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FRACTIONATION OF THE RAT LIVER ENZYMES THAT HYDROLYZE BENZOYL-ARGININE-2-NAPHTHYLAMIDE

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Summary

1. The enzyme activity in the particulate fraction from rat liver that hydrolyzes α-*N*-benzoyl-DL-arginine-2-naphthylamide (Bz-Arg-NNap) has been separated into two approximately equal components by chromatography on DEAE-cellulose. One component (peak II) is completely retained by the column at low ionic strength while the other component (peak I) passes through.

2. In contrast to the enzyme in peak I, the enzyme in peak II is extremely sensitive to inhibition by leupeptin, it will hydrolyze carbobenzoxy-alanylarginylarginyl-4-methoxy-2-naphthylamine, and it will inactivate aldolase.

3. There appears to be also a minor high molecular weight component of the α-*N*-benzoyl-DL-arginyl-2-naphthylamine-hydrolyzing activity that is retained by the DEAE-cellulose but which has properties similar to those of the peak I enzyme.

Introduction

The results of earlier studies [1] suggested that cathepsin D was the major enzyme involved in primary proteolytic breakdown in lysosomes. However, more recent studies have shown that cathepsin Bl is able to attack a number of different native proteins [2,3]. Cathepsin Bl may be the major protease involved in the digestion of proteins other than hemoglobin. α-*N*-Benzoyl-arginine-2-naphthylamide (Bz-Arg-NNap) is frequently used as substrate for the measurement of cathepsin Bl. But Distelmaier et al. [4] showed the presence, in partially purified enzyme preparations from a number of tissues including liver, of hydrolase activity acting on Bz-Arg-NNap but not on aldolase. Conse-

Abbreviations: Bz-Arg-NNap, α-*N*-benzoyl-DL-arginyl-2-naphthylamine; Z-Ala-Arg-Arg-NNap-OMe, carbobenzoxy-alanylarginylarginyl-4-methoxy-2-naphthylamine

quently we investigated the chromatographic properties of the enzymes from rat liver lysosomes that inactivate aldolase and that hydrolyze Bz-Arg-NNap and carbobenzoxy-alanylarginylarginyl-4-methoxy-2-naphthylamine (Z-Ala-Arg-Arg-NNap-OMe), a new synthetic substrate introduced by Smith et al. [5].

Materials and Methods

A large granule fraction in 0.25 M sucrose was prepared from the livers of female Sprague-Dawley rats by the method of de Duve et al. [6]. This fraction was resuspended in buffer (starting buffer for chromatography as indicated in figure legends) containing 1% Triton X-100. This suspension was centrifuged for 20 min at 36 000 rev./min in a Spinco rotor 40. The clear supernatant, containing 90—95% of the hydrolase activity on Bz-Arg-NNap and on Z-Ala-Arg-Arg-NNap-OMe, was used in all the experiments to be described below except for the demonstration of the linearity of the assay method (Fig. 1).

The hydrolysis of Bz-Arg-NNap and of Z-Ala-Arg-Arg-NNap-OMe was measured by the method of Barrett [7]. Substrate concentrations used were 1 mg/ml (2.27 mM) for Bz-Arg-NNap and 0.2 mg/ml (0.26 mM) for Z-Ala-Arg-Arg-NNap-OMe. Standards containing either 2-naphthylamine or 4-methoxy-2-naphthylamine were routinely included with all enzyme assays. The Brij (Ruger Chemical Co., Irvington, N.J.) that we used in the coupling reagent was found to contain substantial amounts of acidity. We found that coupling was more reliable when this was neutralized immediately before the preparation of the reagent. A chloride-sensitive electrode (Orion Research Co., Cambridge, Mass.) was used to measure chloride concentration. All protein determinations were performed with an automated Lowry method [8] using bovine serum albumin as standard.

Bz-Arg-NNap and 2-naphthylamine were purchased from the Sigma Chemical Co. (St. Louis, Mo.). 4-Methoxy-2-naphthylamine was purchased from Fox Chemical Co. (Los Angeles, Calif.). We are indebted to Professor H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) for the gift of leupeptin and to Drs R.E. Smith and E.L. Smithwick (Eli Lilly Co., Indianapolis, Ind.) for the gift of Z-Ala-Arg-Arg-NNap-OMe.

Results

Liver enzyme activity on Bz-Arg-NNap and Z-Ala-Arg-Arg-NNap-OMe

Fig. 1 shows the linear dependence on the amount of enzyme preparation added of the rate of hydrolysis of the two substrates. A homogenate of 1 g of rat liver hydrolyzed 1 μmol per min of Bz-Arg-NNap and 4 μmol per min of Z-Ala-Arg-Arg-NNap-OMe. All measurements reported below were performed with enzyme dilutions within the linear range of the assay method. The activity on both substrates was completely inhibited by 50 μM *p*-chloroercuribenzoate.

Binding to DEAE-cellulose

Various amounts of the enzyme preparation in 10 mM phosphate buffer, pH 6.7, with 5 mM 2-mercaptoethanol were mixed with equal amounts of DEAE-cellulose equilibrated with the same buffer in calibrated centrifuge

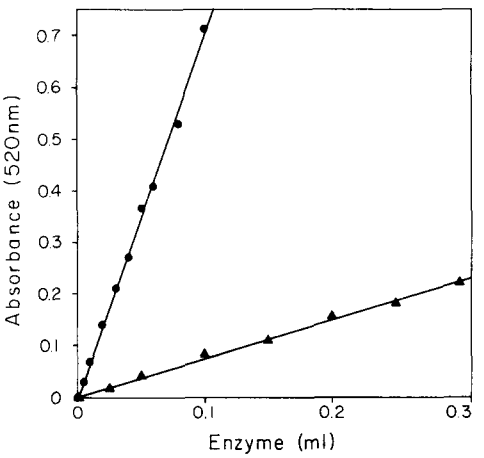

Fig. 1. Dependence of hydrolysis rate on amount of enzyme. The enzyme preparation was a post-nuclear supernatant containing 4.0 mg protein per ml. ▲, hydrolysis of Bz-Arg-NNap; ●, hydrolysis of Z-Ala-Arg-Arg-NNap-OMe.

tubes. After 1 min the tubes were centrifuged and the supernatants were assayed for their ability to hydrolyze Bz-Arg-NNap. The activity in the supernatant expressed in units of μmol per min (after correction for dilution by fluid in the DEAE-cellulose) is plotted in Fig. 2 against the amount of activity added. Over a wide range of added enzyme, a constant fraction (approximately half) was adsorbed on the DEAE-cellulose and the rest of the activity appeared in the supernatant. These results suggested that there were at least two components of the enzymic activity, only one of which was bound by the DEAE-cellulose.

DEAE-cellulose chromatography

A sample of the enzyme preparation in 10 mM phosphate, pH 6.7, 5 mM 2-mercaptoethanol was applied to a column of DEAE-cellulose equilibrated with the same buffer. After washing with the starting buffer, the column was

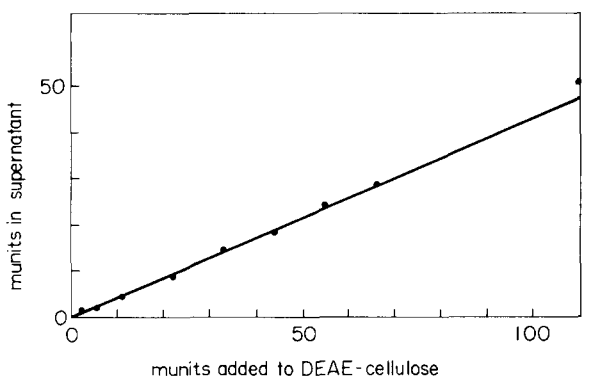

Fig. 2. Binding of Bz-Arg-NNap hydrolase activity from rat liver by DEAE-cellulose. For details, see text.

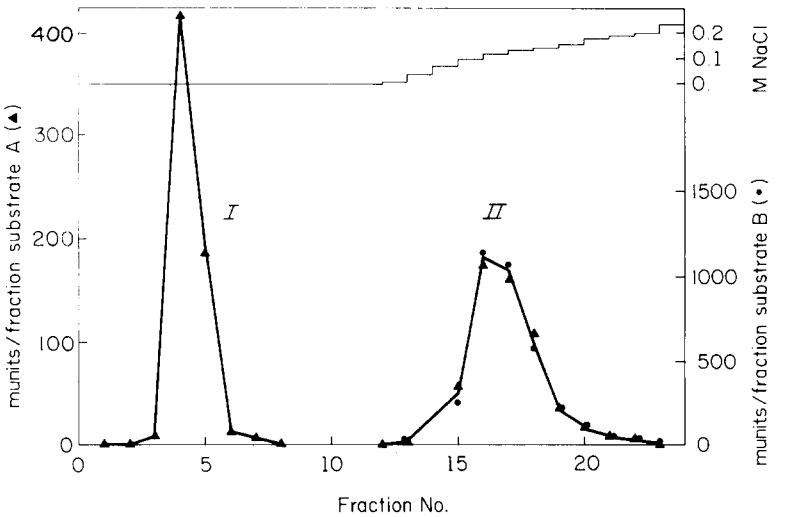

Fig. 3. Chromatography of enzymes on DEAE-cellulose. The enzyme preparation contained 45 mg protein. Fractions of 6.3 ml were collected every 20 min from a 14.0 × 1.55 cm column. Starting buffer was 10 mM phosphate, pH 6.7, 5 mM β-mercaptoethanol. A 200-ml linear gradient of NaCl concentration from 0 to 0.5 M was used. ▲, hydrolysis of Bz-Arg-NNap (substrate A) (recovery 120%); •, hydrolysis of Z-Ala-Arg-Arg-NNap-OMe (substrate B) (recovery 94%).

eluted with a concentration gradient of NaCl. Hydrolase activity on Bz-Arg-NNap and on Z-Ala-Arg-Arg-NNap-OMe was measured on all the eluate fractions.

The results of this experiment are plotted in Fig. 3. The Bz-Arg-NNap-hydrolyzing activity emerged in two approximately equal peaks, one that passed through the column without any apparent retardation (peak I) and one that emerged with the salt gradient at about 0.15 M NaCl (peak II). The activity on Z-Ala-Arg-Arg-NNap-OMe, however, was absent from peak I but eluted exactly like the activity on Bz-Arg-NNap in peak II. For convenience of comparison the two activities in peak II have been normalized to the same scale.

The activity in peak I was rechromatographed on a similar column and again passed through without retardation. The activity in peak II was dialyzed against starting buffer and rechromatographed and again it was retained on the column.

Both peak I and peak II were tested for their ability to inactivate aldolase [4]. Only peak II was active.

The enzymes in the peak fractions I and II were assayed in the presence of various concentrations of the peptide antibiotic leupeptin (mixed forms) [9]. The results of this experiment are plotted in Fig. 4. The ability of the enzyme in peak II to hydrolyze both substrates was severely inhibited even by very low concentrations of leupeptin. In contrast the enzyme in peak I was almost completely unaffected by leupeptin at these same low leupeptin concentrations, although it also is strongly inhibited at a 10-fold higher leupeptin concentration. While the activity on Z-Ala-Arg-Arg-NNap-OMe could be reduced essentially to zero by high enough concentrations of leupeptin, a small residual activity on Bz-Arg-NNap remained. This indicated the possibility of a minor

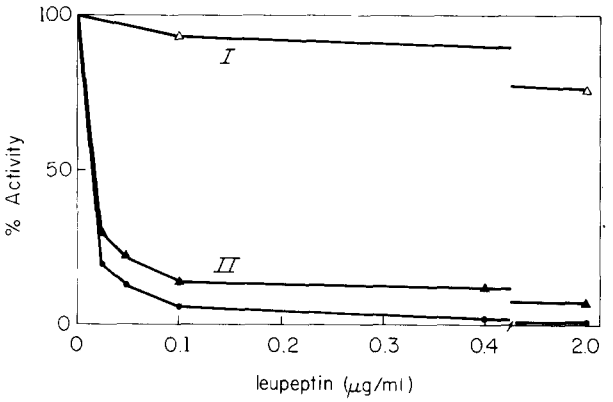

Fig. 4. Inhibition by leupeptin of enzymes in peak fractions from DEAE-cellulose. △, Bz-Arg-NNap hydrolase in peak I (pooled fractions 4—5); ▲, Bz-Arg-NNap hydrolase in peak II (pooled fractions 16—18); ●: Z-Ala-Arg-Arg-NNap-OMe hydrolase in peak II.

component enzyme in peak II that could hydrolyze Bz-Arg-NNap but that was insensitive to leupeptin.

With very low concentrations of inhibitors, the possibility always exists that competitive binding of inhibitor by other proteins may distort the results. However this is most unlikely here since even in very crude preparations such as post-nuclear supernatants, the degree of inhibition we have observed at a given concentration of leupeptin was independent of protein concentration.

Sephadex G-200 chromatography

When the enzyme preparation was chromatographed on Sephadex G-200 all the activity on Z-Ala-Arg-Arg-NNap-OMe and almost all the activity on Bz-Arg-NNap emerged as expected at an elution volume corresponding to a molecular weight around 25 000. While the activity on Z-Ala-Arg-Arg-NNap-OMe showed a clear symmetrical peak, the activity on Bz-Arg-NNap showed a broader peak indicating by its elution volume that some of the activity had a slightly lower molecular weight than the enzyme hydrolyzing Z-Ala-Arg-Arg-NNap-OMe. In addition to the main peak of activity on Bz-Arg-NNap, there was a small peak that eluted just after the void volume.

Discussion

It is clear from the results presented above that the Bz-Arg-NNap hydrolase activity in rat liver is due to two major components, only one of which inactivates aldolase. This enzyme satisfies all the criteria for cathepsin Bl and it is almost certainly the enzyme responsible for the degradation of a number of native proteins [2,3]. This enzyme from rat liver is retained on DEAE-cellulose and is very sensitive to inhibition by leupeptin. On the basis of the results of Sephadex chromatography, the molecular weight of this enzyme seems to be slightly higher than that of the other Bz-Arg-NNap-hydrolyzing enzyme which is less sensitive to leupeptin. The minor component enzyme that emerges first from the G-200 column has not been studied extensively. It probably corresponds to the component of peak II from DEAE-cellulose (Fig. 3) that is

insensitive to leupeptin (Fig. 4). Distelmaier et al. [4] found an even larger proportion of Bz-Arg-NNap hydrolase from rat liver that was unable to inactivate aldolase, but these authors subjected the rat liver homogenate to preliminary protein separations by acid autolysis and $(NH_4)_2SO_4$ precipitation. We have found that these procedures result in substantial losses of activity. Consequently their sample of enzyme was probably not representative of rat liver.

De Lumen and Tappel [10] fractionated rat liver lysosome proteins on DEAE-cellulose. These authors reported only one peak of Bz-Arg-NNap-hydrolyzing activity eluting from the column at about the same concentration of NaCl as our peak II (Fig. 3). Either they removed or destroyed the peak I activity during preliminary $(NH_4)_2SO_4$ fractionation or they did not assay the early eluate fractions for Bz-Arg-NNap-hydrolyzing activity. In any case the activity applied to the column was only 35% of that initially present in the lysosomal preparation.

Z-Ala-Arg-Arg-NNap-OMe seems to be hydrolyzed only by the component of the Bz-Arg-NNap-hydrolyzing activity that inactivates aldolase. This should make it a very useful substrate in the study of the protease activity in lysosomes, particularly since it allows a great increase in sensitivity when assaying the "true" cathepsin Bl that seems to attack native proteins. Almost all this increase in sensitivity is a consequence of a greater rate of hydrolysis, although 4-methoxy-2-naphthylamine gives slightly more color than 2-naphthylamine when coupled to Fast Garnet at pH 6. Similarly leupeptin may be useful since it is so effective in inhibiting this protease activity.

Acknowledgements

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