CATHEPSIN D: CLEAVAGE OF SOLUBLE COLLAGEN AND CROSSLINKED PEPTIDES

Paul G. SCOTT and C. Harold PEARSON

Department of Oral Biology, University of Alberta, Edmonton, Alberta T6G 2N8, Canada

Received 9 January 1978

1. Introduction

Cathepsin D is a lysosomal proteinase of wide distribution [1]. It has been shown to occur extracellularly in connective tissues [2] where, notwithstanding its low pH optimum, it has been implicated in the destruction of proteoglycans [3]. Previous authors reported little [4] or no [5] effect on gelatin and no cleavage of the β or γ components of soluble collagens [5]. During studies on the effects of cathepsin D on non-collagen components of the NaCl-insoluble fraction of bovine skin [6] a specific dissolution of collagen was noted, with maximal effect occurring near pH 4. Cleavage of intermolecular crosslinks was considered the most plausible mechanism for such an effect and we therefore decided to examine this possibility using as model substrates, soluble collagen, gelatin, CNBr peptides of collagen and a peptide containing a crosslink, made available through a related investigation. This paper reports that there is a cathepsin D-susceptible peptide bond within the extra-helical carboxy-terminal sequence of the α1(I) chain, located on the amino-terminal side of the (δ-hydroxy-) α-amino adipic acid-δ-semialdehyde residue which is involved in the formation of intermolecular crosslinks.

Abbreviations: CNBR, cyanogen bromide; SDS, sodium dodecylsulphate; EDTA, ethylenediaminetetra-acetic acid

* The CNBr peptide α1CB6 contains a residue of lysine or hydroxylysine which may be converted to the aldehyde and become involved in crosslinking [15]. In this work it was isolated after reduction to the alcohol

2. Materials and methods

Cathepsin D was prepared from bovine thymus as in [7], with the addition of chromatography on Sephadex A-50 in 5 mM phosphate buffer, pH 7.0, between the haemoglobin–agarose affinity column and gel chromatography on Sephadex G-100. This removed haemoglobin and other inert proteins which co-eluted with cathepsin D from the first column. The specific activity of the final preparation was 50 units/mg (units [7]). Electrophoresis in 10% polyacrylamide gels containing 0.1% (w/v) SDS, after reduction with 2-mercaptoethanol, revealed one major band with mol. wt 45 000 ± 1000 and a minor component of 28 000 + 1000. The specific activity and electrophoretic properties of the enzyme preparation are closely comparable to [7]. Pepstatin [3], in approx. 20-fold molar excess over the enzyme, completely inhibited the hydrolysis of haemoglobin.

Acid-soluble collagen was prepared from the corium layer of a freshly-flayed foetal calf-skin. Extractants contained 3 mM sodium azide and four proteinase inhibitors: 5 mM benzamidine hydrochloride, 5 mM *N*-ethyl maleimide, 10 mM 6-amino hexanoic acid and 10 mM EDTA. Procedures were identical in other respects to [8]. Carboxymethyl cellulose chromatography was used to isolate α1 and α2 chains [9] which were purified by chromatography on Agarose A5m eluted with 1 M $CaCl_2$ / 0.05 M Tris, pH 7.4 [10]. Cyanogen bromide (CNBr) peptides were prepared from purified α1 and α2 chains as in [11]. Fractions containing α1CB6ald* and the crosslinked peptide β12CB6 × 4 were isolated from uncrupted bovine dentine collagen [12] which had been reduced with tritiated sodium borohydride

(820 mCi/mmol) at a reductant to substrate ratio of 1:40 in 0.15 M NaCl/0.1 M sodium phosphate buffer, pH 7.4, for 2 h at room temperature. The reduced collagen was dialyzed against 0.1 M acetic acid, then lyophilized and digested with CNBr [13] and fractionated on Agarose A5m, as in [14]. Tritium-labelled fraction 8p, which contains β12CB6 × 4, was isolated by re-chromatography on Agarose A5m whereas α1CB6ald was isolated by re-chromatography of an A5m fraction on two coupled 2.5 × 100 cm columns of Agarose A1.5m.

Digestions with cathepsin D were performed at pH 3.9–4.0 in sodium phosphate buffer of about 0.15 M, in the presence or absence of pepstatin at 10 μg/ml, at substrate conc. 1–2 mg/ml and enzyme conc. 0.1 or 1.5 units/ml with other conditions as noted in figure legends. Digestion was terminated by freezing to −20°C. For analysis by electrophoresis in 7.5% polyacrylamide gels containing 0.1% (w/v) SDS, the pH of the digests was raised to 6.5–7.0 by addition of 0.1 M NaOH and 2/3 vol. concentrated sample buffer (5 M urea, 0.55% (w/v) SDS, 0.3 M sodium phosphate pH 7.2). Other procedures for electrophoresis were as in [11]. Gels stained with Coomassie blue R250 were scanned at 560 nm in a Gilford 252 spectrophotometer. Peak areas were measured with an Ott planimeter. Radioactivity was determined in slices of the gels after digestion with NCS solubilizer [14].

3. Results

When foetal calf-skin acid-soluble collagen was incubated with cathepsin D (1.5 units/ml), at 25°C for 18 h, there was no measurable change in the ratio of α/β components. A difference of 5% would have been readily detectable with the present techniques. This finding agrees with [5]. At 45°C, however, smaller fragments (3 and 6, fig.1B) were formed. This effect was completely inhibited by pepstatin (fig.1A). Again, no change in α/β ratio was observed but there was a significant decrease in the individual contents of α2 and β12, suggesting that the fragments were derived from the α2 rather than the α1 chain. This was confirmed by results obtained when the CNBr peptides of isolated and purified α1 and α2 components were employed as substrates (fig.2). Three new components

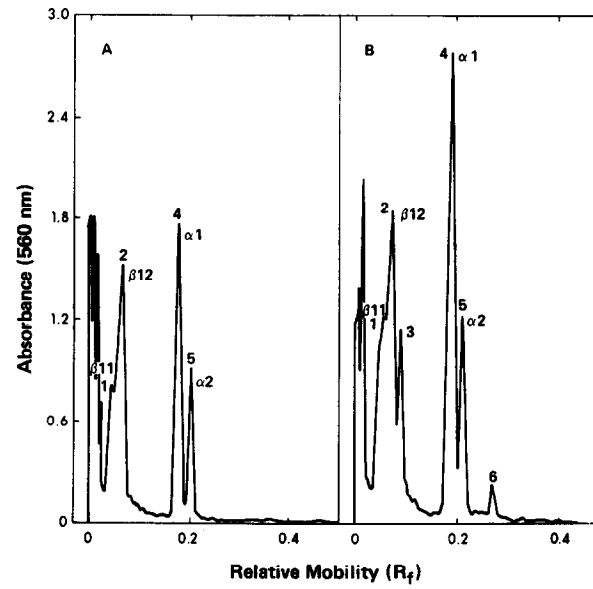

Fig.1. Soluble collagen was incubated for 2.5 h at 45°C with cathepsin D (0.1 units/ml) in the presence (A) or absence (B) of pepstatin (10 μg/ml). After neutralization with 0.1 M NaOH, aliquots were electrophoresed on 7.5% polyacrylamide gels containing 0.1% SDS. Buffer controls (no enzyme or pepstatin) gave identical results to (A).

(2, 4 and 6, fig.2B) were formed from the α2 chain, the molecular weights of which were determined from a calibration based on gels of untreated α2CB peptides run in parallel. No low molecular weight fragments were detected but by planimetry there were significant decreases in the contents of α2CB 3.5 (mol. wt 60 000) and uncleaved α2, (mol. wt 94 200) which were quantitatively accounted for by the appearance of components 4 (mol. wt 38 000) and 6 (mol. wt 22 000) and 2 (mol. wt 73 000) and 6, respectively. α2CB4 was unaffected. Since the order of CNBr peptides in the α2 chain is 1–0–4–2–3.5, with α2CB3.5 accounting for 660 residues [16], we conclude that cathepsin D hydrolyzes this sequence at a position about 240 residues from the carboxy-terminus (average residue weight in collagen is approx. 92).

The only α1CB peptide affected by cathepsin D was α1CB6, the apparent molecular weight of which decreased from 19 000–18 600 (fig.2A). Such a change is outside experimental error (± 2–3%). An identical result was obtained when soluble collagen

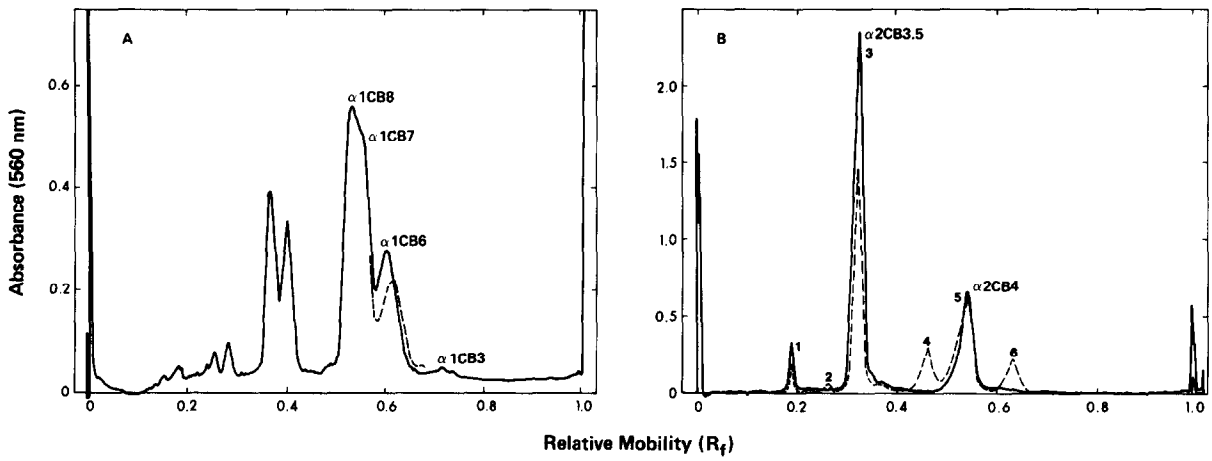

Fig.2. CNBr peptides of the α1 chain (A) and α2 chain (B) were incubated with cathepsin D (0.1 units/ml) in the presence (——) or absence (- - - -) of pepstatin, 10 μg/ml. Other conditions were as in fig.1.

which had been digested at 25°C was converted to CNBr peptides and run on SDS gels. This places the cleavage of the α1 chain within the 25 residue extra-helical sequence at the carboxy-terminal end of the molecule, usually designated residues C1-25 [16].

When α1CB6, labelled in position C17 by reduction of the δ-hydroxy-α-amino adipic acid-δ-semi-aldehyde with tritiated $NaBH_4$, was digested with cathepsin D (fig.3A,B) much of the radioactivity was lost from the major component which was reduced in molecular weight by 1000. A fraction (8p), isolated from bovine dentine collagen and shown to contain a dimeric peptide formed from the α1CB6 and α2CB4 peptides of adjacent collagen molecules joined by a tritiated dihydroxylysinonorleucine residue [14], was cleaved by cathepsin D as shown in figure 3C,D. The fragment labelled with highest specific activity is that identified as α2CB4, a result which is consistent with cleavage of the crosslinked peptide only within the extra-helical region of α1CB6. Explicit characterization of these fragments will be reported elsewhere.

4. Discussion

We have described hitherto unreported effects of cathepsin D on collagen. There seems little doubt that these are due to cathepsin D, rather than to a contaminant. Other enzymes which are inhibited by pepstatin, such as cathepsin E or renin, seem to be of much more restricted distribution and are unlikely to be present in our preparation. A synergistic effect with traces of cathepsin B1 can probably be ruled out because this latter enzyme would almost certainly have caused detectable conversion of β to α components [5,17].

No effect of cathepsin D on gelatin was noted [5], in contrast with results reported here. This apparent discrepancy may possibly be explained by the use of a different collagen (rat skin) by those authors. It should be emphasized that the susceptible peptide bond within the α2 chain was accessible only in denatured collagen and that the α2 chain was a much poorer substrate for cathepsin D than was the extra-helical carboxy-terminal sequence of the α1 chain, being cleaved to a much smaller extent under equivalent conditions.

In order to demonstrate cleavage by cathepsin D in the extra-helical sequence of the α1 chain it was necessary to employ a relatively short peptide, α1CB6, as substrate or to separate the modified α1CB6 after treatment of native collagen with the enzyme. It should also be noted that this sequence is usually lost from collagen extracted into acetic acid, presumably due to the action of proteolytic enzymes [18]. The present work used collagen extracted with solutions containing a combination of proteinase inhibitors which has been shown to substantially prevent this loss (P.G.S., unpublished).

The ability to depolymerize crosslinked peptides, together with its known extracellular occurrence [2], make cathepsin D a strong candidate for involvement in collagen degradation in vivo. In support of this we have recently found (P.G.S. and C.H.P., unpublished) that the enzyme is able to solubilize a significant proportion of native acid-insoluble bovine foetal-skin collagen with concomitant shortening of the carboxy-terminal extra-helical sequence of the α1 chain.

The enzyme may be used to discriminate between crosslinks involving amino-terminal and carboxy-terminal sequences, and this property, together with

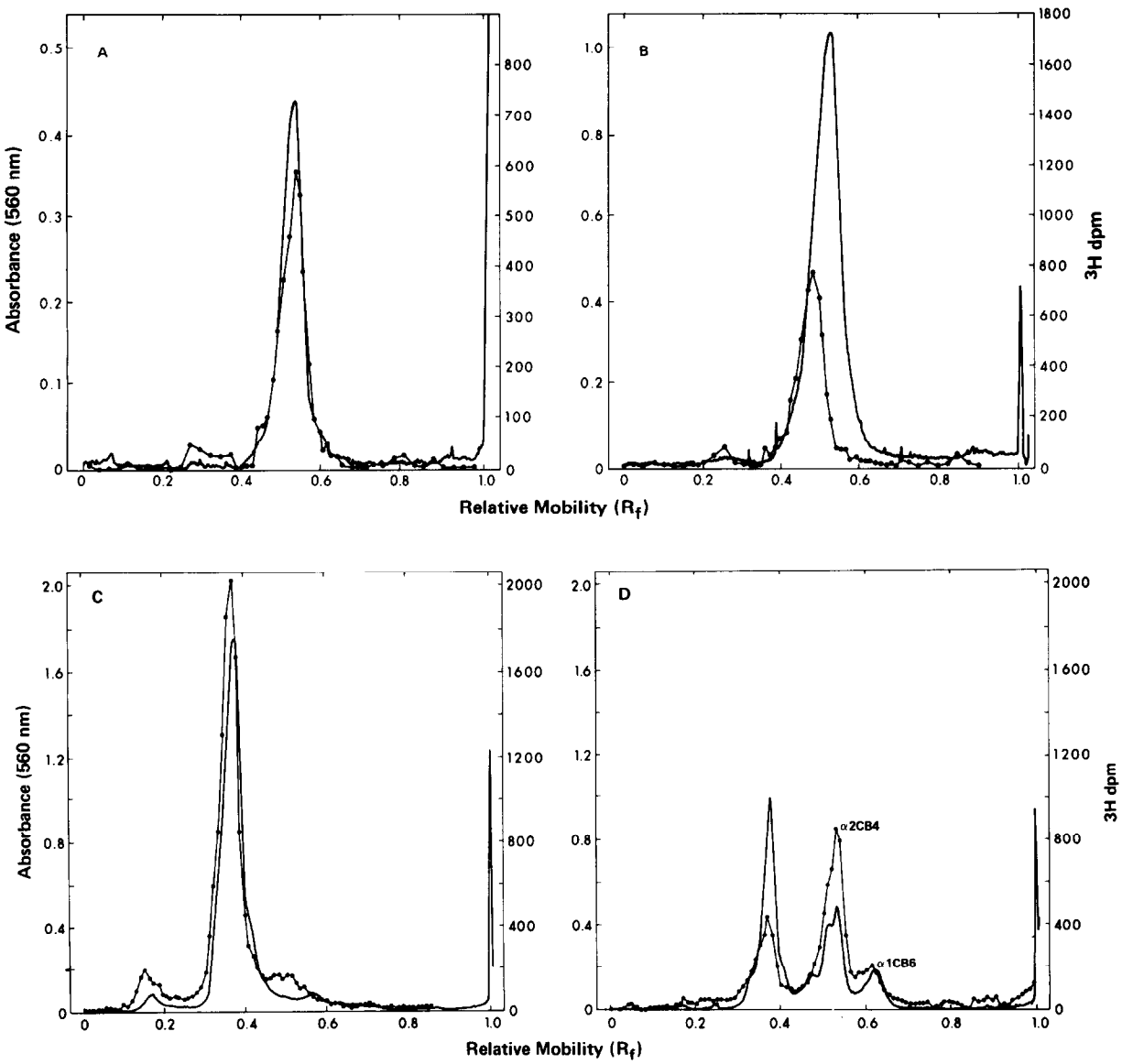

Fig.3. The CNBr peptide α1CB6, labelled in the extra-helical residue C17, incubated with cathepsin D (0.1 units/ml) in the presence (A) or absence (B) of pepstatin. The partially purified crosslink-containing peptide β12CB6 × 4 was incubated with cathepsin D (1.5 unit/ml), 45°C, 3 h in presence (C) or absence (D) of pepstatin (10 μg/ml). Other conditions as in fig.1. (——) $A_{560\ nm}$ (•——•) ^3H dpm.

is very limited action on denaturated collagen, recommend it as a probe in the study of intermolecular crosslinking of insoluble collagens.

Acknowledgements

We thank Miss Oy-Wah Yau and Mrs Sophie Lehocky for excellent technical assistance. Supported by the Medical Research Council of Canada. P.G.S. is an MRC Scholar.

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