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Action of rat liver cathepsin B on bradykinin and on the oxidized insulin A-chain

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Rat liver cathepsin B was tested for its peptide-bond specificity against bradykinin and the oxidized insulin A-chain. Bradykinin was shown to be resistant to the action of cathepsin B. One possible reason for this resistance is the proline content of the peptide and the discrimination against proline residues at three or four subsites of cathepsin B. Oxidized insulin A-chain was degraded by a peptidyl dipeptidase activity. Three dipeptides were cleaved from the C-terminal part of the insulin A-chain after having been incubated for 2 h (molar ration E:S = 1:2800) and six dipeptides were released after a longer digestion (10 h, E:S = 1:575).

Cathepsin B; Peptidyl dipeptidase; Substrate specificity; Bradykinin; Oxidized insulin A-chain

1. INTRODUCTION

The lysosomal cysteine proteinase cathepsin B (EC 3.4.22.1) is considered to be important in intracellular protein degradation. The proteinase acts as an endopeptidase [2] and as a peptidyl dipeptidase [3]. One reason for these two different modes of action may be found in the primary structure of the substrates, in particular in the presence of proline residues. It has been shown that cathepsin B accepts proline in its subsite S_3 but discriminates against proline at the subsites S_2, S_1 [4] and S_1' [5]. The presence of proline residues in the positions P_1, P_2 and P_1' should therefore prevent the peptidyl dipeptidase activity of the enzyme. Interestingly, at the C-terminus of oxidized insulin B-chain and of proparathormone, proline residues are present in the third-last and penultimate positions, respectively. Both polypeptides were degraded by the endopeptidase activity of cathepsin B [6,7], the proline residues present in the P_1 and P_1' positions prevented a peptidyl dipeptidase attack. Therefore, the action of cathepsin B on the proline-free oxidized insulin A-chain and the proline-rich bradykinin was investigated.

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Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane. For the discussion of the interactions between the proteinase and the substrate the nomenclature of Schechter and Berger [1] is used.

2. EXPERIMENTAL

2.1. *Materials*

Oxidized bovine insulin A-chain, fluorescamine, dansyl chloride and dansyl amino acid standards were purchased from Serva (Heidelberg, FRG). Bradykinin triacetate and dithioerythritol were obtained from Reanal (Budapest, Hungary). Hg^{2+}-inactivated cathepsin B was prepared from rat liver lysosomes as described by Krischke et al. [8]. The

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molarity of enzyme stock solution was determined by active-site titration with E-64 according to Barrett et al. [9]. UV_{254}-silica and lucifol cellulose sheets were obtained from Kavalier (Votice, Czechoslovakia). Acetonitrile and trifluoroacetic acid, both in spectrophotometric grade, were purchased from Merck-Schuchardt (Darmstadt, FRG). All other solvents and chemicals used were of reagent grade and obtained from Laborchemie (Apolda, GDR).

2.2. *Incubation of the oxidized insulin A-chain and bradykinin with cathepsin B*

The digestion of oxidized insulin A-chain by cathepsin B followed the procedure of McKay et al. [6]. Hg^{2+}-inactivated cathepsin B was reactivated in 0.2 ml of 0.2 M triethylamine/acetate, pH 6.0, containing 1.25 mM dithioerythritol and 1.25 mM EDTA · Na_2, at 37°C for 5 min. The reaction was started by addition of 3 μmol of oxidized bovine insulin A-chain, dissolved in 0.8 ml of 0.2 M triethylamine/acetate, pH 6.0. After a digestion time of 10 h at 37°C and an enzyme-substrate (E:S) molar ratio of 1:575 the incubation mixture was freeze-dried. During the 2 h digestion 4 μmol of oxidized insulin A-chain were used at an E:S molar ratio of 1:2800.

4 μmol bradykinin were incubated in 0.9 ml of 0.2 M triethylakine/acetate, pH 6.0, at 37°C with activated cathepsin B at a molar E:S ratio of 1:2800 under conditions analogous to those used for the insulin A-chain digestion. The incubation was allowed to proceed for 120 min and was stopped by freeze-drying.

2.3. *Separation and analysis of the split products*

The freeze-dried 10-h digest of the oxidized insulin A-chain was separated from the proteinase by suspension in 1 ml trifluoroacetic acid and centrifugation for 5 min in a high-speed T-55 centrifuge (Janetzki, GDR). The clear supernatant was applied in 200 μl portions to a Lichrosorb 10 RP-18 column of an HPLC apparatus (Knauer, Berlin, Germany). The cleavage products were eluted from the column with a linear gradient (0–50% by vol.) of acetonitrile in 1% (by vol.) trifluoroacetic acid at a flow rate of 1 ml/min. The eluate was monitored at 205 nm and collected in 1 ml portions. All fractions representing peaks of absorbance were characterized for homogeneity by TLC on cellulose sheets. Nonhomogeneous fractions were separated by ascending chromatography in *n*-butanol/pyridine/acetic acid/water (15:10:3:12 by vol.). The peptides were located by fluorescamine-staining and finally eluted with 6 N HCl [10].

The 120-min digest of oxidized insulin A-chain and the bradykinin incubation mixture were separated by high-voltage paper electrophoresis at 25 V/cm for 120 min in acetic acid/formic acid/water (80:30:890 by vol.), pH 1.9 (MIM, Budapest, Hungary), followed by TLC on cellulose sheets in *n*-butanol/pyridine/acetic acid/water (15:10:3:12 by vol.). The peptides were located by fluorescamine-staining and eluted from the paper sheets with 0.1 N acetic acid and from the TLC sheets with 6 N HCl.

The isolated peptides were hydrolyzed with 6 N HCl in a refluxing condenser for 24 h. The amino acids were determined with a Microtechna amino acid analyzer AAA 339 (Prague, Czechoslovakia). The determination of the N-terminal amino acid of the purified peptides was done by dansylation followed by TLC on UV_{254} silica sheets [11].

3. RESULTS AND DISCUSSION

The effects of cathepsin B on the nonapeptide bradykinin and the oxidized insulin A-chain were quite different. Bradykinin (Arg-Pro^2-Pro^3-Gly^4-Phe-Ser-Pro^7-Phe-Arg) proved to be resistant to an attack of cathepsin B. No hydrolysis of the peptide could be detected after an incubation time of 120 min. The resistance of bradykinin to cathepsin B action derived from the presence of proline residues, since it has been shown in our laboratory that proline residues are not tolerated at the subsites S_1 and S_2 of cathepsin B but are accepted in S_3 and S_2' [4]. The tolerance of proline at the S_2' pocket is somewhat in contrast with results of Takahashi et al. [5]. They supposed from studies on oligopeptides and polypeptides that in addition to the S_1' pocket, the S_2' pocket is also unfavourable for proline residues [5]. If all four subsites (S_2,S_1,S_1',S_2') really discriminate against proline, then no productive binding mode with bradykinin is possible without at least one pocket being occupied by a proline residue. If one considers the binding of Pro^7 in S_2' then Gly^4 would be placed in the S_2 pocket. This is described as the most impor-

Table 1

Analysis of peptides obtained by digestion of oxidized insulin A-chain with cathepsin B

Peptide no.	Amino acid analysis	N-terminal residue	Recovery (nmol)	Peptide
120-min digest				
I-1	Gly 1.0(1), Ile 0.8(1), Val 2.2(2), Glu 4.0(4), Cys 3.9(4), Ala 0.8(1), Ser 2.3(2), Leu 1.8(2), Asp 2.5(2), Tyr 2.0(2)	Gly	240	Gly^{1}-Asn^{21}
I-2	Gly 1.0(1), Ile 0.6(1), Val 2.2(2), Glu 3.2(3), Cys 2.6(3), Ala 0.7(1), Ser 1.8(2), Leu 1.2(1), Tyr 0.9(1)	Gly	61	Gly^{1}-Gln^{15}
II-1	Cys 1.1(1), Asp 1.0(1)	N.D.	314	Cys^{20}-Asn^{21}
III-1	Leu 1.0(1), Glu 1.3(1)	Leu	83	Leu^{16}-Glu^{17}
III-2	Asp 1.0(1), Tyr 0.8(1)	Asp	99	Asn^{18}-Tyr^{19}
10-h digest				
1.1	Cys 0.8(1), Asp 1.0(1)	N.D.	740	Cys^{20}-Asn^{21}
1.2	Cys 0.9(1), Val 1.0(1)	Val	440	Val^{10}-Cys^{11}
2.1	Tyr 0.9(1), Glu 1.0(1)	N.D.	310	Tyr^{14}-Gln^{15}
3.1	Gly 1.0(1), Ile 0.8(1), Val 1.0(1), Glu 1.9(2), Cys 1.7(2), Ala 1.0(1), Ser 1.1(1)	Gly	272	Gly^{1}-Ser^{9}
3.2	Tyr 0.9(1), Asp 1.0(1)	Asp	241	Asn^{18}-Tyr^{19}
3.3	Glu 0.8(1), Leu 1.0(1)	Leu	439	Leu^{16}-Glu^{17}
4.1	Ser 1.1(1), Leu 1.0(1)	Ser	277	Ser^{12}-Leu^{13}

Hydrolysis was carried out at 37°C with 4 μmol of oxidized bovine insulin A-chain for 120 min at an E:S molar ratio of 1:2800 and with 3 μmol for 10 h at an E:S molar ratio of 1:575. Further information is given in section 2. (Cys, cysteic acid; N.D., not determined)

tant subsite of cathepsin B and since there exists a clear preference at this subsite for bulky hydrophobic amino acid residues [3], glycine in the S_2 pocket is particularly unfavourable. Both considerations can explain the resistance of Des-Arg^{9}-[Leu^{8}]-bradykinin towards cathepsin B from porcine spleen as demonstrated by Takahashi et al. [5].

The oxidized insulin A-chain was hydrolyzed by rat liver cathepsin B by a sequential cleavage of dipeptides from the carboxyl-terminus of the polypeptide. After an incubation time of 120 min (E:S = 1:2800) the dipeptides $Cys(SO_3)^{20}$-Asn^{21}, Asn^{18}-Tyr^{19}, and Leu^{16}-Glu^{17}, the N-terminal peptide Gly^{1}-Gln^{15} and nondigested insulin A-chain were isolated. After a 10-h digestion time at an almost 5-fold higher enzyme concnetration (E:S = 1:575), 6 C-terminal dipeptides ($Cys(SO_3H)^{20}$-Asn^{21}, Asn^{18}-Tyr^{19}, Leu^{16}-Glu^{17}, Tyr^{14}-Gln^{15}, Ser^{12}-Leu^{13}, Val^{10}-$Cys(SO_3H)^{11}$), and the appropriate N-terminal nonapeptide Gly^{1}-Ser^{9} were indentified. However, no complete insulin A-chain could be found here (table 1, fig.1). This indicates that cathepsin B operates as a peptidyl dipeptidase towards this substrate. It can be supposed that the $Cys(SO_3H)^{6}$-$Cys(SO_3H)^{7}$ sequence in the oxidized insulin A-chain may act to block a further sequential cleavage. A glutamic acid residue is probably located close to the end of subsite S_2 in cathepsin B [12]. It would repel a negative charge such as the cysteic acid residue.

In addition to the hydrolysis of glucagon [13] and rabbit muscle aldolase [14], the hydrolysis of the oxidized insulin A-chain provides another example of the peptidyl dipeptidase activity of cathepsin B on polypeptide or protein substrates.

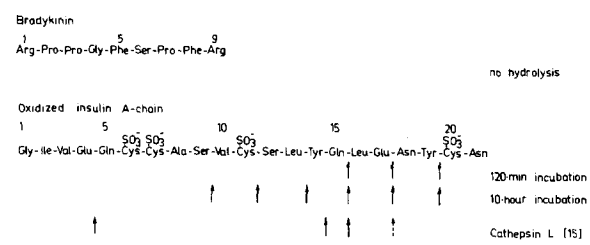

Fig.1. Action of rat liver cathepsin B on bradykinin and on the oxidized insulin A-chain (arrows indicate the sites of cleavage).

This cleavage patterns is well distinguished from that of rat liver cathepsin L (EC 3.4.22.15) [15] (fig.1), but also from the endopeptidase attack of cathepsin B on the oxidized insulin B-chain [6].

The cleavage of the oxidized insulin A-chain by rat liver cathepsin B is somewhat in contrast with results of Takahashi et al. [5]. They could not detect any hydrolysis of the peptide fragment of oxidized insulin A-chain (Gln^{15}-Asn^{21}) by porcine spleen cathepsin B. It is not possible to say, whether these differences are due to species differences in the source of cathepsin B preparations or to different peptide conformations of the substrate used.

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