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

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Received 9 April 1980

1. Introduction

Four cysteine proteinases have been detected in lysosomes: cathepsin B [1], cathepsin H [2], cathepsin L [3], and cathepsin N [4]. Cathepsin L has the highest specific activity against proteins as substrates and only weak action on synthetic substrates tested, except α-*N*-benzoyl-L-arginine amide [3].

Here the action of cathepsin L on the oxidized insulin B-chain has been studied in order to elucidate the specificity of cleavage by this proteinase. The data allow a direct comparison with the known splitting positions in the insulin B-chain by the action of cathepsin B [5,6] and cathepsin N [7], and suggest the amino acid sequence of possible specific synthetic substrates and inhibitors.

2. Experimental

2.1. *Materials*

Cathepsin L was isolated from rat liver lysosomes and purified according to [3]. Oxidized insulin B-chain was prepared by treatment of bovine insulin with preformed performic acid [8] and separated on cation-exchange resin M 27 (Beckman Instruments, München) as in [9]. Spheron phosphate 1000 resin (40–63 μm) was a kind gift from Dr J. Čoupek, Factory of Laboratory Instruments, Prague. Thin-layer cellulose sheets Polygram CEL 300 were from Macherey and Nagel, Düren.

2.2. *Digestion of the oxidized B-chain of insulin*

Oxidized B-chain (1 μmol) was dissolved in 1 ml 0.2 M triethylamine (pH > 12) and titrated back to pH 6.0 with diluted acetic acid. Cathepsin L (4.7 μg) was added to the B-chain. The incubation mixture (1.5 ml; pH 6.0) was made 5 mM in dithioerythrite and 5 mM in EDTA. The enzyme:substrate ratio was 1:750 (w/w). Proteolysis was at 37°C and stopped after 10 and 30 min, respectively, by addition of 40 μl triethylamine, raising to pH > 11.

2.3. *Separation of peptides*

The lyophilized digest was dissolved in 300 μl formic acid and 200 μl 20% acetic acid and applied to a column (0.8 × 20 cm) of Spheron phosphate [10], equilibrated with 10% acetic acid. By using an amino acid analyser (AAA 6020, CSSR Academy of Sciences), modified for peptide separations, the column was developed similar to [9]. The first gradient was made by mixing 95 ml 10% acetic acid with 95 ml 0.2 M pyridine/acetic acid buffer (pH 3.1) in a 2-chamber gradient mixer with a 1:1 ratio of diameters. Subsequently the column was eluted with 40 ml 0.2 M buffer (pH 3.1), then with a gradient made by mixing 50 ml 0.2 M buffer (pH 3.1) with 50 ml of 2.0 M pyridine/acetic acid buffer (pH 5.0). The column was pumped at a 40 ml/h flowrate at 40°C, and 2.7 ml fractions were collected. The peptides in an aliquot of the effluent were continuously determined with ninhydrin after alkaline hydrolysis [11] at 130°C [12]. Tubes containing peptides were combined and dried down by rotary evaporation under reduced pressure at 40°C.

The fractions were re-dissolved in 50–100 μl 0.15 M triethylamine and further purified by electrophoresis on thin-layer cellulose sheets in formic acid–acetic acid–water (50:150:800, by vol., pH 1.9) at 600 V and 5–6 mA for 90 min [13]. The peptide bands were detected by fluorescamine [14] and eluted 3 times with 1 ml 50% acetic acid.

2.4. *Amino acid analyses*

The peptides were hydrolysed with 6 M HCl in evacuated sealed tubes for 24 h at 110°C. Analyses were performed on a Mikrotechna AAA 881 amino acid analyser (Prague) adapted for the application of microbore columns.

2.5. *Identification of N-terminal residues*

N-terminal residues were identified by the dansyl chloride method [15], with separation of the dansyl-amino acids on polyamide sheets [16].

Table 1

Analysis of peptides isolated from 37°C digest of the oxidized B-chain of bovine insulin (1 μmol) at pH 6.0 with cathepsin L using an enzyme:substrate ratio of 1:750 (w/w) – Experimental details are given in the text

Peptide number	Amino acid analysis	N-terminal residue	Recovery (nmol)	Peptide
30 min digest				
I	Asp (1.0); Val (0.9); Phe (0.9)	Phe	100	Phe_1 ... Asn_3
II	$CySO_3H$ (0.8); Glu (1.2); Gly (2.1); Val (0.9); Tyr (1.0); Phe (2.0); Arg (1.1)	Val	300	Val_{18} ... Tyr_{26}
III-1	$CySO_3H$ (1.0); Glu (1.3); Gly (2.2); Ala (0.9); Val (1.0); Leu (2.0); Tyr (1.8); Phe (1.8); Arg (1.0)	Ala	7	Ala_{14} ... Tyr_{26}
III-2	$CySO_3H$ (0.9); Glu (1.3); Gly (2.0); Val (1.0); Phe (1.8); Arg (1.0)	Val	15	Val_{18} ... Phe_{25}
IV	$CySO_3H$ (0.9); Asp (1.1); Ser (1.0); Glu (2.2); Gly (1.0); Val (2.1); Leu (2.2); Phe (0.9); His (2.0)	Phe	73	Phe_1 ... Glu_{13}
V-1	Ala (1.0); Leu (2.0); Tyr (0.9)	Ala	130	Ala_{14} ... Leu_{17}
V-2	$CySO_3H$ (0.9); Ser (1.0); Glu (2.2); Val (0.9); Gly (1.2); Leu (1.9); His (2.0)	Glu	20	Gln_4 ... Glu_{13}
VI	Thr (0.9); Pro (0.9); Ala (1.1); Lys (1.0)	Thr	485	Thr_{27} ... Ala_{30}
10 min digest				
I	Asp (1.0); Val (1.0); Phe (0.9)	Phe	39	Phe_1 ... Asn_3
II	$CySO_3H$ (1.0); Glu (1.3); Gly (2.1); Val (1.0); Tyr (1.0); Phe (2.0); Arg (1.2)	Val	51	Val_{18} ... Tyr_{26}
III	$CySO_3H$ (0.9); Glu (1.3); Gly (2.0); Ala (1.0); Val (1.0); Leu (2.0); Tyr (1.7); Phe (1.9); Arg (0.9)	Ala	15	Ala_{14} ... Tyr_{26}
IV	$CySO_3H$ (0.9); Asp (1.0); Ser (1.0); Glu (2.1); Gly (1.1); Val (1.9); Leu (2.1); Phe (0.9); His (1.9)	Phe	58	Phe_1 ... Glu_{13}
V-1	$CySO_3H$ (1.1); Asp (0.9); Ser (1.0); Glu (2.3); Gly (1.1); Ala (1.1); Val (1.8); Leu (3.7); Tyr (1.1); Phe (1.0); His (1.7)	Phe	12	Phe_1 ... Leu_{17}
V-2	Ala (1.0); Leu (2.0); Tyr (0.9)		67	Ala_{14} ... Leu_{17}
VI	Thr (0.9); Pro (1.0); Ala (1.0); Lys (1.0)	Thr	323	Thr_{27} ... Ala_{30}
VII	$CySO_3H$ (1.8); Asp (1.1); Thr (1.1); Ser (1.2); Glu (3.3); Pro (0.9); Gly (3.1); Ala (2.0); Val (3.1); Leu (4.1); Tyr (1.9); Phe (3.0); His (2.0); Lys (0.9); Arg (1.0)	Phe	52	undegraded B-chain

3. Results

Because of the high specific activity of cathepsin L splitting proteins and polypeptides, it was possible to use a low enzyme:substrate ratio and short incubation times. Repetition of the experiments gave identical results, also obtained with several enzyme preparations.

3.1. *Digestion for 30 min*

Preliminary experiments. in which the products were separated by ion-exchange chromatography, indicated that no undigested B-chain remained after 30 min digestion under the conditions specified. As shown in table 1 (upper part) 6 peptide peaks were detected by ion-exchange chromatography and these were numbered in order of increasing elution time from the column (I–VI). The peptides, separated by thin-layer electrophoresis, were then additionally numbered in order of increasing mobility towards the cathode [1,2].

As calculated from the amino acid analysis, the peptides containing the residues 18–26 and 27–30, respectively, dominate. This indicates major cleavages between Leu_{17} and Val_{18}, and between Tyr_{26} and Thr_{27}. In addition, the bonds between Asn_3–Gln_4 and Glu_{13}–Ala_{14} are hydrolysed to a relative great extent. The cleavage site between Phe_{25} and Tyr_{26} is a minor one (table 1). Taken together the peptides found account for the whole B-chain structure.

3.2. *Digestion for 10 min*

As shown in table 1 (lower part) in the experiment 7 peptide peaks were detected by ion-exchange chromatography (VII is undegraded B-chain). This short time proteolysis confirms clearly the major cleavage site between Tyr_{26} and Thr_{27} of the B-chain. Whereas the amount of peptide containing residues 27–30 is nearly the same after 10 and 30 min, the quantity of the peptide consisting of the residues 18–26 is still small after 10 min (table 1).

The yield of peptide containing the residues 1–3 is quite small after 10 min. One could suppose that it is formed by splitting of the larger peptides 1–13 and 1–17. The fact that the quantity of peptide 1–13 does not change may be explained by its simultaneous production from undegraded B-chain and peptide 1–17 (table 1).

4. Discussion

In contrast to cathepsin B [5,6] and cathepsin N [7], cathepsin L shows a very clear specificity splitting the insulin B-chain, in that the positions P_2 and P_3 of the substrate [17] should be occupied by particularly hydrophobic amino acids, such as: Phe–Val, Leu–Val, Leu–Tyr, Phe–Phe (fig.1). The peptide bond between Tyr_{26} and Thr_{27} was hydrolysed most preferentially by cathepsin L (table 1). This shows that in addition to P_3 and P_2 a hydrophobic residue

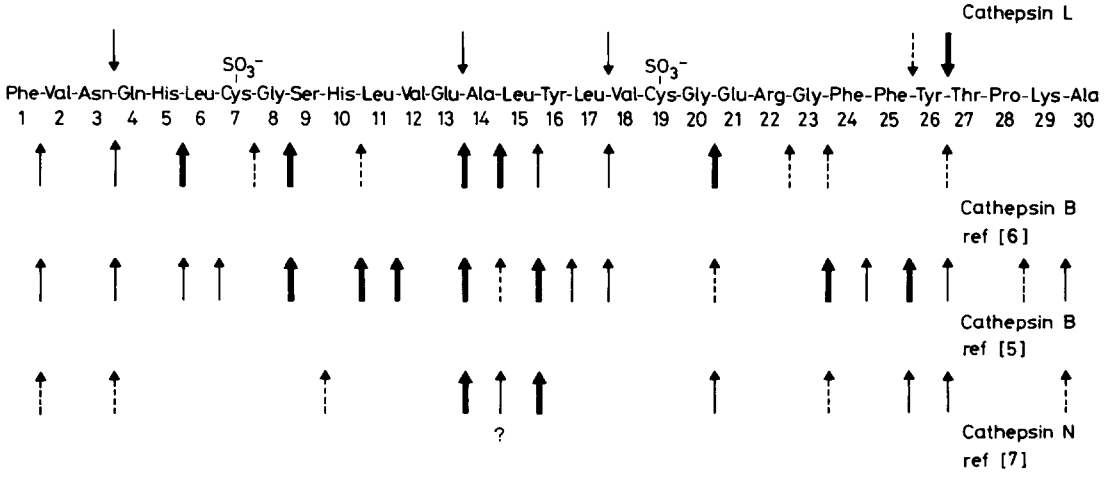

Fig.1. Hydrolysis of the B-chain of oxidized insulin with cathepsin L, cathepsin B, and cathepsin N. The degree of susceptibility of each cleavage site is indicated as: (⬇) Major; (↓) moderate; (↓) minor.

also in P_1 can increase the susceptibility of the peptide bond for cathepsin L (although peptide bonds with Asn or Glu in P_1 are hydrolysed, too. In accordance with this substrate specificity of cathepsin L for hydrophobic amino acids at least in P_2 and P_3, Z–Phe–PheCHN$_2$ was found to be a powerful inhibitor of cathepsin L, whereas cathepsin B was less sensitive to inhibition by this diazomethane [18].

The composition of the peptides (table 1) represents the whole B-chain. Overlapping peptides have been found for all split positions with one exception: Tyr_{26} and Thr_{27}. The recovery of the split peptides in comparison to the amount of B-chain used in the experiments is diminished by losses during the separation procedure. Some very small amounts of peptides, not sufficient for amino acid analysis, have been observed.

Although the specificity of cathepsin L for hydrophobic amino acids at least in P_2 and P_3 determined with the insulin B-chain as substrate is in accordance with the action of Z–Phe–PheCHN$_2$ as a very powerful inhibitor of cathepsin L, the results cannot be generalized until they have been confirmed using different polypeptide chains as substrates. Cathepsin B for instance splits glucagon [19] in a quite different manner than the insulin B-chain [5,6].

Acknowledgement

We thank Mrs D. Fiedler for performing the accurate amino acid analyses.

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