AN INHIBITOR OF MAMMALIAN COLLAGENASE ACTIVE AT MICROMOLAR CONCENTRATIONS FROM AN ACTINOMYCETE CULTURE BROTH

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Anti-collagenase activity was detected in the culture supernatant of an Actinomycete strain S 4373. The molecule was purified by solvent extraction, medium pressure and high pressure reverse phase chromatography and finally by HPLC gel filtration. The pure product was analyzed by mass spectroscopy and was identified as actinonin, a known pseudopeptide antibiotic. The *Ki* was determined as 1.4 μM and this value was confirmed using pure synthetic actinonin.

Mammalian collagenase (EC 3.4.23.7) is a metallo-endopeptidase which specifically cleaves mammalian triple helical collagen at a unique cleavage site[1]. Degradation of collagen is observed in several pathological situations such as in arthritis, corneal ulceration, periodontal disease and tumor proliferation and may represent a key step in the development of these pathologies[2,3]. For this reason there is presently considerable interest in the development of effective inhibitors of the enzyme.

A potent natural protein inhibitor of collagenase (tissue inhibitor of metalloprotease, TIMP) has recently been described which forms an irreversible molar stoichiometric complex with active collagenase[4]. The amino acid sequence of this protein has recently been elucidated after cloning of the gene[5]. The *Ki* of pure TIMP is below 10^{-9} M in our assay system. (Y. LELIÈVRE — unpublished results).

The known low-molecular-weight inhibitors of mammalian collagenase are much less potent. Peptide inhibitors based on the known amino acid sequence around the scissile bond in collagen attached to groups potentially capable of interaction with the active center zinc atom of collagenase have been designed by several groups. Thus GRAY *et al.* produced tripeptide inhibitor of *Ki* 10^{-5} M[6] and MCGREGOR obtained inhibitors of similar potency[7,8]. More recently, MCCULLAGH *et al.* obtained a IC_{50} of 0.8 μM with carboxyalkyl peptides presumably acting as transition state inhibitor[9]. Very recently MOORE and SPILBURG have described hydroxamate bearing peptides active at submicromolar concentrations[10].

The activity of the fermentation product isolated in the present study approaches that of the most potent rationally designed molecules so far described.

Materials and Methods

Enzyme Preparation and Assay

Collagenase was produced from porcine synovial cell culture as described by DAYER *et al.*,[11] and was purified by the method of CAWSTON and TYLER[12]. ^{14}C-Labeled rat skin collagen was prepared and assayed in the "diffuse fibril assay" as described by CAWSTON and BARRETT[13].

Production of Actinomycete Culture Extract

Actinomycete strains were fermented in 2 liters Biolafitte fermentor using a medium comprising distiller's solubles 25 g, glucose 10 g, soya oil 5 g, $CaCO_3$ 5 g and $(NH_4)_2SO_4$ 2 g, per liter. Fermentation was for 90 hours at 28°C.

Extraction of Culture Medium

100 ml of methanol was mixed with 200 ml of culture fluid, agitated for 30 minutes at room temp then centrifuged for 10 minutes at 4,000×*g*. The supernatant was evaporated to dryness under reduced pressure. The residue was redissolved in water and applied to a medium pressure C18 silica gel (20~40 μm) column (210×25 mm). The column was developed at a flow rate of 2.5 ml/minute using a Duramat LFG pump at 3 bars pressure. Polar material was washed off with water and bound material was then eluted as a single fraction using pure acetonitrile. This material was evaporated to dryness and redissolved in water at a ten-fold higher concentration.

Purification by HPLC

500 μl-samples of active material were then rechromatographed by reverse phase HPLC using a 300×7.6-mm C18 Nucleosil column using a Varian model 5500 HPLC system. Elution was at 2 ml/minute using a gradient from 10 to 50% acetonitrile in water.

A second HPLC separation of the active fraction was performed using two TSK SW 2000 columns (300×7.6 mm) after passage through a TSK pre-column. Water at 0.5 ml/minute was used as eluant.

The purity of the active fraction from the TSK column was verified by analytical reverse phase HPLC (C18 Nucleosil, column size 250×4.6 mm).

In all HPLC systems detection was at 215 nm using a Varian UV 200 detector. 1 ml-fractions were collected.

Characterization by Mass Spectroscopy

Spectral analysis was performed by chemical ionization using ammonia as the reactive gas for desorption on a Normag model R 1010. Analysis using electron bombardment was on a Finnigan model 3,000 mass spectrometer.

Results

Purification

Collagenase inhibitory activity in the culture supernatant was recovered quantitatively in the methanol extract and in the bound fraction eluted from the C18 column with pure acetonitrile. Chromatography of this material on the HPLC reverse phase column resulted in the elution profile shown in Fig. 1.

The active fraction from this column was further purified on the TSK column (Fig. 2). Each column run yielded around 1.5 mg of apparently pure inhibitor as judged by analytical HPLC (Fig. 3).

Spectral Analysis

The spectrum obtained by chemical ionization showed a peak MH^+ at *m/z* 386 and fragments at mass *m/z* 201 and 186.

The spectrum obtained from the electron bombardment analysis showed peaks at *m/z* 386 (MH^+), 355, 322, 312 and ions of mass *m/z* 224 and 213.

The exact masses were determined and correspond to the empirical formula $C_{13}H_{22}O_2N$ and $C_{12}H_{25}ON_2$ (data not shown). The molecule was identified from reference to in house data files as actinonin. Comparative inhibition assays with the isolated molecule and synthetic samples of actinonin confirmed that both preparations inhibited mammalian collagenase with the same *Ki* of 1.4×10^{-6} M.

Fig. 1. Reverse phase HPLC of Actinomycete extract.

500 μl of sample from the preparation column was injected into a C18 Nucleosil column (300× 7.6 mm) and eluted with linear acetonitrile gradient (10~37%) in water. The dotted line represents eluted anti-collagenase activity.

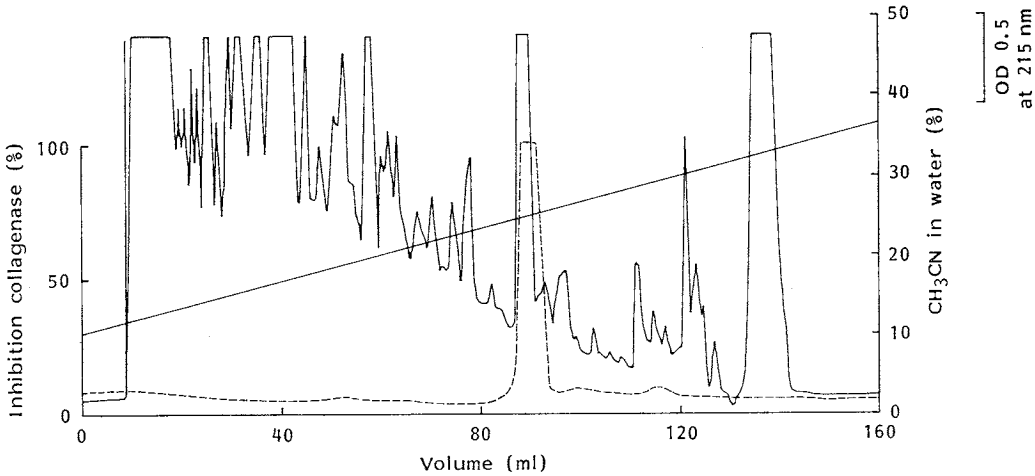


Fig. 2. HPLC gel filtration of partially purified collagenase inhibitor.

Column: 2×TSK SW 2000 (300×7.6 mm) developed using water at 0.5 ml/minute.

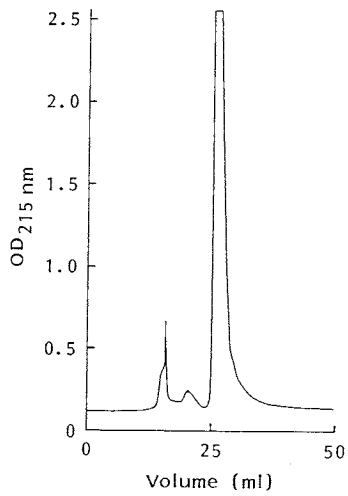
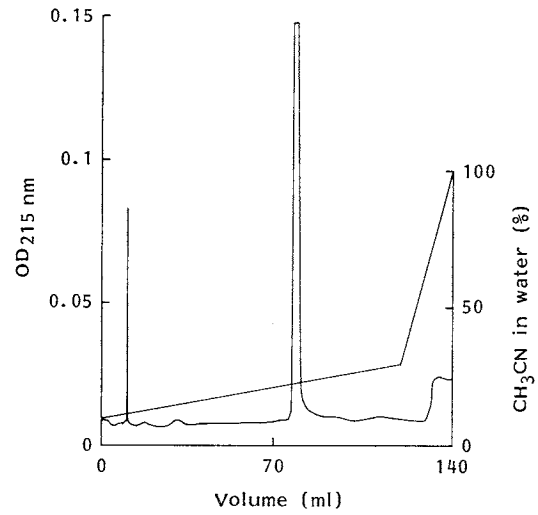


Fig. 3. Analytical HPLC of purified collagenase inhibitor.

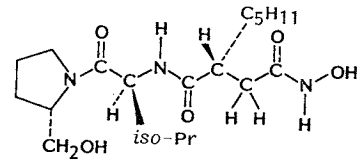
20 μl were injected on a C18 column developed using an acetonitrile gradient (15~30%) in water.



Discussion

Actinonin is a non-toxic pseudopeptide antibiotic first isolated from a *Streptomyces* culture by GORDON *et al.*[14]. The structure of actinonin is shown in Fig. 4. The relationship of structure to antibiotic function has been extensively studied[15].

Fig. 4. Structure of actinonin.



Metalloproteases like collagenase are known to be inhibited by molecules capable of chelation with the enzyme's essential zinc atom. Hydroxamate moieties such as that of actinonin are in principle capable of forming such chelates, and several hydroxamate inhibitors of metalloproteases have been described, for example in the case of the metalloproteases thermolysin[16], enkephalinase[17] aminopeptidase[18] and collagenase[10]. Very recently UMEZAWA *et al.*[19] have described the inhibition of aminopeptidase M and leucine aminopeptidase by actinonin.

It is likely that the collagenase-inhibitory activity of actinonin is mediated by its hydroxamate moiety but more specific interaction with other enzyme subsites is probable since we have shown that Gly-NHOH and *tert*-butoxycarbonyl-Leu-NHOH do not significantly inhibit collagenase (unpublished results). Since actinonin exhibits anti-collagenase activity similar to that of the best currently available low-molecular-weight inhibitors[9,10] we are now investigating the collagenase inhibitory activity of actinonin analogues.

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