CHARACTERISTICS OF GELATINASE – A PROTEOLYTIC ENZYME ASSOCIATED WITH PIG PEPSIN

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As investigations carried out mainly in recent years have shown, the gastric juice of some species of animals contains not one but several proteolytic enzymes. Two proteinases – pepsin and gastricsins – have been isolated from human gastric juice [1]. From gastric mucosa extract of the hen, three proteolytic enzymes, called pepsins I, II, and III [2], have been obtained. Data exist on the nonuniformity of rennin [3].

As early as 1931, Northrop isolated from unpurified pig pepsin a small amount of an enzyme possessing a well-defined capacity for liquifying gelatin and therefore called gelatinase [4]. The presence of proteolytically active impurities in pig pepsin has been established by the method of paper electrophoresis [5].

In 1959, Ryle and Porter, using chromatography on diethylaminoethylcellulose, found that unpurified preparations of pig pepsin contained, in addition to pepsin proper, comparatively small amounts of two other proteinases – parapepsin I (pepsin B) and parapepsin II (pepsin C). These authors assumed that parapepsin I (pepsin B) was identical with the gelatinase found by Northrop [6]. Subsequently, pepsinogens corresponding to pepsins B [7] and C [8], and also pepsinogen D [9], were found.

In 1961, we showed that paper electrophoresis in combination with the development of proteolytic activity with respect to the liquifaction of the gelatin layer of photographic film easily permits the detection of a second proteolytic enzyme in commercial preparations of pig pepsin [10]. Since this enzyme had an extremely high activity with respect to gelatin, we have provisionally called it "gelatinase" and have put forward the hypothesis that it is related to Northrop's gelatinase and to Ryle and Porter's parapepsin I (pepsin B). In the present paper, the gelatinase isolated from commercial preparations of pig pepsin is characterized in more detail.

Experimental

Materials and methods. Pepsin and gelatinase. Both enzymes were obtained from an unpurified preparation of pig pepsin by chromatography on diethylaminoethylcellulose by a method described previously [11]; they were electrophoretically homogeneous.

Paper electrophoresis. The experiments were carried out in an apparatus of the Durrum type at 300 V and a potential gradient of 3.7 V/cm in volatile buffer mixtures [12]. The positions of the proteolytically active fractions on the electrophoretegrams were determined from the liquifaction of the gelatin layer of photographic film [10, 13] at pH 3.7 or 1.5. In order to show up the protein fraction, the electrophoretegrams were strained with a 0.1% solution of Bromophenol Blue in ethanol (Table 1).

Table 1

Composition of the Buffer Mixtures Used in Electrophoresis*

pH	CH_3COOH (glacial)	Pyridine	85% HCOOH	NH_4OH
	ml			
2.0	—	—	70	—
2.35	58	—	8	—
3.12	15	0.3	—	—
3.66	11	1	—	—
4.04	7	2	—	—
4.29	8	4	—	—
4.65	6.5	6.5	—	—
5.09	6	9	—	—
5.36	2	10	—	—
5.60	1	8.1	—	—
6.19	0.3	8	—	—
7.0	260 (2 N)	—	—	135 (1 N)
8.3**	260 (2 N)	—	—	50 (Conc.)

*Volume of the solutions made up to 2 liters with water.

**Solution made up to 1 liter with water. Before the experiment, the buffer mixture was diluted by a factor of 100 with water.

Comparison of the activities of pepsin and gelatinase on synthetic substrates. 0.25 ml of 0.088 N hydrochloric acid and then 0.25 ml of a solution of the substrate in 0.01 N caustic soda were added to 0.5 ml of a solution of the enzyme in water. The resulting mixture, with pH 1.8-2.0, was incubated for 16 hr at 37°, after which 1 ml of ninhydrin reagent [14] was added, the temperature was raised to 100° for 15 min, 5 ml of 60% alcohol was added, and colorimetry was carried out at 570 mμ. To perform experiments at pH 3.6, the substrate was dissolved in 0.25 ml of a mixture of 5 ml of 0.01 N caustic soda, 5 ml of 0.0088 N hydrochloric acid, and 10 ml of water.

Determination of the activity with respect to hemoglobin. The activities of pepsin and gelatinase were determined by the method of Anson and Mirsky [15]. The experiment was carried out with 0.2 ml portions of solutions of the enzymes at concentrations of 50, 100, and 200 γ/ml and 1 ml of a 2% solution of bovine hemoglobin.

Determination of the viscosity. The viscosity was measured in an Ostwald viscometer. 0.1 ml of a solution of the enzyme (500 γ) was added to 1.5 ml of a 2.5% solution of isoelectric gelatin in the appropriate buffer at 35.5°, and the issuing time of the solution was determined after 1 min and then after each 5 min. Pyridine-acetate mixtures were used to dissolve the gelatin (see above), the pH values of the resulting solutions being 2.1, 3.55, and 4.75.

Determination of the N-terminal amino acids in the products of the hydrolysis of gelatin with pepsin and gelatinase. 200 mg of lyophilized gelatin was dissolved with stirring in 10 ml of pyridine-acetate buffer at pH 4.8 and 37°. The solution was treated with 3 mg of enzyme and the mixture was left in the thermostat at 37° for 5 or 45 min. Then the pH of the mixture was brought to 8.5 with a saturated solution of sodium bicarbonate, 200 mg of dinitrofluorobenzene was added, and the mixture was left with stirring for 1 hr at 40° and then for 20 hr at 20°. When the reaction was complete, concentrated hydrochloric acid was added to 5.7 N and the mixture was hydrolyzed in a sealed tube at 110° for 24 hr. The hydrolyzate was diluted with water by a factor of 3, the DNP-amino acids were extracted with ether, the ethereal extract was evaporated to dryness, and the dinitrophenol was eliminated by vacuum sublimation at 60°. The residue was dissolved in formic acid and transferred to a chromatogram. Chromatography and determination of the DNP-amino acids were carried out by a published method [11]. Control experiments were carried out concurrently to determine the content of N-terminal amino acids in the gelatin after the corresponding incubation without the enzyme and in the enzymes after incubation without gelatin.

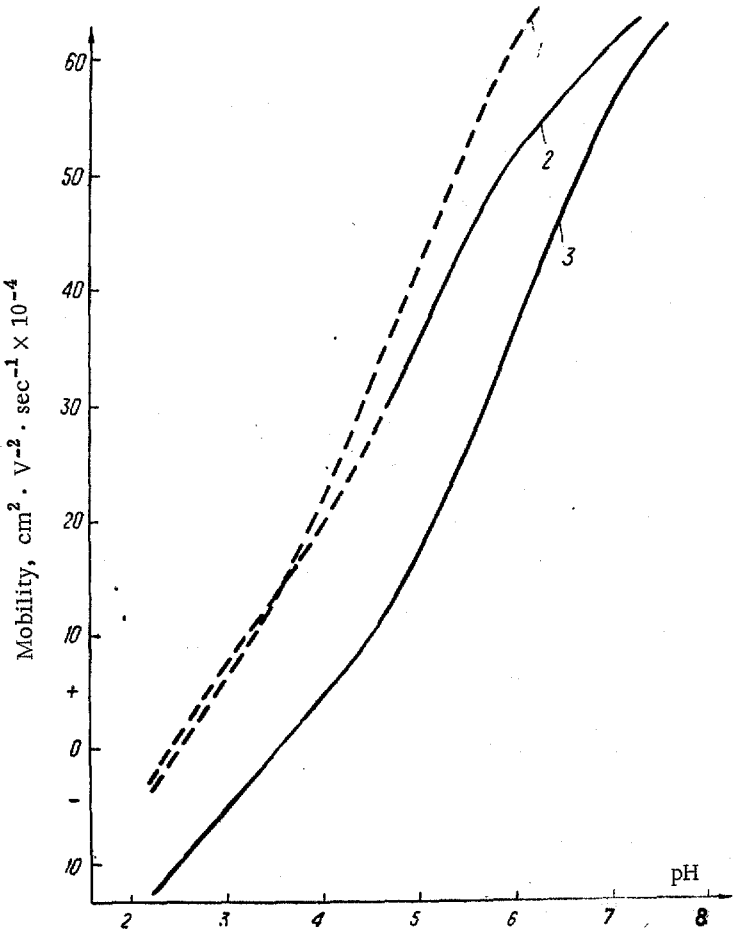

Fig. 1. Electrophoretic mobilities of pepsin (1), pepsinogen (2), and gelatinase (3) as functions of the pH. At pH 4-5, pepsinogen is activated and at lower pH values its mobility coincides with that of pepsin.

Results and discussion.

By investigating electrophoretic mobilities over a wide range of pH values, it is possible to obtain an idea of the ratio of the cationic and anionic groups in the molecule of the protein investigated. In order to characterize the gelatinase, its mobility was compared with the electrophoretic behavior of pepsin. The latter belongs to the class of proteins with well-defined acidic properties, this being explained by the features of its amino acid composition.

According to Perlmann's data [16], one molecule of pig pepsin contains about 36 free carboxyl groups and a phosphate residue, to which are opposed two arginine residues, one lysine residue, and one histidine. The overwhelming predominance of anionic groups is responsible for the low isoelectric point of pepsin.

In our experiments, this proved to be 2. 4-2. 5. The isoelectric point of gelatinase is at pH 3. 5. Thus, this enzyme possesses the properties of an acidic protein, but they are less pronounced than those of pepsin. It can be seen from Fig. 1, which shows the electrophoretic mobility of gelatinase as a function of the pH, that at all pH values it moves towards the anode considerably more slowly than pepsin. In addition, in the pH range of 5-8, the mobility of gelatinase is less than that of pepsinogen, the molecule of which differs from pepsin by having a larger number of cationic groups (15 instead of 5). If it is assumed that the molecules of pepsin and gelatinase are of similar dimensions, the difference in their electrophoretic mobilities must be ascribed to a greater relative content of cationic groups in the gelatinase molecule.

Gelatinase, having a higher isoelectric point than pepsin, is, in contrast to the latter, stable in neutral and weakly alkaline media [10] and in this respect resembles pepsinogen. It may be assumed that the "balance" of cationic and anionic groups in the gelatinase molecule gives rise to a greater stability of the spatial structure of the enzyme.

There is no detailed electrophoretic characterization of parapepsin I in the literature. It is known that it is an anion at pH 4. 0 and 5. 6. On electrophoresis on starch gel at pH 5. 6, parapepsin I has a mobility which is approximately half that of pepsin [6]. The ratio of the mobilities of pepsin and gelatinase at pH 5. 6 that we have found is close to 2, which again confirms the possibility of the identity of gelatinase and parapepsin I.

Particular interest was attached to a comparison of the proteolytic properties of pepsin and gelatinase. As mentioned in a preceding communication [10], the presence of gelatinase is very easily revealed by the liquifaction of gelatin; however, no proteolytic activity with respect to the degradation of hemoglobin when small amounts of the enzymes

Table 2

Action of Gelatinase and Pepsin on Synthetic Substrates

Substrate	Enzyme	pH	Concentration of enzyme, γ/ml	Ratio of enzyme to substrate (by weight)	Hydrolysis, %
Acetyl-L-phenylalanyl-L-di-iodityrosine	Gelatinase	1.8	75	1:4	64
		3.6			100
	Pepsin	1.8			100
		3.6			60
Acetyl-DL-phenylalanyl-L-tyrosine	Gelatinase	1.8	400	1:3	64
		3.6			13
	Pepsin	1.8			100
		3.6			20
Acetyl-DL-phenylalanyl-DL-phenylalanine	Gelatinase	1.8		2:1	5
		3.6			0
	Pepsin	1.8			20
		3.6			9.5

eluted from electrophoregrams are used has been observed. Having acquired the possibility of working with very small amounts of material, we have studied its action on gelatin, hemoglobin, and synthetic peptide substrates (Table 2).

As can be seen from Table 2, synthetic peptides are hydrolyzed by both enzymes, although in some cases gelatinase exhibits a somewhat lower activity than pepsin. However, it must be noted that the results given have only a semi-quantitative character, since hydrolysis with pepsin was excessive, making an accurate comparison of activities difficult.

Although gelatinase degrades hemoglobin, its specific activity on this substrate is almost four times lower than that of pepsin. Moreover, gelatinase far exceeds pepsin in its capacity for reducing the viscosity of solutions of gelatin. This property of gelatinase is so characteristic that it deserves special consideration. Figure 2 shows the fall in the relative viscosity of gelatin solutions occurring as a consequence of the action of pepsin or gelatinase at various pH values. As can be seen from the figure, gelatinase causes a sharp fall in viscosity even in the first few minutes of incubation, while pepsin acts far more slowly and the final decrease in viscosity is not so great. The difference in the nature of the actions of the two enzymes is particularly pronounced at pH 3. 55-4. 75.

In order to compare the mechanism of the action of pepsin and gelatinase on gelatin in more detail, we used the dinitrophenylation method to determine the N-terminal amino acids of the peptide fragments formed in the degradation of gelatin by these enzymes at pH 4. 8 at 37° (Table 3).

The results of the determination, given in Table 3, show that the action of gelatinase, like that of pepsin, leads to the rupture of the peptide bonds. It is characteristic that after the first 5 min gelatinase has split considerably more

peptide bonds than pepsin. In the subsequent 40 min, this difference evens out. These results agree well with the fall in viscosity of gelatin in analogous experiments (see Fig. 2).

No essential differences in the specificity of the hydrolysis of peptide bonds by pepsin and gelatinase were observed. The only exception worth mentioning is the more intensive splitting, in the first stages of the action of gelatinase, of bonds formed by the amino group of serine.

Table 3

Increase in the Content of N-Terminal Amino Acids During the Hydrolysis of Gelatin with Pepsin and Gelatinase (in Moles of DNP-Amino Acids per 10^5 g of Gelatin)

DNP-amino acids	Time of hydrolysis			
	5 min		45 min	
	Pepsin	Gelatinase	Pepsin	Gelatinase
Glycine	—	0.022	—	0.022
Serine	0.016	0.238	0.157	0.238
Threonine	0.004	0.034	0.025	0.053
Aspartic acid	0.033	0.045	0.176	0.194
Glutamic acid	—	—	—	—
Alanine	0.021	0.043	0.113	0.190
Valine	0.015	0.048	0.096	0.099
Leucine	0.072	0.126	0.306	0.143
Phenylalanine	0.026	—	0.029	0.006
Lysine	0.062	0.061	0.114	0.077
Proline	0.003	0.006	0.054	0.045
Hydroxyproline	—	0.006	0.005	0.036
Total	0.252	0.629	1.075	1.103

Thus, there are no grounds for explaining the specificity of the action of gelatinase by the presence in it of some special "gelatinase" activity not connected with the splitting of peptide bonds. The considerable difference in the activity of gelatinase with respect to hemoglobin and gelatin depends mainly not on the nature of the substrates but on the differences in principle between the methods used for determining activities on these substrates. In establishing activities with respect to hemoglobin, treatment of the enzymatic hydrolyzate with trichloroacetic acid, which precipitates proteins and large peptides, is specified. In this case, the content of soluble low-molecular-weight hydrolysis products in the filtrate is used as a measure of the degree of degradation.

If it is assumed that the enzyme ruptures a peptide bond approximately in the central part of the molecule, then because of the precipitation of the large fragments formed the very fact of the rupture of the peptide bonds may remain unnoticed.

In contrast to this, measurement of the viscosity of solutions of gelatin readily permits the detection of the splitting of the molecule into large fragments. From the results obtained it may be considered that it is just this kind of decomposition which predominates in the action of gelatinase on gelatin.

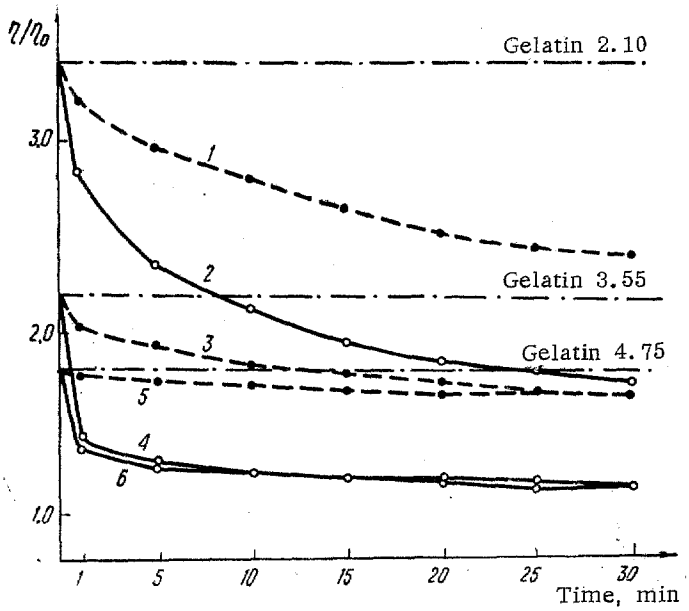

Fig. 2. Relative viscosity of solutions of gelatin as a function of the time of incubation with enzymes: 1, 3, and 5) Addition of pepsin at pH 2.10, 3.55, and 4.75, respectively; 2, 4, and 6) Addition of gelatinase at the same pH values.

The results given in the present paper once again indicate that pig pepsin is accompanied by a proteolytic enzyme of similar nature. However, the content of this enzyme is extremely small, which complicates its detailed study. In view of this, it is not possible to make a final conclusion about the homogeneity of gelatinase, although a number of

indications (paper electrophoresis, chromatography on DEAE-cellulose) indicate that the enzyme may be considered homogeneous. The present experimental data show the similarity of gelatinase to parapepsin I, but additional investigations of both enzymes are necessary in order to confirm their identity.

Summary

1. A comparative study of pig pepsin and the proteolytic enzyme which accompanies it (gelatinase) has been carried out.

2. As shown by paper electrophoresis, gelatinase is a less acidic protein than pepsin.

3. Like pepsin, gelatinase ruptures peptide bonds in hemoglobin, gelatin, and some dipeptides. A special feature of gelatinase is its well-defined capacity for reducing the viscosity of gelatin solutions. The possible causes of the specific nature of the action of gelatinase on gelatin have been considered.

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