IMMUNOCHEMICAL INVESTIGATION OF THIOL PROTEINASES OF THE BOVINE SPLEEN: CATHEPSINS B, H, AND L

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UDC 612.015.1:577.152.344/-088.1

KEY WORDS: liver; cathepsins; thiol proteinases; spleen.

Many different kinds of tissue thiol proteinases, similar in properties to cathepsin B have been discovered recently [9]. Cathepsins H and L [9, 10], T [7], and M [12] have been isolated from the liver of rats and some other animals. Enzymes with similar properties, described as proteinases I and II [3, 4] and cathepsins N [6] and S [11], have been isolated from bovine spleen. The present writers have developed a scheme for the isolation of proteinase I and cathepsin B in a highly purified form from bovine spleen and have studied their enzymic and physicochemical properties [3]. On the basis of comparison of the properties of enzymes with the cathepsins that have been described, we have concluded that proteinase I is evidently cathepsin L, and proteinase II is cathepsin H of the spleen, which were not previously known [3].

The question of similarities and differences in the structure of intracellular thiol proteinases is still not clear. An immunochemical approach can be adopted for a study. It was used in the present investigation to study antigenic properties of proteinases I and II and cathepsin B from bovine spleen.

EXPERIMENTAL METHOD

Proteinases I and II and cathepsin B were isolated by the method in [3]. The proteinase I had a degree of purification of about 3500 times [3]. On polyacrylamide gel electrophoresis (PGE) one principal and two minor components were found: On PGE in the presence of sodium dodecylsulfate (DDS) a component with a molecular weight (mol. wt.) of 23,000 daltons and traces of contamination with a component with mol. wt. of about 15,000 daltons were obtained. The proteinase II (degree of purification about 200 times) was electrophoretically homogeneous: mol. wt. about 30,000 daltons [3]. Preparations of proteinase II from two different isolation procedures were used for immunization. The cathepsin B was partially denatured; PGE revealed one wide zone, whereas PGE in the presence of DDS yielded components with mol. wt. of about 28,000 and 25,000 daltons respectively. Bearing in mind that proteinases I and II may be toxic for animals, they were partially inactivated before being used for immunization by dialysis against water.

Rabbits were immunized intradermally at several points in the dorsal region, 150-200 μg of proteinase in 0.3-0.4 ml of 0.15 M NaCl, emulsified in an equal volume of Freund's complete adjuvant, being injected. The animals were reimmunized twice with an interval of 15 days, and 100 μg proteinase in Freund's complete adjuvant was injected intradermally each time. In the case of cathepsin B, reimmunization was given twice. Rabbit antisera (AS) were obtained on the 8th and 10th day after the last injection of enzyme. The double diffusion test in agar was carried out in Gusev's micromodification [1].

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Laboratory of Biochemistry and Chemical Pathology of Proteins, Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR. Laboratory of Immunochemistry, N. F. Gamaleya Research Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 96, No. 10, pp. 50-53, October, 1983. Original article submitted December 13, 1982.

0007-4888/83/9610-1399$07.50

EXPERIMENTAL RESULTS

The results of immunodiffusion analysis, characterizing the specificity of the AS obtained, are given in Fig. 1. They show that each AS reacted specifically with the corresponding antigen and did not give a positive precipitation reaction with the other proteinases. AS against cathepsin B (AS_B) reacted better with the enzyme when denatured in an alkaline medium than with the original cathepsin (Fig. 1c). This was evidently due to denaturation of cathepsin B at physiological pH values during immunization. Similar results were obtained previously for cathepsin B from human liver [5, 13]. AS against proteinase I (AS_I) and AS against proteinase II (AS_{II}) reacted with neither native nor the denatured form of cathepsin B (Fig. 1a, b). These results are evidence of a difference in the antigenic properties of proteinases I and II and cathepsin B and they indicate a difference in the structure of these proteins.

All three AS possessed species specificity, for they did not give a precipitation reaction with the corresponding partially purified proteinases from pig kidney, but they reacted with homologous enzymes from bovine kidney. These findings agree with previous results showing species specificity of AS against cathepsin B from bovine spleen [2].

The study of the effect of certain inhibitors and denaturing agents on the antigenic properties of proteinases I and II showed that after the reaction with p-chloromercuribenzoate (p-CMB), which inactivates enzymes [3], proteinase I lost much of its ability to react with specific AS, whereas proteinase II preserved its reactivity (Fig. 2). Heat treatment also affected the antigenic properties of proteinase I more than those of proteinase II. Interaction with the low-molecular-weight inhibitor leupeptin and an inhibitor present in normal serum did not affect the ability of either proteinase I or II to react with the homologous AS (Fig. 2). The results indicate that groups of the active center in the proteinase II molecule need not take part in the formation of antigenic determinants. This conclusion is not so evident for proteinase I and can be drawn only on the basis of the experiments with leupeptin and serum inhibitor. Loss of the antigenic properties of proteinase I after the reaction with p-CMB can be explained not only by blocking of an SH-group essential for activity, but also by conformational changes in the molecule, leading to destruction of antigenic determinants. Support for this hypothesis is given by the results of experiments with heat treatment of proteinase I.

The study of the ability of specific AS to inhibit activity of the two proteinases showed that serum of both control and immunized animals, even in high dilutions, inhibited activity of the enzymes practically completely. This was due to the presence of inhibitors, including a specific inhibitor of thiol proteinases, which is found in the α_2-globulin fraction [14]. To study the question of specific inhibition of proteinase activity by the action of AS, pure antibodies must be used.

AS_I was used to compare two preparations of proteinase I isolated by different methods [3, 4]. It will be clear from Fig. 3a that the two preparations were immunologically identical. This is in agreement with previous views that the preparation described in [4], which had lower proteolytic activity and a lower molecular weight, contained the partially denatured form of proteinase I, having undergone limited proteolysis [3]. Antigenic analysis of several fractions isolated in the course of its purification by the method in [4] indicate the possible existence of partial degradation products of proteinase I (Fig. 3a).

An immunodiffusion study of products obtained during isolation of proteinases I and II by the method in [3] revealed a component giving a precipitation reaction with AS_I (Fig. 3b). This component was contained in the protein fraction with mol. wt. 30,000-40,000 daltons (appreciably higher than mol. wt. of proteinase I, which is about 23,000 daltons [3]) but with lower proteolytic activity than proteinase I. From the antigenic point of view it was richer than proteinase I, with which it was partially identical (Fig. 3b). Whether this component is a different enzyme from proteinase I or one related to it in structure, or whether it is a form of proteinase I with higher molecular weight, formed as a result of activation of some common precursor, is not yet clear.

The results of the present investigation are evidence that the three thiol proteinases of bovine spleen — cathepsin B, proteinases I and II — differ in their substrate specificity [3] and differ also in their immunochemical properties. This conclusion is in agreement with the results of an investigation [10] which showed that cathepsins B, H, and L from rat liver differ antigenically. As was pointed out above, proteinase II was identified by the present

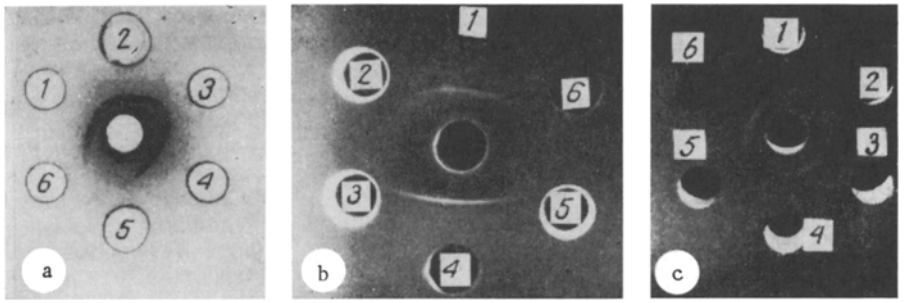

Fig. 1. Analysis of specificity of AS against proteinase I (a), proteinase II (b), and cathepsin B (c). Central well contains AS; 1, 4) antigen, 50 and 100 μg/ml respectively (a, b, c). a: 2) Proteinase from another isolation procedure, 50 μg/ml; 3) cathepsin B, 250 μg/ml; 5) cathepsin B, denatured at pH 8.5, 125 μg/ml; 6) proteinase II, 120 μg/ml; b: 2) proteinase II from another isolation procedure (inactive), 100 μg/ml; 3) proteinase I, 100 μg/ml; 5, 6) cathepsin B, original and denatured respectively, 125 μg/ml; c: 1, 4) cathepsin B used for immunization, 125 and 62 μg/ml respectively; 2) proteinase I, 100 μg/ml; 3) proteinase II, 100 μg/ml; 5) cathepsin B, 100 μg/ml; 6) cathepsin B denatured at pH 8.5, 125 μg/ml.

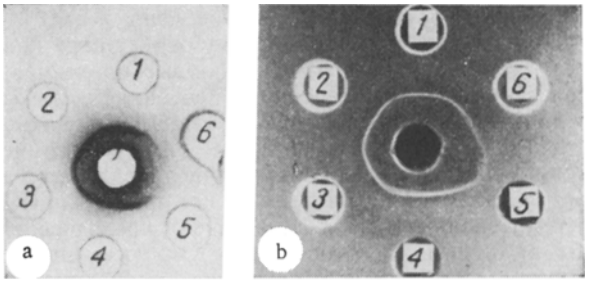

Fig. 2. Effect of inhibitors and denaturing agents on antigenic properties of proteinase I (a) and proteinase II (b). Central well contains AS; 1, 4) antigens, 50 and 100 μg/ml respectively; 2) proteinase (50 μg/ml) incubated at pH 8.5 for 2 h at 20°C, 5) proteinase incubated at 60°C for 30 min; 3) proteinase (50 μg/ml) + leupeptin (40 μg/ml), pH 7.2, 1 h, 20°C; 6) proteinase (50 μg/ml) + p-CMB (10^{-3} M), pH 7.2, 1 h, 20°C.

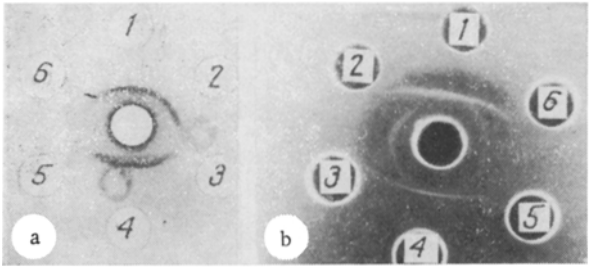

Fig. 3. Antigenic analysis of preparations of proteinase I isolated by different methods (a) and fractions obtained during purification of the enzyme (a, b). Central well contains As_I; a: 1, 4) proteinase I, isolated by method [3], antigen, 50 and 100 μg/ml respectively; 2) proteinase I isolated by method [4]; 5, 6) fractions on isolation by method [4]; b: 1, 4) component (fraction isolated by method in [3]). 100 and 50 μg/ml respectively; 2, 3) antigen, 100 and 50 μg/ml respectively; 5, 6) proteinase I from another isolation procedure, 100 and 50 μg/ml respectively.

writers as splenic cathepsin H [3]. AS against this enzyme from another source has been described in only one paper [10], and was obtained against enzyme from the spleen by the present writers for the first time. Proteinase I is evidently splenic cathepsin L [3], but its properties differ somewhat from the cathepsin L from rat liver, whose properties have been most completely described [9]. These differences are perhaps due to the tissue- and species-specificity of the enzyme. For exact identification of proteinase I we must know its intracellular localization, and progress toward this end may be attained by the use of specific AS, which has not previously been obtained against cathepsin L.

It must be pointed out that immunochemical studies of cathepsin B and the related thiol proteinases began only recently [8, 10, 13]. They are very promising, for they can explain not only similarities and differences in the structure of enzymes of similar type, but can also establish their intracellular and intercellular localization. This last feature is extremely important, for processes of metastasization of tumors [8, 13, 15] and tissue destruction in various diseases [9, 13] have been connected with the secretion of cathepsin B and other intracellular thiol proteinases. The development of specific immunochemical methods of determination of these enzymes may be of considerable diagnostic importance.

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