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ON THE SPECIFICITY OF BOVINE SPLEEN CATHEPSIN B2 *

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Summary

The specificity of bovine spleen cathepsin B2 has been investigated by means of some natural oligo- and polypeptides, i.e. glucagon, melittin, insulin A and B chain, bradykinin, angiotensin I and II, oxytocin ACTH, clupein and salmin. The enzyme is primarily a carboxypeptidase which hydrolyzes peptide linkages of most amino acids common to proteins.

In addition, cathepsin B2 displays amidase and esterase activity without requiring a free carboxyl group. The main pH optimum is between 4 and 5, in some cases higher.

Introduction

It has been shown that 'cathepsin B' originally described by Greenbaum and Fruton [1], consists of two separate enzymes which have been named cathepsins B1 and B2 [2,3]. Cathepsin B1 is an endopeptidase [4], and we originally thought that cathepsin B2 was also an endopeptidase. The purpose of the present paper is to report that cathepsin B2, whose purification has been described recently [5], is primarily a carboxypeptidase. This has already been shown by Ninjoor, Taylor and Tappel [6] with a large number of synthetic and some natural peptides and also by McDonald and Ellis [7] with mainly Cbz-substituted dipeptides. Furthermore, an amidase and esterase activity of cathepsin B2 could be proven.

Experimental

Materials. All chemicals and biochemicals used were of analytical grade. Bz-Arg-NH_2 was from Bachem, Liestal, Switzerland. Bz-Arg-OEt, glucagon, insulin,

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Abbreviations: Bz-Arg-NH_2, α-*N*-benzoyl-L-arginineamide; Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethylester.

bradykinin, melittin, oxytocin, ACTH as well as salmin were purchased from Serva, Heidelberg; angiotensin I and II from Calbiochem, and clupein from Roth, Karlsruhe. The oxidized B chain of insulin was either obtained from Boehringer, Mannheim, or prepared from insulin according to the method of Humbel et al. [8], as were the oxidized A chain and the aminoethylated forms of A and B chain.

Cathepsin B2 was purified according to the method of Otto and Riesenkönig [5] having a specific activity in the range of 10—15 μmol (*V*) towards Bz-Arg-NH_2. The preparation was electrophoretically homogeneous, yielding two bands both of which were active towards Bz-Arg-NH_2 and the polypeptide melittin. It was stored in 50 mM acetate buffer (pH 4.6) containing 20 mM KCl and 1 mM EDTA (but no dithioerythritol), at −15°C.

Methods. Enzyme assays were carried out with Bz-Arg-NH_2 as described [5]. Esterase activity of cathepsin B2 was determined spectrophotometrically at 254 nm according to the method of Schwert and Takenaka [9] with 0.8 mM Bz-Arg-OEt in 40 mM phosphate buffer (pH 6) containing EDTA and dithioerythritol (each 0.8 mM). The time-course of the hydrolysis of the various peptides by cathepsin B2 was followed up with the ninhydrin technique or by means of an amino acid analyzer. A suitable amount of substrate (1—6 mg per ml) was dissolved in acetate or acetate/pyridine buffer (100—200 mM) containing EDTA and dithioerythritol (each approx. 1 mM) in a final volume of 0.5—1.5 ml. After withdrawal of a 10—50 μl sample the reaction was started by the addition of cathepsin B2 (10—50 μg). During the following incubation (38°C) samples were withdrawn and mixed with either 5% (w/v) trichloroacetic acid (950—990 μl) for ninhydrin tests [10] or adjusted to pH 2 with a small volume of 2 M HCl for amino acid analysis. The amino acid analyzer was a model BC 200 of Bio-Cal, Munich, and was run with a sodium citrate buffer system. Peaks were evaluated with a computer PDP 8/I from Digital Equipment Corp., Maynard, Mass. USA. Ammonia liberated from the amide group of melittin was estimated enzymatically by means of the glutamate dehydrogenase reaction in a combined optical test. The cuvette contained in a final volume of 1.25 ml, 80 mM triethanolamine buffer (pH 6.9), 2 mM EDTA, 0.5 mM dithioerythritol, 10 mM α-ketoglutarate, 2 mM NADH, 280 μg of melittin and 300 μg of glutamate dehydrogenase (Boehringer). The reaction was started by the addition of cathepsin B2 which hydrolyzed 1 mol of ammonia from the terminal glutamic acid diamide. The coupled reaction was optimal at the pH used (6.9).

Results

Preliminary experiments on the action of cathepsin B2 on the oxidized B chain of insulin showed the formation of a large number of small peptides and single amino acids difficult to analyze. We therefore chose to work with glucagon as another often-used substrate. Fig. 1 shows that under the conditions used this peptide hormone becomes almost completely hydrolyzed after 2h. Of the theoretically obtainable 27 mol of amino acids (plus 1 mol of the final dipeptide) approximately 25 mol could be determined. The time-course of the hydrolysis shows an intermediate lag phase which, perhaps, could be ascribed

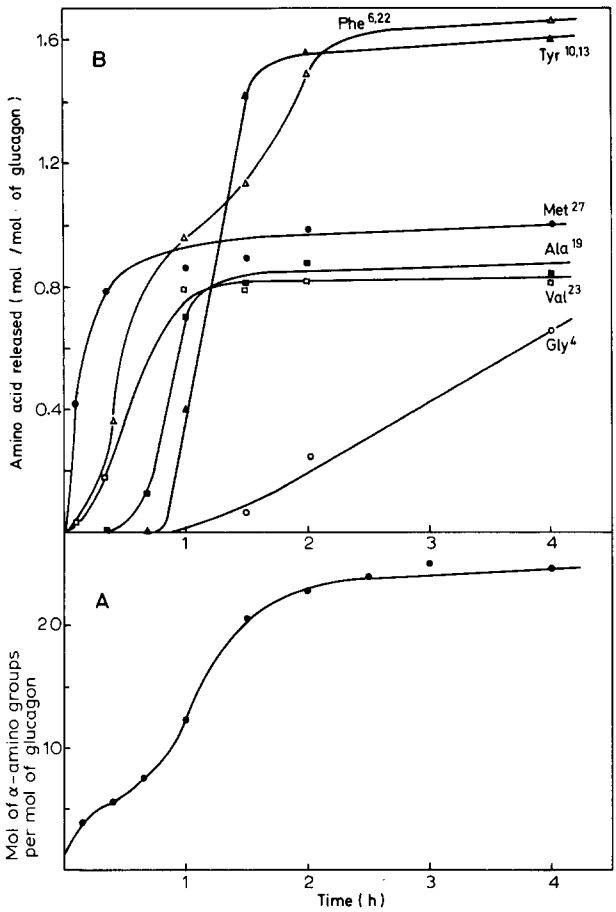

Fig. 1. Time course of hydrolysis and release of amino acids from glucagon by cathepsin B2. 1 μmol of glucagon was incubated with 75 μg of cathepsin B2 in 1 ml of 100 mM pyridine/acetate buffer (pH 4.1) containing EDTA and dithioerythritol (each 1 mM). A. 10-μl samples were withdrawn for determination of total ninhydrin-positive material. B. 100-μl samples were withdrawn, acidified with 10 μl of 2 M HCl and used for amino acid analysis.

to Trp-25. Analysis of the amino acids reveals that their release and appearance follows exactly their sequence in the polypeptide, counted from the carboxyl terminus. Those amino acids which occur twice appear consequently at double concentration and with a time-course depending on their distance from the carboxyl terminus. These results point to a carboxypeptidase activity of cathepsin B2. Its pH optimum is 4.4 (acetate buffer); the K_m value calculated from the initial velocity (5 min) is approximately 0.5 mM.

The investigation of short-chained and proline-containing peptides like bradykinin, angiotensin I and II with the ninhydrin technique and by means of the amino acid analyzer shows that hydrolysis is halted when proline occupies the terminal or the penultimate position of the original or the partly-hydrolyzed peptide. Consequently, the release of amino acids from these substrates is 1, 2 or 0 mol, respectively (Fig. 2A). No further hydrolysis such as might have

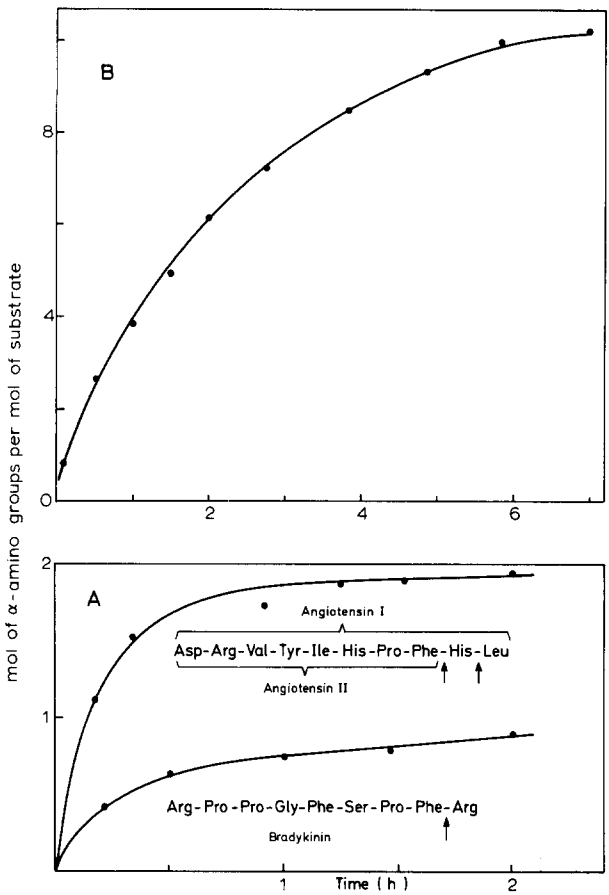

Fig. 2. Time courses of the hydrolyses of some natural peptides by cathepsin B2. Samples were withdrawn at certain intervals and used for determination of total ninhydrin-positive material or for analysis of released amino acids. A. 0.5 μmol of bradykinin as well as angiotensin I and II in 0.5 ml of pyridine/acetate buffer, pH 4.0, containing EDTA and dithioerythritol (each 1 mM) were incubated with 8 μg (or 20 μg) of cathepsin B2. B. 0.27 μmol of melittin in 0.5 ml of 200 mM acetate buffer (pH 4.7) containing EDTA and dithioerythritol as above was incubated with 32 μg of cathepsin B2.

resulted from endopeptidase activity could be observed. Angiotensin II does not yield any amino acid at all; nor does oxytocin, with its carboxyl-terminal glycineamide. The oxidized A chain of insulin, which does not contain proline but 4 cysteic acid residues, is only slowly attacked by cathepsin B2.

The arginine-rich peptides clupein and salmin are rapidly hydrolyzed although here, too, hydrolysis comes to a halt with ninhydrin values reaching a plateau when a proline residue is approached. Addition of the main reaction product, arginine, to the incubation mixture does not influence the rate of cleavage. A sequential release of amino acids is not demonstrable due to the uniform composition of these two polypeptides. Evidence for any further endopeptidase activity was not obtained. Rapid hydrolysis of histones by a cathepsin B2 preparation from rat liver lysosomes has been described by De Lumen and Tappel [11].

The carboxypeptidase activity of cathepsin B2 thus established, we reinvestigated its action on the oxidized B-chain of insulin. As expected, the main product was alanine from the carboxyl terminus, together with some lysine from the penultimate position. There were also, particularly after longer incubation periods, a number of amino acids from the central part of the B chain. Similar results were obtained with the aminoethylated B chain.

In order to confirm the blocking effect of a proline residue with another peptide containing proline away from the carboxyl terminus, in the centre of the peptide chain, we chose melittin, a peptide from bee venom containing 26 amino acid residues with proline in position 14 and glutamic acid diamide at the 'carboxyl' terminus. Fig. 2B shows the time course of the hydrolysis, proving at the same time that the terminal glutamic acid diamide does not block the action of cathepsin B2. Amino acid analysis reveals, as with glucagon, the sequential release of residues from the 'carboxyl' terminus. The terminal glutamic acid diamide is partially hydrolyzed to glutamine and ammonia. Ammonia was determined quantitatively by means of the glutamate dehydrogenase

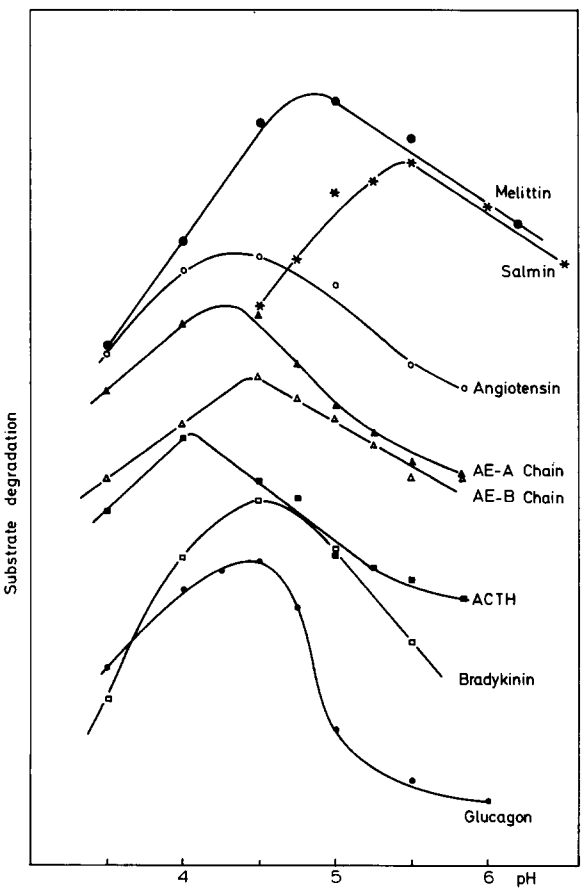

Fig. 3. pH optima of cathepsin B2 with various peptide substrates, determined in 200 mM acetate buffer and under conditions as described in Figs. 1 and 2. Curves are arbitrarily arranged one upon another at approximately equal distances and at comparable ordinate scale.

reaction; glutamine appears together with Gln-25 as 2 mol of amide in the amino acid analyzer. Melittin too, like the insulin B chain, rendered during longer incubations some amino acids from the part 'beyond' proline, i.e. amino acids adjacent to the imino-side of proline. This point requires further investigation.

In contrast to the negligible hydrolysis of the oxidized (acidic) A chain of insulin, but in accordance with the comparatively fast hydrolysis of the arginine- and lysine-containing peptides, the aminoethylated A chain with its modified, lysine-resembling residues was quickly though not completely hydrolyzed, the aminoethylated cysteine residues in position 6 and 7 perhaps still causing a delay. Finally, the polypeptide hormone ACTH yielded mainly the terminal Phe-39 and Glu-38.

A reappraisal of the esterase activity of cathepsin B2, formerly considered to be negligible [3], showed activity with the substrate Bz-Arg-OEt, approximately 30% of the amidase activity towards Bz-Arg-NH_2.

The pH optimum for cathepsin B2 with most of the substrates investigated here is between 4 and 5.5 (Fig. 3), with a tendency for the more basic peptides to higher pH values. The hydrolysis of Bz-Arg-NH_2 proceeds at optimum rate slightly above pH 6, and the amidase activity with the substrate melittin can be conveniently measured at pH 7.

Discussion

The lysosomal cathepsin B2 has been identified, mainly by means of natural peptides as possible substrates, as a carboxypeptidase. It may be assumed that some of the "catheptic carboxypeptidase" activities of Greenbaum et al. [12, 13] (identified in a cathepsin B preparation according to Greenbaum and Fruton [1]) can be ascribed, at least in part, to what is now known to be cathepsin B2. This justifies the recent proposal to classify this enzyme as "lysosomal carboxypeptidase B" [7]. The carboxypeptidase activity of this enzyme is particularly evident with the peptide hormone glucagon which yielded, in some contrast to Tappel's report [6], all its amino acids (except for the N-terminal dipeptide) in a nicely sequential manner. No conspicuous differences in the rate of hydrolysis of the 16 different amino acids (with the possible exception of tryptophan) have been observed. On the other hand, results with angiotensin, bradykinin, B chain of insulin, melittin as well as ACTH show quite clearly that a proline residue in the last or penultimate position of a peptide chain blocks the action of cathepsin B2. The slow hydrolysis of the oxidized A chain of insulin points, in addition, to an impeding effect of cysteic acid, both properties thus a parallel to pancreatic carboxypeptidase A [14]. Conversely, lysine as well as arginine residues, e.g. as in hippurylarginine, are easily hydrolyzed, perhaps even better at slightly higher pH values (5—6), this feature being more a parallel to pancreatic carboxypeptidase B.

In contrast to the pancreatic carboxypeptidases, cathepsin B2 displays amidase activity, e.g. towards the synthetic Bz-Arg-NH_2 as well as toward melittin with its unusual glutamic acid diamide as 'carboxyl terminus', but not toward oxytocin with glycineamide in the terminal position or toward Ac-Gly-Leu-NH_2, as already shown by Ninjoor et al. [6]. The pH optimum for amidase activity is at higher pH values, and higher substrate concentrations are required

(K_m for Bz-Arg-NH_2 = 20 mM) [3]. The esterase activity with Bz-Arg-OEt shows similar properties. Neither activity is completely unusual for a carboxypeptidase; esterase activity in carboxypeptidases A and B is well known although with the decisive difference, that these enzymes require the presence of a free carboxyl group adjacent to the bond to be hydrolyzed (viz. hippuryl-β-phenyllactate). Amidase activity of a carboxypeptidase has been reported in the case of an enzyme from yeast [15,16]. In addition, this latter enzyme showed esterase activity, both activities without the requirement of a free carboxyl group — like cathepsin B2.

Recently, Lazdunski et al. [17] reported about a peptidase with both endo- and exopeptidase activities, an "aminoendopeptidase" from E. coli. De Lumen and Tappel, too, discussed in a former publication [11] the endopeptidase activity of cathepsin B2 as a possibility. In all these and similar cases, the involvement of two active centres should be considered, a topic recently discussed by Zisapel et al. [18] for pancreatic carboxypeptidase B.

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