inhibitor can play a major role in the protection of the cartilage matrix proteins against proteolytic damage by association with the target molecules. Owing to its affinity for the serine proteinases, as indicated by the kinetic data of enzyme inhibition (Thompson & Ohlsson, 1986; Burkhardt *et al.*, 1987; Bieth, 1980), it ought to have a strong protective potential. Further studies are needed to answer the question as to whether disturbances in concentration or function of the inhibitor contribute to cartilage degradation in diseases such as osteoarthritis or rheumatoid arthritis.

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