BBA 66762

PROTEASES OF MACROPHAGES IN RAT PERITONEAL EXUDATE, WITH SPECIAL REFERENCE TO THE EFFECTS OF ACTINOMYCETE PROTEASE INHIBITORS

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(Received May 25th, 1972)

SUMMARY

The glycogen-induced macrophages in rat peritoneal exudate were separated from most other nucleated cells by means of a glass bead column. The lysate of the glass-adhering cells was subjected to characterization of its proteolytic activities using several specific protease inhibitors, recently discovered from the culture media of actinomycetes. The hemoglobin-hydrolyzing activity at pH 4.0 was strongly inhibited by pepstatin, a pepsin (EC 3.4.4.1) inhibitor. The caseinolytic activity at pH 7.0 was sensitive to chymostatin, a chymotrypsin (EC 3.4.4.5) inhibitor. In addition to these two major proteases, *i.e.*, cathepsin D (EC 3.4.4.23) and chymotrypsin-like enzyme, there are two other minor proteolytic activities. One is active at acidic pH and sensitive to leupeptins, a trypsin (EC 3.4.4.4) inhibitor, and the other is active at neutral pH and sensitive to Hg^{2+} but not to leupeptins.

INTRODUCTION

Macrophages, which have phagocytic properties, are associated with defense reactions to infectious agents, inflammatory processes, and immunological mechanisms. Cohn and Wiener[1] demonstrated that macrophages from rabbit peritoneal exudate contained various hydrolytic enzymes such as acid phosphatase (EC 3.1.3.2), β-glucuronidase (EC 3.2.1.31), cathepsin, lysozyme (EC 3.2.1.17), and acid ribonuclease (EC 2.7.7.16). We were interested in characterizing proteases of macrophages in the rat peritoneal exudate collected after intraperitoneal injection of glycogen. For this purpose, it is necessary to obtain macrophages as free as possible from lymphocytes, granulocytes, mast cells, and some erythrocytes which are, in general, simultaneously present in such an exudate. We found that use of a glass bead column gave satisfactory results. The macrophages thus separated were disrupted by freezing and thawing, and the lysate was subjected to enzymatic characterization.

Intracellular proteases are usually characterized as cathepsins A, B, C, *etc.* on

the basis of their capabilities of cleaving certain synthetic substrates and inhibition profiles[2,3]. The procedures involved are not very simple, and the answers have not always been unequivocal. Recent discovery by Umezawa and his associates[4–6] of several actinomycete protease inhibitors seems to provide less complicated procedure with more definite answer as to the characterization of proteases. Thus, Ikezawa *et al.*[7] demonstrated the usefulness of leupeptins, chymostatin, and pepstatin* in characterizing lysosomal proteases from swine liver. They found that cathepsin D (EC 3.4.4.23) was strongly inhibited by pepstatin and cathepsin B was inhibited by leupeptins and chymostatin, while cathepsin A did not undergo appreciable inhibition by these three agents. We have used these three kinds of inhibitors *plus* antipain[8], another inhibitor also isolated from the actinomycete culture media, to study their effects on the hydrolytic activity of the macrophage lysate toward acid-denatured hemoglobin and casein. The present paper describes the results of these experiments. Only recently, Barrett and Dingle[11] have described the inhibition of tissue acid proteinases by pepstatin.

METHODS

Separation of macrophages

Rats (Wister–King Aptakmann/MK) weighing about 250 g were lightly anesthetized with an intraperitoneal injection of Nembutal sodium (sodium pento-barbital), and 15 ml of sterile 0.5% (w/v) glycogen in isoosmotic NaCl was injected into the peritoneal cavity. After 72–96 h, the animals were killed by decapitation and exsanguination, and their peritoneal cavities were washed with phosphate-buffered saline, pH 7.0, using a pipet. In this procedure, it is very important to use silicon-coated glassware. All the peritoneal cells obtained in a single experiment (usually 15–20 donors) were collected by sedimentation at 1000 rev./min for 5 min at room temperature.

The pellet of cells was resuspended in 10 ml of Hank's solution and the number of the total cells was calculated. At the same time, the preparations for microscopical studies were made by Giemsa staining. The cell suspension was then added to a glass bead column, 1 cm × 20 cm. Only the tubing of the column had been siliconized beforehand. The glass beads used were the product of B. Braun Melsungen Apparatebau. A stainless steel net on a rubber stopper was used to hold the beads at the bottom of the column. After incubation for 60 min at 37 °C, the column was washed enough with phosphate-buffered saline. Then, the non-adherent cells were collected by sedimentation at 1000 rev./min for 5 min and resuspended in 10 ml of Hank's solution for the calculation of cell count and microscopic studies. The difference in cell count between influent and effluent cells represents the cell count number for the adherent cells (see below, Table I).

Hank's solution containing both Mg^{2+} and Ca^{2+} is more preferable for isolation of macrophages than phosphate-buffered saline, for these ions are adherence-promoting factors[12,13]. But Hank's solution was unsuitable for the later assay of proteolytic

* Leupeptins, acetyl- and propionyl-L-leucyl-L-leucyl-DL-argininal[3]; chymostatin, an oligopeptide of undetermined structure[5]; pepstatin, *iso*-valeryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid[4,10]; antipain, [(*s*)-1-carboxy-2-phenylethyl]carbamoyl-L-arginyl-L-valyl-argininal[8,9].

TABLE I

DISTRIBUTION OF CELLS IN RAT PERITONEAL EXUDATE BEFORE AND AFTER THE PASSAGE THROUGH A GLASS BEAD COLUMN

Cell count for each kind of the constituent cells in the influent is taken as 100%.

Constituent cells	*Cell counts*		
	Influent $\times 10^8$ (%)	*Effluent* $\times 10^8$ (%)	*Adherent** $\times 10^8$ (%)
Total cells	23.1 (100)	6.3 (27.3)	16.8 (72.7)
Erythrocytes	8.1 (100)	2.8 (34.6)	5.3 (65.4)
Nucleated cells			
Total	15.0 (100)	3.5 (23.3)	11.5 (76.7)
Lymphocytes	4.4 (100)	3.0 (68.2)	1.4 (31.8)
Granulocytes	0.5 (100)	0.4 (80.0)	0.1 (20.0)
Macrophages	10.1 (100)	0.1 (1.0)	10.0 (99.0)

* The difference in cell count between influent and effluent cells was taken as the cell count for the glass-adhering cells.

enzymes, because phenol red, a constituent of Hank's solution, absorbs at 280 nm. So, after the incubation for 60 min at 37 °C, the column must be washed sufficiently with phosphate-buffered saline.

Enzyme extraction

The glass beads with adherent cells were transferred into a round-bottomed flask and subjected to seven or more freezing and thawing treatments. Previous data with specific granules from rabbit polymorphonuclear leucocytes[14], rat liver lysosomes and granules from rabbit peritoneal macrophages[1] indicated that repeated freezing and thawing, treatment with surface-active agents such as saponin, deoxycholate and Triton X-100, mechanical breakage in Waring blender, osmotic breakdown or some kind of autolytic breakdown, *e.g.*, thermal activation, was similarly effective to release the lysosomal enzymes. A preliminary experiment in this laboratory, however, showed that sonic vibrations decrease the proteolytic activity of the macrophage preparation. After several trials of other methods the freezing and thawing treatment was found preferable for the present experiments. The frozen and thawed preparation was centrifuged at 3000 rev./min for 10 min. The residual debris at this stage exhibited no appreciable proteolytic activity on denatured hemoglobin. The supernatant fraction was thus used as the enzyme solution.

Enzyme assay

Proteolytic activity was determined using a 1% solution of acid-denatured hemoglobin as substrate by slightly modifying the method of Anson[16]. The substrate solution was adjusted to the desired pH value between 2.0 and 9.0 with 1 M NaOH or 1 M HCl. The incubation was carried out at 38 °C for 2 h. After the reaction was stopped by the addition of trichloroacetic acid, the absorbance of the filtrate was measured at 280 nm. Control experiments without the substrate were always run, because some activity of cellular ribonuclease must be present[17]. The proteolytic activity at pH 7.0 was also measured using 1% casein as substrate by the method of Kunitz[18] with an appropriate modification. The casein substrate for the assay of crude

enzyme extract was pretreated for 15 min at pH 12 to remove all ribonuclease activity in the commercial casein[19].

Actinomycete protease inhibitors

The isolation and characterization of these inhibitors were described in previous papers[4-6, 8-10]. Stock solutions (2 mg/ml) of leupeptins and antipain were made in water and those of chymostatin and pepstatin in 50% methanol.

RESULTS

Separation of macrophages with a glass bead column

The adhesiveness of macrophages onto the glass surface is the basic principle for the use of a glass bead column. Table I shows that glycogen-induced peritoneal macrophages actually adhere almost completely to glass beads, while approximately 70% and 80% of lymphocytes and granulocytes, respectively, are not retained. Approximately two-thirds of erythrocytes applied to the column were adherent together with macrophages, but this did not seem to cause any serious problems as far as the proteinase assay of these cells was concerned. A separate experiment in this laboratory has shown that the proteolytic activity is undetectable in rat erythrocytes under the standard assay conditions, although human erythrocytes were shown to be capable of splitting protein substrates[20]. Consequently, the results shown in Table I may indicate that the majority of proteolytic activity in the cellular lysate which was later obtained from the processed glass beads is represented by macrophage enzymes.

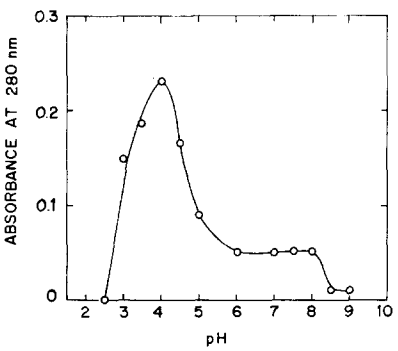

Fig. 1. Dependence on pH of the hemoglobin-hydrolyzing activity of rat peritoneal macrophages. An incubation mixture made of 1 ml of 1% denatured hemoglobin solution adjusted to desired pH, 0.3 ml of buffer at various pH values, and 0.2 ml of the enzyme solution was allowed to stand at 38 °C for 2 h. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid, and the absorbance at 280 nm of the filtrate was measured. The ordinate is for the increment in absorbance at 280 nm after 2 h incubation.

pH-activity curve

As shown in Fig. 1, the protease activity is maximal at pH 4.0 and also detected in the neutral pH range. The present observation is in general agreement with earlier reports on the proteolytic activity of granulocytes in the peritoneal exudate[1]. The very low activity observed at pH 2.5 suggests an almost complete absence of cathepsin E[2,3].

TABLE II

EFFECT OF VARIOUS EFFECTORS ON PROTEASES OF RAT PERITONEAL MACROPHAGES

Effector	*Final concn*	*Percent activity**	
		Hydrolysis of hemoglobin at pH 4.0	*Hydrolysis of casein at pH 7.0*
Leupeptins	13.2 μg/ml	82.8	85.2
	132 μg/ml	83.3	80.5
Pepstatin	13.2 μg/ml	17.9	88.8
	132 μg/ml	19.1	44.5
Chymostatin	13.2 μg/ml	85.5	29.6
	132 μg/ml	56.6	30.4
Antipain	13.2 μg/ml	88.6	91.8
	132 μg/ml	83.0	74.6
$HgCl_2$	1×10^{-4} M	86.8	57.4
	1×10^{-3} M	79.8	27.2
Cysteine	1×10^{-4} M	111.4	101.2
	1×10^{-3} M	114.6	108.3
EDTA	1×10^{-4} M	109.3	78.1
	1×10^{-3} M	103.1	81.1

* Taking the respective activities at pH 4.0 and at pH 7.0 in the absence of effectors as 100%.

Inhibition of proteases at pH 4.0

When four different kinds of actinomycete protease inhibitors were tested for their abilities of inhibition, pepstatin, a pepsin (EC 3.4.4.1) inhibitor, showed an outstanding effect (Table II). This strongly suggests the predominance of cathepsin D action, since cathepsin D, a pepsin-like acid protease, is markedly inhibited by pepstatin, while cathepsins A and B are not inhibited[7]. The concentration of pepstatin required for 50% inhibition of macrophage cathepsin D can be calculated from the sigmoid portion of the curve shown in Fig. 2, corresponding to 39% inhibition of the total proteolytic activity. The obtained value is $4.7 \cdot 10^{-3}$ μg/ml, which is even smaller than the value ($1.1 \cdot 10^{-2}$ μg/ml) for cathepsin D from swine liver[7]. The right-hand flat portion of the curve in Fig. 2 suggests the presence of additional protease or proteases which could not be inhibited by pepstatin. As shown in the figure, the residual activity (approx. 20%) found in the presence of a large amount of pepstatin was further inhibited by leupeptins, chymostatin or antipain. The latter finding together with the partial susceptibility of the original activity to Hg^{2+} and the small but significant increase in activity by cysteine (Table II) may be consistent with the presence of cathepsin B activity as a minor component of the acid proteases in the macrophage lysate.

Inhibition of proteases at pH 7.0

As shown in Table II, approx. 70% of the caseinolytic activity at pH 7.0 was inhibited by chymostatin, a chymotrypsin (EC 3.4.4.5) inhibitor[5]. The concentration of chymostatin required for 50% inhibition can be obtained from the sigmoid portion of the curve shown in Fig. 3. A value of 1.32 μg/ml was obtained. This value is higher than the reported value for chymotrypsin (0.15 μg/ml)[5]. The residual activity (approx. 30%) found in the presence of excess amount of chymostatin was found to be

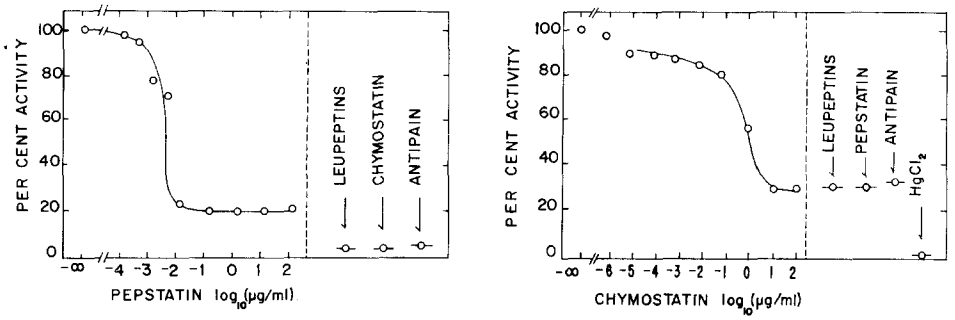

Fig. 2. Effect of actinomycete protease inhibitors on the hemoglobin-hydrolyzing activity of rat peritoneal macrophages at pH 4.0. The sigmoid curve represents the effect of concentration of pepstatin. On the right are shown the results obtained after the addition of leupeptins (1.32 μg/ml), chymostatin (1.32 μg/ml), or antipain (1.32 μg/ml) together with pepstatin ($1.32 \cdot 10^{-1}$ μg/ml). The results of enzyme assays are expressed in terms of percent activity, taking the activity in the absence of inhibitor as 100%.

Fig. 3. Effect of actinomycete protease inhibitors on the casein-hydrolyzing activity of rat peritoneal macrophages at pH 7.0. The sigmoid curve represents the effect of concentration of chymostatin. On the right are shown the results obtained after the addition of leupeptins (13.2 μg/ml), pepstatin (13.2 μg/ml), antipain (13.2 μg/ml), or mercuric chloride ($1 \cdot 10^{-3}$ M) together with chymostatin (13.2 μg/ml). The results are expressed as in Fig. 2.

insensitive to further additions of other three kinds of actinomycete inhibitors, but sensitive to $HgCl_2$ (Fig. 3). These results indicate the simultaneous presence of a chymotrypsin-like enzyme (or enzymes), which makes up the major portion of the original activity, and a thiol protease (or proteases) as a minor constituent.

DISCUSSION

The lysate preparation used in the present study was obtained from the peritoneal exudate cells that adhered onto the glass beads. The majority of the adhering cells were macrophages, but smaller numbers of lymphocytes and a minute amount of granulocytes could not be eliminated (Table I). The purification of macrophages by polysucrose density-gradient centrifugation has recently been described[21].

The results of the present experiments indicate that at both pH 4.0 and 7.0 at least each two different kinds of proteases can be discerned. The question then arises whether there are in fact four different kinds of proteases or there are only two or three enzymes which actually have two pH optima. The pepstatin-sensitive protease, which was found to be active at pH 4.0 and one may call cathepsin D, seems to be different from the protease functioning in the digestion of casein at pH 7.0, because the latter digestion was not as readily inhibited by pepstatin as the hemoglobin hydrolysis at pH 4.0 was (Table II). Likewise, the chymotrypsin-like activity found at pH 7.0 can not account for the major part ability of hemoglobin to be digested at pH 4.0. The non-identity between these two major proteases, one acting at pH 4.0 and the other at pH 7.0, is thus apparent. Weston *et al.*[22] demonstrated that acid protease, cathepsin D, could function also at neutral pH in the degradation of chick embryonic cartilage.

The second and minor activity responsible for casein digestion at pH 7.0 was

found to be insensitive to leupeptins and antipain (Fig. 3), and in this respect it does not seem to be identical with the second enzyme found at pH 4.0. The latter resembles cathepsin B with an acidic pH optimum, whereas the former is less well-defined at the present moment. These lines of evidence so far available may support the presence of at least four different kinds of proteases in the lysate preparation used.

ACKNOWLEDGMENTS

We wish to thank Drs Hamao Umezawa and Takaaki Aoyagi, Institute for Microbial Chemistry, Tokyo, for their generous supply of actinomycete protease inhibitors, and Dr Takahito Yoshida, Aichi Cancer Center Research Institute, Nagoya, for his valuable advice in designing the project.

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