Biochimica et Biophysica Acta, 334 (1974) 179–186

BBA 67110

EFFECT OF PAPAIN INHIBITOR FROM CHICKEN EGG WHITE ON CATHEPSIN B_1

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(Received August 9th, 1973)

SUMMARY

We have examined the effect of papain inhibitor from chicken egg white on the proteolytic activity of cathepsin B_1. We have found that this intracellular animal proteinase is blocked by this inhibitor. The inhibition is of the pseudo-irreversible type. The enzyme–inhibitor complex is formed even if the inhibitor is allowed to react with the mercuri form of cathepsin B_1, i.e. with the inactive cathepsin B_1 in which the free SH-group of the catalytic site has been blocked by mercury.

INTRODUCTION

Only a few naturally occurring inhibitors of intracellular proteinases have been known. Finkenstadt [1] has inhibited the proteolytic activity of cathepsin B and C by the supernatant obtained after differential centrifugation of a homogenate of rat liver tissue. The inhibitor was a thermostabile high-molecular-weight product, other characteristics, however, were not reported by the author. Snellmann [2] has described the inhibition of cathepsin B from calf liver by the haptoglobin fraction of human serum. Starkey and Barrett [3] have studied the inhibition of cathepsin B_1 from human liver by human serum and found that the main inhibitory effect shows the α_2-macroglobulin fraction of human serum whereas the inhibition by immunoglobulin G is less pronounced. These authors were not able to demonstrate the inhibition of the proteolytic activity of cathepsin B_1 by the haptoglobin fraction of human serum. A fact of interest is that α_2-macroglobulin inhibits not only cathepsin B_1 but proteinases in general. We have shown in our laboratory that cathepsin E from rabbit bone marrow is inhibited by the pepsin inhibitor isolated from the cell walls of the roundworm *Ascaris lumbricoides*. This inhibitor shows a narrow specificity, inhibits only pepsin and cathepsin E, does not inhibit either cathepsin D or rennin [4]. An inhibitor of mol. wt 14 000 has been isolated from the rabbit skin during the disappearance of the so-called Arthus phenomenon; this inhibitor was found to inhibit a thiol dermoproteinase which has been isolated also from the skin during the initial stage of this reaction. Moreover this inhibitor also inhibited the proteolysis by papain [5].

A number of relatively low-molecular-weight inhibitors (mol. wt less than 1000)

have been isolated from the cultivation media of various *Streptomyces* strains. Of these inhibitors pepstatin inhibits the activity of cathepsin D and E [6, 7], leupeptin and chymostatin inhibit in addition to other enzymes also cathepsin B_1 [7]. An almost complete inhibition of the proteolytic activity of lysozomal proteinases from the macrophage homogenate can be brought about by a combination of several of these inhibitors [8].

Fossum and Whitaker [9] have isolated an inhibitor from chicken egg white which unlike the ovoinhibitor and ovomucoid, described earlier, does not inhibit either trypsin or chymotrypsin but only papain, ficin, and partly bromelain.

Cathepsin B_1 considerably resembles in some of its enzymatic characteristics papain. Since we have been comparing the properties of these two enzymes in our laboratory for some time, we decided to investigate the effect of the papain inhibitor from chicken egg white on the proteolytic activity of cathepsin B_1.

MATERIALS AND METHODS

Cathepsin B_1 was isolated from bovine spleen as described earlier [10]. Cathepsin B_1 was used in the inactive mercuri form.

Cathepsin C was prepared from bovine spleen. The final purification of the enzyme was achieved by affinity chromatography on a mercurial Sepharose column. The enzyme was stored in the inactive mercuri form (Keilová, H. and Tomášek, V., unpublished).

Cathepsin D was isolated from bovine spleen [11].

Cathepsin E was isolated from rabbit bone marrow by the method of Lapresle [12].

Chymotrypsinogen was prepared by 5-fold crystallization of a commercial product (Léčiva, Praha). Chymotrypsin was prepared by the activation of chymotrypsinogen by trypsin. It was recrystallized three times.

Human serum albumin was purchased from the Institute of Sera and Vaccines, Praha.

Ovalbumin was prepared from chicken egg white [13].

Ribonuclease was a commercial preparation from Koch-Light.

Carboxymethyl-cellulose (CM-Cellex) was from Calbiochem, A.G. Luzerne.

Sephadex was a product of Pharmacia, Uppsala.

N_α-Benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride was synthetized according to Tuppy et al. [14]. Glycyl-L-phenylalanine-*p*-nitroanilide was synthetized by Dr Kasafírek, Institute for Pharmacy and Biochemistry, Praha.

The inhibitor was prepared from fresh eggs essentially by the method of Fossum and Whitaker [9]. The whites of fresh eggs were diluted 1:1 by 0.25% NaCl. The precipitate formed after the adjustment of the pH to 6.0 was centrifuged off and the clear supernatant was saturated to 50% with $(NH_4)_2SO_4$. The precipitate was suspended in water) one fifth of the original volume) and dialyzed against water. The clear supernatant was subjected to gel filtration on a Sephadex G-75 column equilibrated with 0.1 M acetate buffer at pH 5.0. The inhibitor-containing fraction was chromatographed on CM-cellulose equilibrated with the same buffer. The inhibitor was eluted from the column with an increased volume of the same buffer whereas most of the proteins remained adsorbed under these conditions. The inhibitor solution was concentrated

by pressure dialysis using a UM 10 Amicon Ltd (Great Britain) filter and stored at −20 °C.

Assay of Proteolytic Activity

Cathepsin B_1. The enzyme solution (100 μl) was activated for 10 min at 40 °C in 1 ml of the activating buffer (0.1 M phosphate buffer, 25 mM cysteine–HCl, 1 mM EDTA, pH 6.0). The substrate (50 μl of a solution of 40 mg of N_α-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride in 1 ml of dimethylformamide) was then added to the mixture. The hydrolysis was allowed to proceed at 40 °C for 10–20 min according to the intensity of the arising color. The hydrolysis was discontinued by the addition of 100 μl of glacial acetic acid. The *p*-nitroaniline released was determined spectrophotometrically at 405 nm. For each measurement a blank experiment was made in which the substrate was added after the acidification by acetic acid. The quantity of enzyme which liberates 1 μmole of *p*-nitroaniline in 1 min under the conditions of the experiment was taken as one unit.

Cathepsin C. The same procedure as that used for cathepsin B_1 was used with the exception that glycyl-L-phenylalanine-*p*-nitroanilide (2 mg in 1 ml of dimethylformamide) was used as substrate.

Cathepsin D and E. The activity was determined by the hemoglobin assay (2 % solution of hemoglobin at pH 3) as described in our preceding paper [4].

Chymotrypsin. The activity of chymotrypsin was determined spectrophotometrically using as substrate 0.001 M acetyl-L-tyrosine ethyl ester in 0.05 M phosphate buffer at pH 7 and 25 °C [15].

Determination of inhibitory activity

The solution (100 μl) of the corresponding enzyme was mixed with 100 μl of the dilute inhibitor solution. The proteolytic activity was determined after 2 min as described above. The quantity of inhibitor added is given in μl of the original concentrated solution. The inhibition is expressed in percent with respect to the control experiment carried out in the absence of the inhibitor.

Determination of molecular weight

The molecular weight was determined by gel filtration on a column of Sephadex G-75 in 0.1 M KCl. A 2.3 cm × 67 cm column in the upward-flow arrangement was used. Samples containing 5 mg protein in 1.5 ml were applied to the column and fractions of 4.5 ml were collected at a flow rate of 33 ml per h. The column was calibrated by the determination of the elution volumes of serum albumin (mol. wt 68 000), ovalbumin (mol. wt 45 000), chymotrypsinogen (mol. wt 25 000), and ribonuclease (mol. wt 13 700).

RESULTS AND DISCUSSION

The thiol intracellular tissue proteinase cathepsin B_1 resembles in many respects the plant thiol proteinase papain. Both enzymes have the same specificity as regards the digestion of the B-chain of oxidized insulin. Even though both enzymes cleave simple arginine substrates, they split the Arg-Gly bond in the B-chain of oxidiz-

ed insulin only to a negligible degree [16]. In the studies on the mechanism of the enzymatic hydrolysis by papain, a group ionizable at pK 3.9–4.3 was found. A group ionizable at pK 4 was found in cathepsin B_1 under identical conditions [17].

In view of the similarities described above we made an effort to compare the behavior of these two enzymes with the naturally occurring papain inhibitor from chicken egg white.

Firstly we examined in orienting experiments the effect of the whole chicken egg white on the proteolytic activity of cathepsin B_1, C, D, and E. As shown in Table I only cathepsin B_1 and cathepsin C are inhibited, whereas cathepsin D and E remain unaffected. The interaction of cathepsin C with the inhibitor from chicken egg white will be described later (Keilová, H. and Tomášek, V., unpublished).

TABLE I

INHIBITION OF CATHEPSIN B_1, C, D, AND E BY WHOLE CHICKEN EGG WHITE

The proteolytic activity without the inhibitor (control) and in the presence of the inhibitor was assayed as described in the experimental part. 100 μl of undiluted whole chicken egg white was used as an inhibitor.

Enzyme	% of proteolytic activity	
	In the presence of egg white	Control
Cathepsin B_1	4	100
Cathepsin C	8	100
Cathepsin D	100	100
Cathepsin E	100	100

If the fraction of chicken egg white obtained by saturation with $(NH_4)_2SO_4$ to 50% is subjected to gel filtration on Sephadex G-75, we find a weak activity inhibiting cathepsin B_1 in the first high-molecular-weight fraction. The main portion of the inhibitor, however, emerges from the column in an elution volume considerably higher which corresponds to its lower molecular weight. The result is demonstrated in Fig. 1 and is in full agreement with the data reported by Fossum and Whitaker [9]. The inhibitor present in the first fraction (I) inhibits besides papain also cathepsin B_1 and chymotrypsin, is, however, without effect on cathepsin C. The inhibitor which shows the lower molecular weight (II) inhibits papain, cathepsin B_1, and cathepsin C, does not inhibit, however, chymotrypsin. There is, therefore, no doubt that these two inhibitors are entirely different.

Since the inhibitor of lower molecular (II) weight is very labile on freeze–drying and looses up to 50% of its activity during this operation, the samples of this inhibitor were stored as small batches of frozen solutions at −20 °C.

In our opinion our data indicate that we have isolated the same inhibitor as that used for the inhibition of papain by Fossum and Whitaker.

Since the effect of this inhibitor on cathepsin B_1 has not been studied, we consider our first task to determine the type of inhibition, either reversible or irreversible. We examined, therefore, the degree of inhibition at different enzyme concentrations

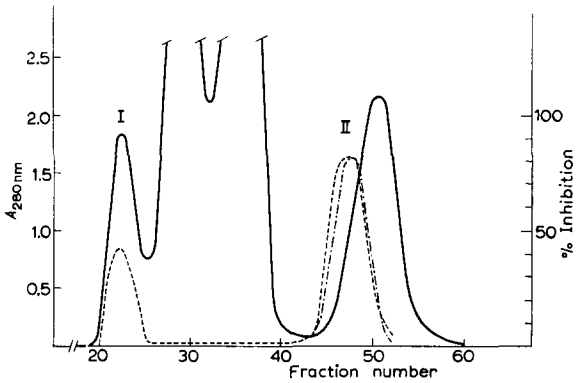

Fig. 1. Gel filtration on Sephadex G-75 of a fraction of chicken egg white after precipitation with $(NH_4)_2SO_4$. Column, 2.5 cm × 150 cm; fractions, 7.7 ml per 28 min; ———, $A_{280\,nm}$; — — —, percent of inhibition of activity of cathepsin B_1; — · — ·, percent of inhibition of activity of cathepsin C.

as a function of the concentration of the inhibitor. Our results provide unambiguous evidence that (as in the case of many other naturally occurring inhibitors) here, too, the inhibition is of the pseudo-irreversible type. As can be seen in Fig. 2, all curves start at the origin; at higher enzyme concentrations, however, they are more or less parallel. This is typical of the so-called tight-binding inhibitors [18].

The problem of the interaction of proteinases with naturally occurring inhibitors has been studied mostly on proteinases of the serine type. It is assumed that the formation of the enzyme–inhibitor complex requires the presence of the fully

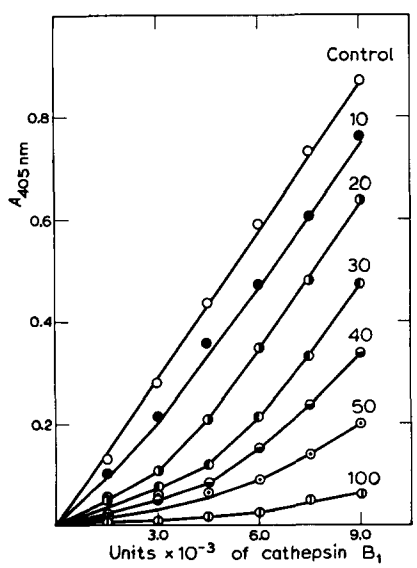

Fig. 2. Cleavage of *N*-benzoyl-D,L-arginine-*p*-nitroanilide by various concentrations of cathepsin B_1 in the presence of different concentrations of papain ovoinhibitor. Time of incubation is 15 min at 40 °C. The figures on the individual curves indicate the quantity of the inhibitor solution ($A_{280\,nm}$ = 0.2) in μl.

active form of the enzyme [19]. The problem, however, is far from being solved since it has been reported that the inhibitor can bind even to the inactive forms of the enzyme. Feinstein and Feeney [20], e.g. have described a complex of TPCK-chymotrypsin with potato inhibitor and with turkey and golden pheasant ovomucoids. TLCK-trypsin reacts with turkey and chicken ovomucoids [19, 20]. Similarly papain, a thiol proteinase, binds even in the inactive mercuri form the papain ovoinhibitor [9]. We assume that the latter observation may be closely related to the results of the experiments carried out by Sluyterman [21] which show that the thiol group of active papain is not essential for the binding of the substrate.

We have, therefore, tried to determine whether a complex of the papain ovoinhibitor with cathepsin B_1 is formed even in the case when the sulfhydryl group of the enzyme, essential for its enzymatic activity, has been blocked. In all experiments we used cathepsin B_1 in its mercuri form which is entirely inactive unless treated with cysteine or other reagents containing a sulfhydryl group. The formation of the enzyme–inhibitor complex was studied by the method of gel filtration. We determined the elution volumes of cathepsin B_1, of the free papain ovoinhibitor, and of the mixture of the two compounds and assumed that the formation of the complex, whose molec-

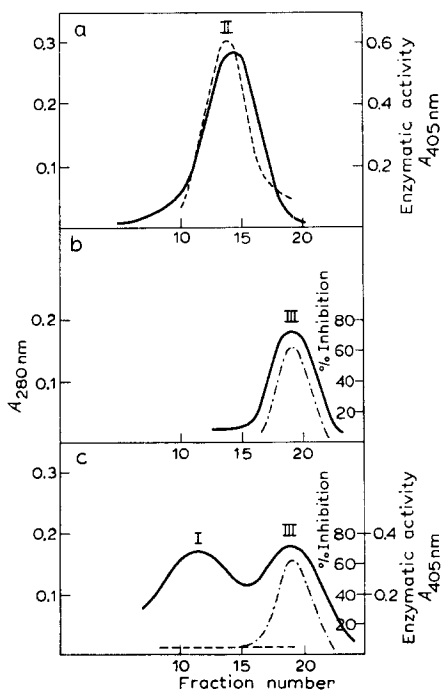

Fig. 3. Determination of the elution volumes of cathepsin B_1 (a), papain ovoinhibitor (b), and an enzyme–inhibitor complex (c) by gel filtration on Sephadex G-75. The experiments were run under the conditions given in the experimental part for the determination of molecular weight. a, 3 mg of cathepsin B_1 in the mercuri form in 1.5 ml of water; b, 1.5 ml of solution ($A^{1cm}_{280\,nm} = 1.2$) of papain ovoinhibitor; c, 4 mg of cathepsin B_1 in the mercuri form in 1.5 ml of water was mixed with 1.5 ml of concentrated solution ($A^{1cm}_{280\,nm} = 2.5$) of papain ovoinhibitor. 1.5 ml of this mixture was applied onto the column. ———, A_{280}; – – –, activity of cathepsin B_1; — · — · —, activity of inhibitor (percent of inhibition of cathepsin B_1).

ular weight should be higher than that of the original enzyme, should result in a decrease of the elution volume. As can be seen in Figs 3a–c, our assumption was correct. When cathepsin B_1 alone was subjected to gel filtration, only one peak (II) was obtained (Fig. 3a) and the curve of the absorbance at 280 nm was identical with the curve of the enzymatic activity. By contrast, two peaks (I and III) were obtained by gel filtration of a mixture of cathepsin B_1 with the papain ovoinhibitor (Fig. 3c). None of the material in these two peaks showed cathepsin B_1 activity and neither was this activity found in fractions corresponding to the elution volume of cathepsin B_1. Inhibitory activity was found in Peak III. Hence, the excess of inhibitor, which had been taken for the reaction and had not been attached to cathepsin B_1, was separated by gel filtration.

By using a Sephadex G-75 column, calibrated by the elution volumes of proteins of known molecular weight, we were able to confirm the molecular weight of 24 000 for cathepsin B_1 and 12 500 for the inhibitor (Fig. 4). The elution volume of

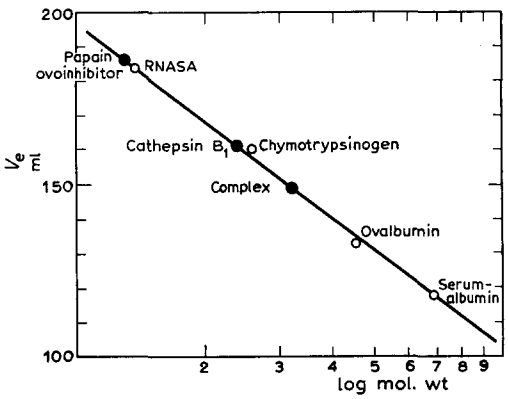

Fig. 4. Dependence of elution volumes on the logarithm of the molecular weight.

the peak obtained by gel filtration of the mixture of cathepsin B_1 and the inhibitor corresponds to a molecular weight of approx. 31 000. The molecular weight of the complex determined by gel filtration is, therefore, lower than would correspond to the theoretical value obtained by the addition of the molecular weights of the two components. One possible explanation of this discrepancy is the fact that this method virtually determines the molecular volume of the product which can also be affected by the shape of the molecule.

The data presented here indicate that the formation of the complex of cathepsin B_1 and the papain ovoinhibitor does not require the presence of a free sulfhydryl group essential for the catalytic function of the enzyme. Mercuri attached to this essential sulfhydryl group does not sterically hinder the binding of the inhibitor. The results presented also provide additional evidence for the enzymatic similarity between the plant thiol proteinase papain and the animal thiol proteinase cathepsin B_1.

ACKNOWLEDGEMENT

The authors thank Mrs J. Zaoralová for her technical assistance.

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