

Proteinase Inhibitors of Bovine Nasal Cartilage

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Extracts from bovine nasal cartilage with 1 M-guanidinium chloride were fractionated by ultrafiltration. Gel chromatography of the low-molecular-weight material resolved three distinct fractions with inhibitory activity against (a) collagenases (22000 mol.wt.), (b) thiol proteinases cathepsin B and papain (13000 mol.wt.), and (c) trypsin and other serine proteinases (7000 mol.wt.).

Bovine nasal cartilage contains inhibitory activity against trypsin and mammalian collagenase (Kuettner *et al.*, 1974; Sorgente *et al.*, 1975), and it has been suggested that these activities are attributable to a cationic protein of mol.wt. 11000 (Kuettner *et al.*, 1976). It has been proposed that the inhibitor might be responsible for the cartilage inhibition of tumour invasion (Kuettner *et al.*, 1977), tumour neovascularization (Langer *et al.*, 1976) and angiogenesis (Kaminski *et al.*, 1977).

We have re-examined the inhibitory activities of an extract of bovine nasal cartilage, and found that the inhibitory activity against proteinases is attributable to distinct components.

Materials and Methods

All chemicals were either analytical grade or the best grade commercially available.

Papain (EC 3.4.22.2; twice-crystallized), pig trypsin (EC 3.4.21.4; type IX) and pig pancreatic elastase (EC 3.4.21.11; type III) were supplied by Sigma (London) Chemical Co., Kingston upon Thames KT2 7BM, Surrey, U.K. Bovine chymotrypsin (EC 3.4.21.1) was supplied by Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Leucocyte elastase (EC 3.4.21.11) and cathepsin G (EC 3.4.21.20) were prepared from human leucocytes by a method similar to that for human spleen described by Starkey & Barrett (1976a). Cathepsin B (EC 3.4.22.1) was kindly supplied by Dr. W. Schwartz of this laboratory, having been obtained from human liver by a method based on that of Barrett (1973).

Abbreviations used: Bz-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester; Bz-Arg-2-NNap, *N*-benzoyl-DL-arginine 2-naphthylamide; Z-Arg-Arg-2-NNap, *N*-benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide.

Extraction and fractionation of cartilage components

Fresh bovine nasal cartilage was cut into slices (about 0.2 mm thick) and extracted with 100 mM-Tris/HCl, pH 7.5, containing 1 M-guanidinium chloride (1:10, w/v), at 4°C for 48 h. The extract was filtered through a porosity-1 glass sinter, solid guanidinium chloride (0.4 g/ml of extract) was added (final concentration about 4 M) and the solution filtered through a porosity-3 glass sinter. Cartilage proteins were separated from proteoglycans by ultrafiltration through an XM-100A membrane (Amicon, Lexington, MA 02173, U.S.A.) at 4°C under pressure of N₂. The filtrate was concentrated about 20-fold by dialysis against Carbowax 20m (Raymond A. Lamb, London NW10 6JL, U.K.). Dialysis was carried out in Visking tubing (Medicell International, London EC4N 4SA, U.K.) that had been acetylated in pyridine/acetic anhydride (3:1, v/v) for 16 h at 22°C. The concentrated solution was then dialysed four times against 500 vol. of 100 mM-Tris/HCl, pH 7.5, at 4°C for 16 h, the precipitate was removed by centrifugation at 2500 g_{av}. for 5 min and discarded, and the clear supernatant used for inhibition studies and further fractionation.

The low-molecular-weight components of the concentrated ultrafiltrate were further fractionated according to molecular size by chromatography on Ultrogel AcA 54 (LKB Instruments, Croydon, Surrey CR2 8YD, U.K.) at 4°C. The column (85 cm × 1.5 cm; 150 cm³) was equilibrated with 50 mM-Tris/HCl, pH 7.5, containing 1 M-NaCl, 5 mM-CaCl₂ and 0.05% Brij 35 (BDH, Poole, Dorset, U.K.), and chromatography performed in the same buffer. The sample (5 ml) containing 1 M-NaCl was chromatographed by downward elution at 17 ml/h, and fractions (3 ml) of the eluate were collected for measurement of A₂₈₀ and inhibitory activity. The column was calibrated by elution of a mixture of Blue Dextran [Pharmacia (G.B.) Ltd., London

W5 5SS, U.K.; mol.wt. 2×10^6], bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 45000), soya-bean trypsin inhibitor (mol.wt. 21500), cytochrome *c* (mol.wt. 13400), Trasylol (a gift from Dr. Schmidt-Kastner, Bayer A.G., Wuppertal, Germany; mol.wt. 6500) and potassium ferricyanide (mol.wt. 329) under the above conditions.

Assays of inhibitory activity

Inhibitory activity against trypsin and other serine proteinases was measured in the azo-casein assay described by Starkey & Barrett (1976a) with 0.2M-Tris/HCl, pH 7.5, as buffer. For greater sensitivity, inhibitory activity against leucocyte elastase and cathepsin G was also measured with synthetic naphthyl ester substrates (Starkey & Barrett, 1976a). Bz-Phe-2-ONap (Sigma) was used in cathepsin G assays, and Z-Ala-2-O-Nap, kindly supplied by Dr. C. G. Knight of this Laboratory, was used in elastase assays. Inhibitory activity against cathepsin B and papain was measured with Bz-Arg-2-NNap (Bachem, Liestal, Switzerland) or Z-Arg-Arg-2-NNap (kindly

supplied by Dr. C. G. Knight) as substrate by the method described by Barrett (1976). Inhibitory activity against mammalian collagenase (EC 3.4.24.3) was measured with enzyme from rabbit skin cultures or from specific granules of human neutrophil leucocytes in the reconstituted ^{14}C -labelled collagen fibril assay, as described by Murphy *et al.* (1977).

Results

Inhibitory activity of cartilage components

The concentrated mixture of low-molecular-weight cartilage components showed inhibitory activity towards a variety of serine and thiol proteinases, and also mammalian collagenases (metallo-proteinases). In the azo-casein assay, the inhibitory activity towards trypsin was shown to be linear with increasing inhibitor concentration, as described by Kuettnner *et al.* (1975). The extract from 100mg of cartilage, sufficient to give 80% inhibition of 1 μg of trypsin, showed less than 10% inhibition of 4 μg of chymotrypsin and no detectable inhibition of 2 μg of

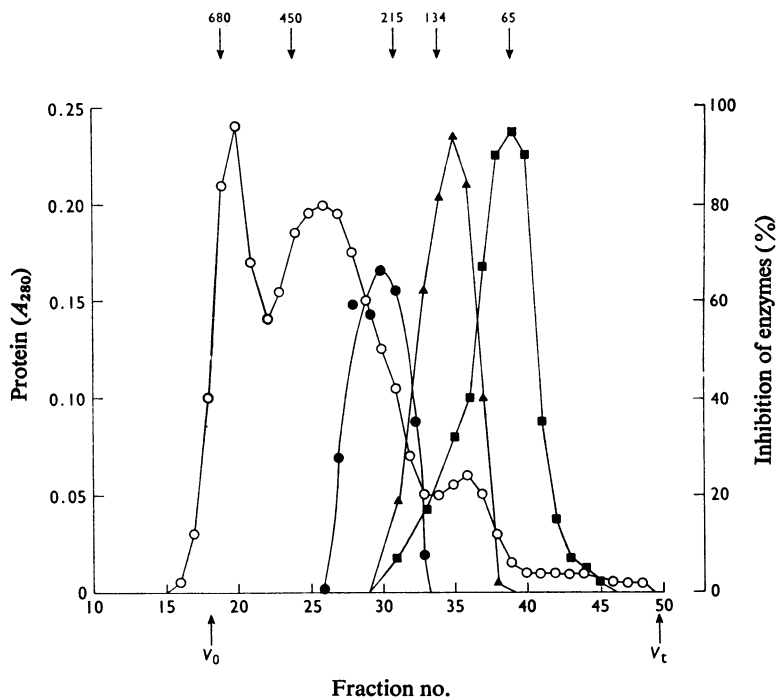


Fig. 1. Separation of the proteinase inhibitors of bovine nasal cartilage by gel chromatography

The concentrated low-molecular-weight fraction from the cartilage was run on Ultrogel AcA 54 as described in the text. Effluent fractions were analysed for protein (A_{280}) (○) and inhibitory activity (as a percentage of the uninhibited control) against collagenase (●) (50 μl of sample against 17 munits of enzyme), cathepsin B (▲) (400 μl against 1.3 munits) and trypsin (■) (200 μl against 0.5 μg). The molecular-weight calibration was obtained with the reference proteins listed in the Materials and Methods section. V_0 , void volume; V_t , total bed volume.

pancreatic elastase. (These enzyme concentrations give equivalent ΔA values when used alone in the azo-casein assay.) No inhibitory activity was detectable against either leucocyte elastase or cathepsin G in the azo-casein assay. Weak inhibitory activity was detectable, however, against the leucocyte enzymes when the synthetic substrates were used. A concentration sufficient to give 80% inhibition of 1 μg of trypsin gave only 15% inhibition of 0.5 μg of cathepsin G and 60% inhibition of 0.1 μg of leucocyte elastase.

Inhibitory activity was detected against both cathepsin B and papain with Bz-Arg-2-NNap as substrate. In each case the degree of inhibition increased linearly with concentration of inhibitor to complete inhibition. An inhibitor concentration sufficient to produce 40% inhibition of 0.6 μg of cathepsin B gave complete inhibition of 0.3 μg of papain. The azo-casein assay was not sensitive enough for the detection of inhibitory activity with the thiol proteinases.

In the reconstituted collagen fibril assay, linear inhibition of rabbit skin collagenase was obtained with increasing extract concentration up to 70% inhibition. Extract equivalent to 1 g of the original cartilage was found to contain 1.3 units of collagenase inhibitory activity, as defined by Murphy *et al.* (1977). The inhibitory activity was completely stable to heating at 90°C during 10 min. Collagenase from human polymorphonuclear leucocytes was also inhibited by the extracts. No inhibition of bacterial collagenase was detectable.

The cartilage components were fractionated according to molecular size on Ultrogel AcA 54 (Fig. 1). Most of the material absorbing at 280 nm was eluted in two peaks of mol.wt. above 30000; there was no inhibitory activity for any enzyme tested, in this region. A third minor peak was observed with a mol.wt. of about 10000.

When the column fractions were tested in the azo-casein assay with trypsin, a single peak of inhibitory activity was detected corresponding to the elution position of Trasylol and therefore to a mol.wt. of about 7000. Measurement of inhibitory activity of the fractions against cathepsin B with Z-Arg-Arg-2-NNap showed a single peak of inhibitory activity corresponding to the elution position of cytochrome c, or a mol.wt. of about 13000. Inhibitory activity towards papain was located in the same effluent fractions as for cathepsin B and was presumably due to the same compound. The inhibitor of the thiol proteinases showed thermal stability, with no significant loss of activity during 10 min at 90°C. The inhibitory activity of the fractions towards mammalian collagenase also exhibited a single peak close to the elution position of soya-bean trypsin inhibitor. Thus this inhibitor is distinct from those for the serine and thiol proteinases and has a mol.wt. of about 22000.

Discussion

Kuettner *et al.* (1974, 1976, 1977) and Sorgente *et al.* (1975) have shown that bovine scapula cartilage contains a low-molecular-weight protein fraction that possesses inhibitory activity towards both trypsin and mammalian collagenase, and suggest that the inhibition is due to a single protein of mol.wt. about 11000 (Kuettner *et al.* 1976). Langer *et al.* (1976) found that a similar purification procedure yielded a number of proteins, the major component being about 16000 in mol.wt.

Our results show that the inhibitors of trypsin and collagenase are distinct, and that bovine nasal cartilage also contains a third inhibitor directed against thiol proteinases. In gel chromatography, the collagenase, thiol proteinase and trypsin inhibitors were eluted as though they had mol.wts. of about 22000, 13000 and 7000 respectively.

The trypsin inhibitor appeared at the same elution volume as Trasylol, and was previously shown by Kuettner *et al.* (1974) to be identical in electrophoretic mobility with Trasylol. Trasylol is a commercial preparation of the cationic (Kunitz) proteinase inhibitor widely distributed in bovine tissues and plasma (Anderer & Hörnle, 1968), and we suggest that this is the bovine cartilage inhibitor of trypsin. The relatively weak inhibition of the two leucocyte serine proteinases is characteristic of Trasylol (Starkey & Barrett, 1976*b,c*). The presence of Trasylol in cartilage could be due to its synthesis by bovine chondrocytes, or to uptake from the plasma.

The peak of inhibitory activity against the thiol proteinases, papain and cathepsin B, was attributable to a compound of about 13000 mol.wt. Protein inhibitors of the thiol proteinases with this molecular weight have previously been detected in chicken egg-white (Sen & Whitaker, 1973), rabbit skin (Udaka & Hayashi, 1965*a,b*) and rat skin (Järvinen & Hopsu-Havu, 1975). All these inhibitors may well be related, since thermal stability similar to that reported here for the cartilage inhibitor has previously been reported for those of egg-white and rabbit skin.

The collagenase-inhibitory activity occurred as a single peak of about 22000 mol.wt., a value significantly different from the other lower-molecular-weight inhibitors that have been described from serum (Woolley *et al.*, 1976) or polymorphonuclear-leucocyte cytosol (Kopitar & Lebez, 1975). Preliminary studies suggest that the cartilage inhibitor is distinct from the 30000-mol.wt. inhibitor synthesized by connective tissues in culture (Murphy *et al.*, 1977). Not only does the cartilage inhibitor differ in molecular size, but its effect is not reversed by thiol-binding reagents, and it is also active against the leucocyte specific collagenase (Sellers *et al.*, 1977). The inhibitor had no activity against clostridial collagenase, unlike that extracted from human

cartilage of arthritis patients by Ehrlich *et al.* (1977).

It is not clear why affinity chromatography on trypsin-Sepharose has previously been of use in the purification of the collagenase inhibitor of cartilage (Kuettner *et al.*, 1976) and the inhibitor of neo-vascularization (Kaminski *et al.*, 1977), since Trasylol was found to possess neither of these activities. Possibly some form of non-specific adsorption was involved. We would suggest that the question of whether the interesting biological effects of low-molecular-weight components extracted from cartilage are due to any of the proteinase inhibitors, and if so which ones, will be answered only when biological experiments have been made with more thoroughly purified preparations, including some from cartilage of species, such as man, which lack the Kunitz inhibitor.

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