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Susceptibility of the cartilage collagens types II, IX and XI to degradation by the cysteine proteinases, cathepsins B and L

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We have investigated the susceptibility of both the helical and non-helical regions of isolated rat chondrosarcoma collagens, types II, IX and XI, to degradation by the cysteine proteinases, cathepsins B and L. Both enzymes degrade these collagens at temperatures from 20 to 37°C and pH values from 3.5 to 7.0. Cleavage occurs only within the non-helical domains unless the helix is destabilized. Cathepsin L is more effective than cathepsin B on a molar basis and they appear to cleave at different sites. Since these cathepsins can degrade cartilage collagens at pH values near neutrality, they may contribute to the destruction of cartilage observed in arthritis.

Cysteine proteinase; Cathepsin B; Cathepsin L; Cartilage collagen; Matrix degradation; Arthritis

1. INTRODUCTION

Cartilage has a distinct supramolecular organization which confers it with both tensile strength and resilience. The major collagen, type II, forms a three-dimensional meshwork of fibers which are primarily responsible for the mechanical strength of cartilage. Entrapped within this structure are the proteoglycans which endow the cartilage with its viscoelastic properties due to their ability to bind large amounts of water. In addition other collagens, types VI, IX, X, and XI, are present; however, their precise function is less clear [1]. Recent findings have shown that type XI may be present within type II collagen fibrils and that type IX is located on the fibril surface suggesting that type IX and type XI may regulate the assembly and organization of the type II fibrils and hence influence the mechanical properties of the tissue [2,3].

The ability of cartilage to withstand high pressure and to absorb shock relies on matrix integrity. Any disturbance of the macromolecular organization of cartilage will alter its biomechanical properties and affect biological function. Alterations in the extracellular matrix may occur through the increased presence or action of proteinases, which in cartilage may be derived from two sources: externally by the secretion of proteinases from infiltrating macrophages and synovial cells at their junction with cartilage; and internally through the action of proteinases derived from the chondrocyte itself.

Two classes of proteinases are known to degrade collagens in vitro: the metalloproteinase family, including collagenase, elastase, gelatinase and stromelysin, which function at neutral pH [4,5] and the collagenolytic cysteine proteinases, cathepsins B, L, N and S [6,7] whose action on collagens is usually limited to low pH values, which cause collagen to swell. Both classes of proteinases have been detected in synovial fluids from humans with both osteo- and rheumatoid arthritis and thus may contribute to the loss of extracellular matrix during these diseases [8–11].

Cartilage collagen types II and X can be degraded by human synovial collagenase and neutrophil elastase, while type IX is cleaved only by the latter at elevated temperatures [12,13]. Recently, stromelysin has been shown to partially degrade type IX collagen probably cleaving within one of the non-collagenous domains releasing a small fragment from the molecule [14]. In this paper results of our investigation into the action of purified human cathepsins B and L on cartilage collagens show that collagen types II, IX and XI are degraded by these proteinases even at pH values close to neutrality suggesting that these enzymes may have a destructive role in osteo- and rheumatoid arthritis.

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Abbreviations: Ep-475, L-3-carboxy trans 2,3-epoxypropionyl-leucylamido-(3-methyl) butane; NHMec, 7-(4-methyl) coumaryl-amide; Z, *N*-benzyloxycarbonyl

2. MATERIALS AND METHODS

2.1. *Purification of enzymes*

Cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) were purified from human liver as previously described [15] with the inclusion of a Sephadex G75 gel filtration step after the ionic exchange in CM Sephadex for cathepsin B. Their concentration was determined by ac-

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tive site titration using the synthetic inhibitor Ep-475 and the activity assayed with the synthetic substrate Z-Phe-Arg-NHMec [16]. Analysis by ELISA using antibodies to cathepsins B and L [15] showed that our preparation of cathepsin L was free of cathepsin B and vice versa.

2.2. *Preparation of collagens*

A mixture of cartilage collagens types II, IX and XI was isolated from rat chondrosarcoma tissue by neutral salt fractionation as described previously [17]. A small amount of type I collagen was present as indicated by the α2(I) chain visible on the SDS polyacrylamide gels.

2.3. *Degradation of soluble collagens*

Neutral salt-soluble cartilage collagens were suspended in 0.1 M sodium citrate buffer, of pH values 3.5, 4.5, 5.5 and 7.0, containing 10% (w/v) glycerol, 1.0 mM Na_2EDTA, 10 mM cysteine to a final concentration of 2.5 mg/ml. To 20 μl of this mixture, purified human liver cathepsin B or L (exact concentrations are shown in figure legends), was added and incubated for various lengths of time at 20, 30 or 37°C. Digestion was stopped by carboxymethylation of the samples by the addition of iodoacetic acid to a final concentration of 0.1 M. The samples were made 1% (w/v) SDS and brought to 65°C for 15 min prior to electrophoresis in a 5–10% gradient SDS-polyacrylamide gel [18].

3. RESULTS AND DISCUSSION

Figs 1, 2 and 3 show the time courses of digestion of a mixture of cartilage collagens by cathepsins B and L at temperatures of 20, 30 and 37°C respectively and at 4 different pH values, 3.5, 4.5, 5.5 and 7.0. Individual collagen types in the mixture are resolved into distinct bands upon separation by SDS-PAGE in non-reducing conditions and represent: IX; α1(XI); α2(XI); and a mixture of α1(II) and α3(XI) as indicated in the figures. Cleavage was completely due to cysteine proteinases as total inhibition of this degradation was observed in the presence of Ep-475, a specific cysteine proteinase inhibitor, and no degradation was observed when the collagens were incubated in the absence of enzymes. A slight decrease in band intensity of type IX was occasionally observed after prolonged incubation times (10 to 20 h) especially at lower pH values. This is probably due to the breakage of the disulphide bonds in the type IX molecule during incubation with 10 mM cysteine.

The actions on collagen types II and XI by these cathepsins at 20°C (Fig. 1) and 30°C (Fig. 2) are similar. Digestion results in the cleavage of the α1(II) chain and the three chains of type XI at pH values at or below 5.5. At more neutral pH values this occurs but at a much reduced rate (compare panels C and D in Fig. 2). At all pH values the α1(XI) chain is the most rapidly degraded component. From the time course of enzyme digestion we can deduce that there is a slight decrease in the observed molecular weight of α1(II), α2(XI) and α3(XI) and an apparently larger molecular weight decrease in the α1(XI) chain, suggesting that the enzymes cleave in the teleopeptide regions. Whether this is occurring at the amino and/or the carboxy non-helical domains is not precisely known. However the decreases in molecular weight of about 5% are consistent with cleavage occurring at both termini. The larger decrease in the α1(XI) chain suggests that this chain has a larger non-helical portion than the α2 and α3(XI) chains.

The fragments of II and XI generated by the two cathepsins appear to migrate differently, suggesting that they cleave at non-identical sites within the teleopeptide regions. For instance cathepsin B cleaves a larger fragment form the α1(XI) chain than cathepsin L resulting in the appearance of a discrete fragment near the buffer front (arrow in Fig. 1, pH 4.5). The appearance of this fragment concomitant with the disappearance of the α1(XI) chain was clearly apparent from time course studies (data not shown). Cathepsin L appears to cleave off a slightly larger fragment from the other chains of type XI and type II than cathepsin B (compare in Fig. 1, pH 5.5, the lanes marked L and B).

At 37°C the action on types II and XI collagen shows a marked pH dependency (Fig. 3). At high pH values and short incubation times the cleavage is similar to that observed at lower temperatures. However, after longer incubations the chains disappear, in general without the formation of discrete fragments, suggesting that cleavage within the helical region is occuring. Both enzymes are capable of this type of cleavage. At the lower pH values any initial cleavage in the teleopeptide region is less readily observed before the chains completely degrade, indicating that the triple helix unwinds. Type II collagen appears to be more resistant than type XI chains presumably reflecting a greater thermal stability of its helical region under these assay conditions.

The metalloproteinase action on types II and XI collagen is quite different from that observed here for the cathepsins. Unlike cathepsins B and L these enzymes cleave within the helical domains: for instance, collagenase cleaves at one specific site within the triple helical region of type II collagen and the α3 chain of type XI [13]; while the other two chains of type XI have been found to be degraded by a tumour derived metalloproteinase [12].

It is known that the type IX collagen molecule contains 4 non-collagenous domains comprising 12 non-helical regions [19] each of which may be susceptible to proteolytic attack by these cathepsins. As can be seen from Fig. 1, a number of similar, but not necessarily identical, high molecular weight peptides are generated by both enzymes at pH values of 5.5 and 7.0, suggesting cleavage within the C- and N-terminal domains, NC1 and NC4 respectively [19]. Cathepsin L at pH values of 3.5 and 4.5 also cleaves within the NC2 and NC3 domains producing fragments similar to type M (COL2) and CF2 (COL1), known to be generated by pepsin digestion of type IX [16], as well as two intermediate degradation products labelled X and Y. All these peptides are reducible by mercaptoethanol (data

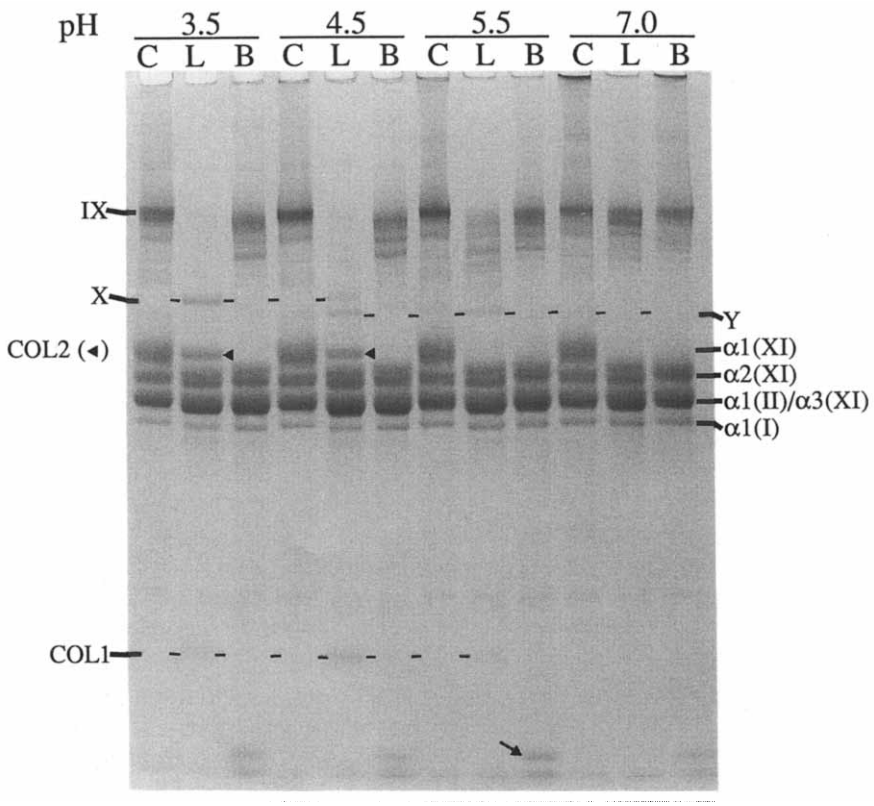

Fig. 1. Digestion of cartilage collagens by cathepsins B and L at 20°C. Neutral salt-soluble cartilage collagens were digested at pH values of 3.5, 4.5, 5.5 and 7.0 for 20 h using either 10 or 40 pmol of cathepsin L (L) or cathepsin B (B) respectively or no enzyme (C), and then separated by SDS-polyacrylamide gel electrophoresis. The peptides labelled X, Y, COL2 (◂) and COL1 are all derived from type IX collagen. α1(XI) fragment derived by digestion with cathepsin B (➡).

not shown) further supporting their identification as being derived from type IX collagen. From the known molecular weights of the collagenous domains of type IX we have tentatively assigned the peptide X as COL1 + COL2 and peptide Y as COL2 + COL3 (Fig. 1). These peptides arise by cleavage in the NC3 and NC2 domains, respectively.

The appearance of these fragments varies with pH. At pH 3.5 most of the type IX is digested to peptide X and COL2 indicating that although cleavage has occurred in both the NC2 and NC3 domains a considerable amount of the NC2 domain remains uncleaved. At pH 4.5. the most prominent bands are COL1 and COL2 indicating extensive digestion of both the NC2 and NC3 domains. Small but equal amounts of peptides X and Y, however, remain undigested. At pH 5.5 the amount of cleavage of both domains is limited; only peptide Y and COL1 are apparent indicating that cleavage has occurred only within NC2.

At higher temperatures (Figs 2 and 3) only small amounts of these resistant peptides are seen as they are probably thermally unstable and would be rapidly degraded into small peptides. None of these bands are generated by digestion with cathepsin B but longer incubation times or higher concentrations of enzyme may be required to produce limited cleavage within the NC2 and NC3 domains. The higher activity of cathepsin L in cleaving these regions may be responsible for its greater efficiency in degrading type IX collagen under all conditions including neutral pH values.

This cleavage within the non-helical domains of type IX by the cathepsins is very different to cleavage by the metalloproteinases, elastase [13] and stromelysin [14]. The latter has been found to cleave initially at one site within a non-helical region, generating a molecule whose triple helical character is still retained [14]. Comparison of the reported activity of stromelysin with our results suggests that cathepsin L is more effective at degrading type IX collagen.

The ability of these cathepsins to cleave at many sites within the non-collagenous domains of these collagens indicates their rather broad substrate specificity. The observed differences between the two cathepsins in the rate of degradation and the size of fragments generated may reflect the more restricted binding of amino acids with large side groups by cathepsin B compared to cathepsin L [20].

In conclusion, these results are the first to demonstrate that the two cysteine proteinases, cathepsins B and L can degrade cartilage collagens and at pH values near neutrality. The ability to degrade within the non-helical domains of these collagens is of major conse-

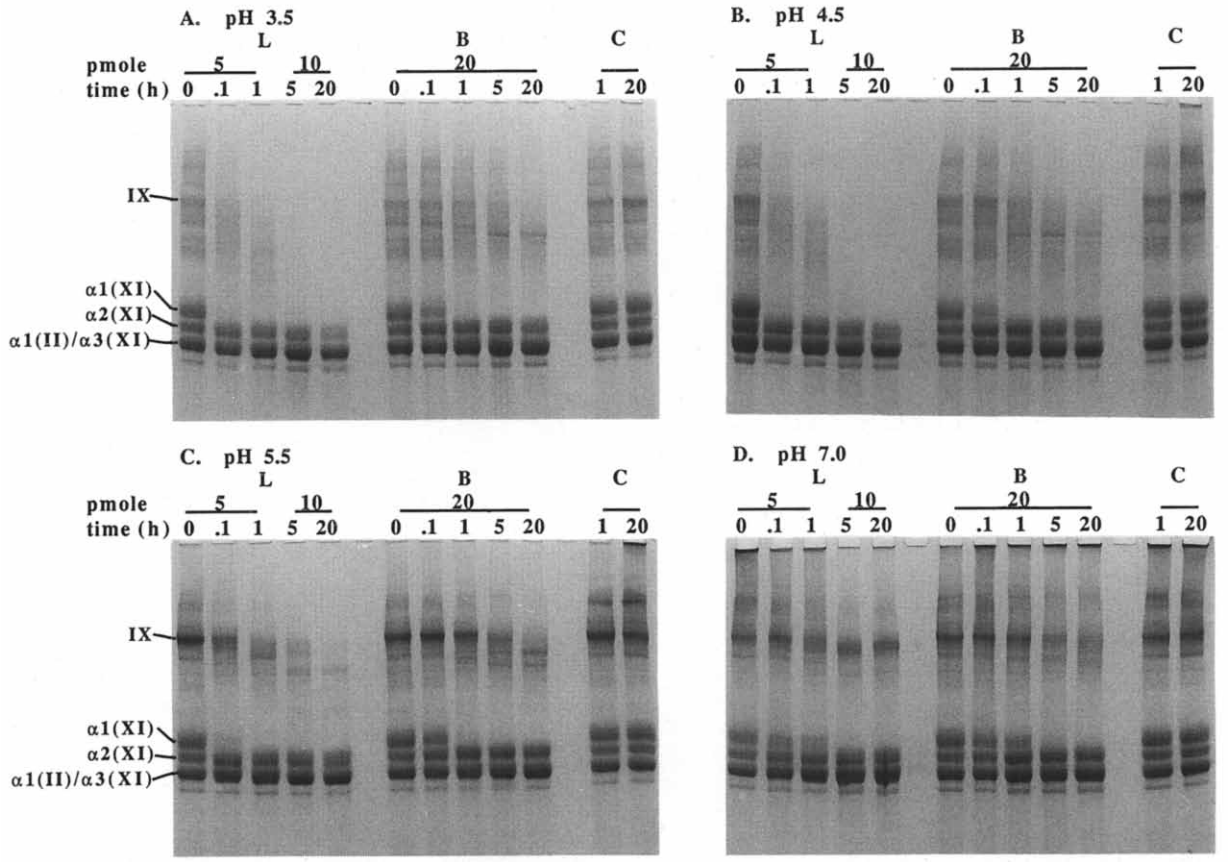

Fig. 2. Digestion of cartilage collagens by cathepsins B and L at 30°C. As Fig. 1 except times and enzyme concentrations as shown.

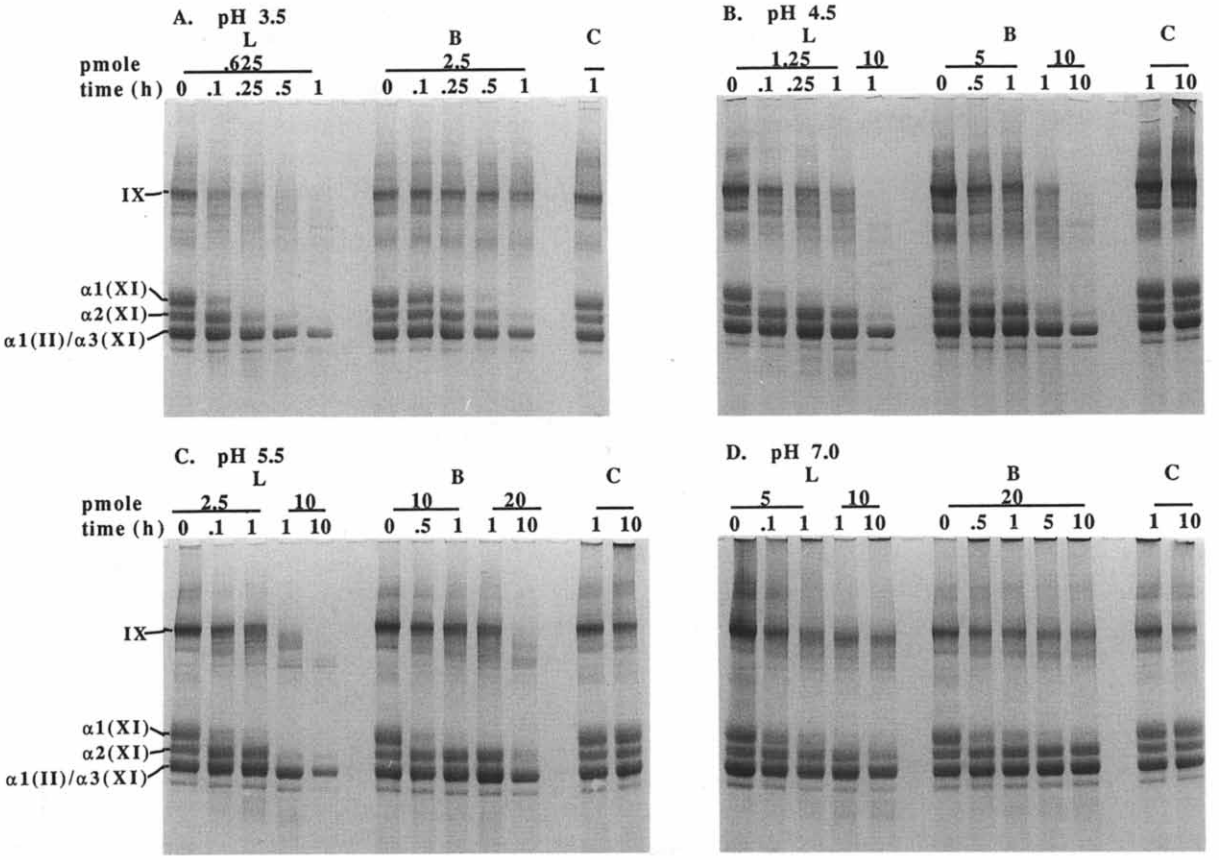

Fig. 3. Digestion of cartilage collagens by cathepsins B and L at 37°C. As Fig. 1 except times and enzyme concentrations as shown.

quence to the cartilage architecture since these regions are involved in collagen crosslinking, which is important in the stabilization of the matrix. In addition, cleavage within the non-helical portions of types IX and XI, particularly by cathepsin L, may have important consequences for type II fibril architecture as it is thought that these collagens are involved in regulating fibril diameter. It is also evident that the cathepsins recognize different regions of the cartilage collagens than the matrix metalloproteinases. As type II fibrils appear to be coated with type IX it may be that collagenase is unable to degrade type II until type IX is removed, perhaps by the action of cathepsin and/or elastase and stromelysin. It is likely therefore that both the cysteine- and metallo-proteinases play important roles in the maintenance and remodeling of the cartilage architecture and also in its destruction as in arthritis.

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