ACTION OF CATHEPSIN N ON THE OXIDIZED B-CHAIN OF BOVINE INSULIN

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1. Introduction

Cathepsin N, formerly known as collagenolytic cathepsin, has been purified from bovine spleen [1] and human placenta [2]. It is a thiol enzyme and, like cathepsin B, is very effective in the degradation of native collagen. In these tissues cathepsin N was found to be the more abundant enzyme. The interrelationship of the lysosomal acid-proteinases with collagenase and other neutral proteinases in the resorption of connective tissue fibres has been described, and here it appears that the cathepsins may act secondarily to these latter enzymes in completing the digestion intracellularly [3].

Despite their similar action in the digestion of collagen fibres in vitro, cathepsin N is less reactive than cathepsin B towards common protein substrates and no low molecular weight substrates have yet been found [4,5]. The specificity of cathepsin N was investigated therefore, using the oxidized B-chain of bovine insulin as a defined polypeptide substrate. These data enable a direct comparison with the specificity of other enzymes thought to be involved in connective tissue breakdown, and may also provide information on the selection of a suitable low molecular weight substrate for this enzyme.

2. Experimental

2.1. *Materials*

Cathepsin N was prepared from full-term human placentae as in [2]. Oxidized B-chain of bovine insulin was obtained from Boehringer Corp. (London) and used without further purification.

2.2. *Methods*

Separation of digestion products of the oxidized B-chain: Oxidized B-chain (10 mg in 3.0 ml ammonium formate (pH 4.0) containing 1 mM 2-mercaptoethanol) was digested with cathepsin N using an enzyme : substrate ratio of 1:25 (w/w) for 10 h or 24 h at 37°C. The digests were then frozen and dried down several times to remove buffer ions. The mixtures were then redissolved in the electrophoresis buffer (300 μl). Initial separation of peptides was performed by preparative high-voltage electrophoresis at 100 V/cm for 30 min on Whatman 3MM paper moistened with a mixture of 8% (v/v) acetic acid and 2% (v/v) formic acid (pH 2.1). The separated bands were stained with fluorescamine [6] and located under an ultraviolet lamp. The peptide zones were then cut out, sewn into a second sheet of chromatography paper and subjected to descending chromatography in *n*-butanol/acetic acid/water (27:8:40). Peptides were again located with fluorescamine, eluted with 0.1 N acetic acid, and dried down on a water pump over NaOH pellets.

2.3. *Amino acid analyses*

Peptides were hydrolysed with constant boiling HCl containing 1% (w/v) phenol in evacuated sealed ampoules for 24 h at 105°C. Analyses were performed on a Rank-Hilger Chromaspek J180 amino acid analyser employing a single column as described by the manufacturers. Losses of aspartic acid, threonine and serine were calculated from hydrolysis of the

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intact oxidized B-chain and the analyses of recovered peptides were then adjusted accordingly.

2.4. *Identification of N-terminal residues*

This was performed by the dansyl chloride method with separation of the dansyl-amino acids on polyamide sheets [7].

3. Results

3.1. *Digestion for 24 h*

Preliminary experiments, in which peptides were separated by high-voltage electrophoresis, indicated that hydrolysis of the oxidized B-chain proceeded most rapidly at pH 4.0 and that no undegraded sub-

Table 1

Analysis of peptides+ isolated from 37°C digests of the oxidized B-chain of bovine insulin (10 mg) at pH 4.0 with cathepsin N (0.4 mg). Experimental details are given in the text

Peptide number	Amino acid composition	N-terminal residue	Recovery (%)	Proposed structure
	24 h digest			
2-2	Cys(O_3H) (1.0), Glu(1.3), Gly(1.9), Val(0.7), Leu(0.7), Tyr(1.2), Arg(1.0)*	Tyr	2.5	Tyr_{16}-Gly_{23}
3-2	Cys(O_3H) (1.1), Asp(1.0)*, Ser(0.8), Glu(1.8), Gly(1.1), Val(1.6), Leu(1.8), Phe(0.8), His(1.8)	Phe	5.2	Phe_1 -Glu_{13}
4-1	Cys(O_3H)(1.0)*, Asp(0.4), Ser(0.7), Glu(1.0), Gly(1.3), Val(1.2), Leu(1.2), His(0.6)	Val	4.0	Val_2 -Ser_9
5-1	Asp(0.9), Val(1.0)*, Phe(1.0)	Phe	2.0	Phe_1 -Asa_3
5-2	Ala(1.0), Leu(1.0)*	Ala	25.2	Ala_{14}-Leu_{15}
5-3	Thr(0.6), Glu(1.2), Pro(0.8), Gly(1.2), Ala(1.0), Tyr(1.1), Phe(1.7), Lys(1.0)*, Arg(0.7)	Glu	12.0	Glu_{21}-Ala_{30}
6-1	Thr(0.9), Pro(1.0)*, Ala(1.1), Lys(1.1)	Thr	17.6	Thr_{27}-Ala_{30}
6-2	Thr(0.7), Pro(1.2), Tyr(0.9), Lys(1.0)*	Tyr	7.2	Tyr_{26}-Lys_{29}
	10 h digest			
3-2	Cys(O_3H)(1.2), Asp(1.0), Ser(0.8), Glu(1.9), Gly(1.3), Val (1.9), Leu(1.7), Phe(1.0)*, His(2.2)	Phe	22.2	Phe_1 -Glu_{13} (+? Phe_1-Ala_{14})
3-3	Cys(O_3H)(1.8), Asp(1.2), Thr(0.7), Ser(1.2), Gly(3.1), Pro(1.0)*, Gly(3.2), Ala(2.1), Val(3.1) Leu(4.0), Phe(2.9), Tyr(2.0), His(2.2) Lys(0.8), Arg(0.9)	Phe	11.8	Undegraded B-chain
3-4	Cys(O_3H)(1.0), Thr(0.4), Glu(0.3), Pro(0.7), Gly (2.2), Ala(1.3), Val(0.95), Leu(1.0), Tyr(1.8) Phe(1.8), Lys(1.0)*, Arg(0.8)	Tyr	7.0	Tyr_{16}-Ala_{30}
5-3	Ala(0.8), Leu(1.0)*	Ala	6.2	Ala_{14}-Leu_{15}

+ Values of amino acid yields corrected for losses during hydrolysis

* Residue arbitrarily taken as unity

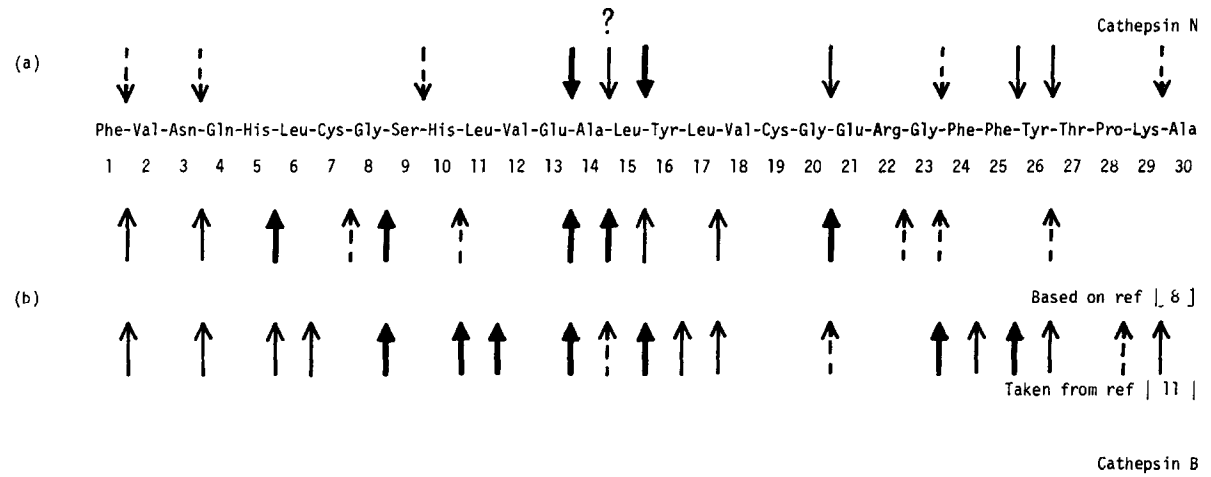

Fig.1. Sites of cleavage in the oxidized B-chain of bovine insulin by cathepsin N (a). The published data for the specificity of action of bovine cathepsin B are given for comparison (b). The degree of susceptibility of each cleavage site is indicated as: (↓) major; (↓) moderate; (↓) minor. The digestion conditions and experimental details for the analysis of the digestion products are given in the text.

strate remained after 24 h. Six peptide zones were identified from the 24 h digest by high-voltage electrophoresis and these were numbered in order of increasing mobility towards the cathode. The zones were fractionated further by chromatography in butanol/acetic acid/water. The separated peptides were then additionally numbered in order of increasing mobility. Eight peptides were isolated in sufficient yield for analysis (table 1). From these data the major cleavage sites were located between Glu_{13}-Ala_{14} and Leu_{15}–Tyr_{16}. In addition, the bonds at Gly_{20}–Glu_{21}, Phe_{25}–Tyr_{26} and Tyr_{26}–Thr_{27}, were also partially hydrolysed. The cleavage sites at Phe_1–Asn_2, Asn_3–Glu_4, Ser_9–His_{10}, Gly_{23}–Phe_{24} and Lys_{29}–Ala_{30}, were relatively minor as judged by the yields of the respective peptides.

3.2. *Digestion for 10 h*

The major cleavage sites were investigated further in a 10 h digest and the products were fractionated by the same techniques. Four peptide components were separated, one of which was due to remaining undegraded substrate (table 1). These initial degradation products confirm that the most susceptible peptide bonds are located at Glu_{13}–Ala_{14} and Leu_{15}–Tyr_{16}. The peptides produced by this initial action were then further degraded at the minor sites during the subsequent period up to 24 h. Cleavage of the bond at Ala_{14}–Leu_{15} was not certainly identified.

The specificity found for cathepsin N using the oxidized B-chain of bovine insulin as the test substrate is shown in fig.1, together with the equivalent published data for bovine cathepsin B.

4. Discussion

The major cleavage sites in the oxidized B-chain of bovine insulin by cathepsin N are situated in the central portion of the molecule. Although Glu_{14} is sited here, the region is otherwise relatively non-polar and is extensively attacked by several acid proteinases [8,9].

The peptide bond specificity of cathepsin N shows several differences from the published specificity of bovine cathepsin B. In studies of human and bovine cathepsin B it was found that the electrophoretic patterns of peptides released from the oxidized B-chain were essentially the same for both enzymes (P.E., D.J.E., unpublished observations). Although the central region of this chain is extensively degraded by both enzymes, the N-terminal half of the molecule is relatively insusceptible to proteolytic cleavage by cathepsin N. Furthermore, whereas cathepsin B

degraded the oxidized B-chain above pH 5, cathepsin N was more effective near pH 4, as found for the digestion of basic histones [5]. This indicates that the susceptible bonds in the telopeptide regions of collagen may also differ for these two enzymes. It has been found previously that cathepsins B and N act synergistically in the solubilization of fibrous type I collagen [10]. This cooperative effect therefore, may arise from these observed differences in enzyme specificity.

Although this investigation confirms the more limited action of cathepsin N, the nature of the active site requirement cannot be established from these data. In particular, substrate sensitivity may vary with the length of the peptide, as in the case of cathepsin B. This shows a preference for those low molecular weight substrates which contain a basic residue yet have very little action on the Arg_{22}–Gly_{23} bond in the oxidized B-chain [8,11]. Previous attempts to classify enzymes on the basis of specificity with defined substrates have met with some criticism. For example, the chymotrypsin-like enzyme of human tissues, as defined from its action on synthetic substrates, exhibited a dissimilar specificity from pancreatic chymotrypsin with the oxidized B-chain as substrate [12]. Cathepsin N may prefer relatively long stretches of non-polar amino acids around the susceptible bonds. Since such areas are extensively cleaved by other acid proteinases in the oxidized B-chain, the possibility of developing a specific substrate based on these sequences is at present limited.

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