THE ACTION OF SOLUBLE AND IMMOBILIZED CARBOXYLIC PROTEINASES ON SERUM PROTEINS

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Proteolytic enzymes are widely used to obtain fragments of immunoglobulin and for the cleavage of other serum proteins. The use for this purpose of proteinases of animal origin (pepsin [1], trypsin [2], and cathepsin B_1 [3]) and of plant origin (papain [4] and bromelin [5]) has been described. Literature information on the use of microbial proteinases in these processes is limited.

The aim of the present work was a comparative study of the action of carboxylic proteinases (porcine pepsin and its analogs) and proteinases of microscopic fungi of the genus *Aspergillus* [*A. awamori* (aspergillopepsin A) and *A. foetidus* (aspergillopepsin F)] in solution and in the immobilized form on immunoglobulin and other serum proteins.

The choice of fungal proteinases for performing the limited proteolysis of immunoglobulin is not fortuitous. They are typical carboxylic proteinases and, just like pepsin, they are inactivated by diazoacetyl inhibitors, the optimum pH for their action being in the acid region at 2.3-2.5, their molecular weights are close to that of pepsin, and they are distinguished by stability in acid solutions [6, 7]. Thus, the proteinases of mold fungi are close analogs of pepsin, which permits their use in the limited proteolysis of immunoglobulin.

The action of carboxylic proteinases on a highly purified preparation of horse antitetanic immunoglobulin or human donor gamma-globulin was evaluated by means of electrophoresis.

In good agreement with literature information [8], the limited proteolysis of preparations of immunoglobulin by porcine pepsin in solution (37°C, pH 4.0, 30 min) leads to the formation of fragments with molecular weights of 90,000 ($F(ab')_2$) and 45,000-50,000 (Fc) (Fig. 1). It is not excluded that in the zone corresponding to a molecular weight of 45,000-

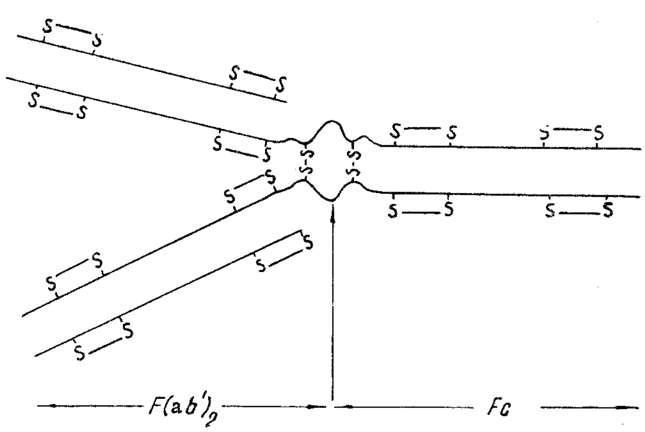

Fig. 1

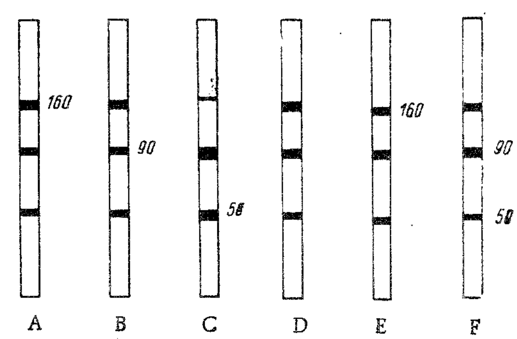

Fig. 2

Fig. 1. Scheme of the structure of immunoglobulin. The vertical arrow denotes the position of action of carboxylic proteinases.

Fig. 2. Electrophoresis of hydrolyzates of immunoglobulin obtained under the action of carboxylic proteinases in solution: A, B) porcine pepsin at pH 3.0 and 4.0; C, D) aspergillopepsin A at pH 3.0 and 4.0; E, F) aspergillopepsin F at pH 3.0 and 4.0. The molecular weights are shown in thousands of daltons.

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TABLE 1. Composition of the Hydrolyzates of Horse Antitetanic Gamma-Globulin (from the results of electrophoresis in polyacrylamide gel)

Main component	Molecular weight	pH of hydrolysis	Amount, % on the total protein	
			hydrolysis with porcine pepsin	hydrolyis with aspergillopepsin A
Uncleaved molecules	160 000	3,0	60	3
		4,0	65	62
		5,0	75	75
$F(ab')_2$ fragments	90 000	3,0	33	55
		4,0	30	22
		5,0	14	17
Fc fragments	45 000–50 000	3,0	6	43
		4,0	5	15
		5,0	10	5

50,000, in addition to the Fc fragments there may also be univalent Fab fragments, although their formation is not characteristic for the action of pepsin. The limited proteolysis of practically all the gamma-globulin molecules to $F(ab')_2$ and Fc fragments, and also to peptides of low molecular weight in a short time (30-60 min), takes place only at high enzyme: substrate ratios of 1:10 to 1:25. However, with an increase in the time of hydrolysis to 2-6 h, the same result is achieved at lower enzyme–substrate ratios of 1:100-1:200.

In addition to the action of pepsin on immunoglobulin we studied the limited proteolysis of this protein by the carboxylic proteinase of microscopic fungi — aspergillopepsin A — in solution. The products formed were analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate followed by densitometry. The hydrolysis of immunoglobulin by this enzyme was performed at various pH values (37°C, 30 min; enzyme : substrate ratio 1:100). It was established that at pH 3.0-5.0 under the action of aspergillopepsin A fragments of gamma-globulin monotypical with those that are obtained by the action of porcine pepsin (Fig. 2) were obtained. The limited proteolysis of immunoglobulin takes place with the formation of high-molecular-weight fragments of the types of $F(ab')_2$ (mol. wt. 90,000) and Fc (mol. wt. 50,000), their ratio depending on the pH of the medium. At pH 3.0 (37°C, 30 min, enzyme:substrate ratio 1:100), the specific cleavage of practically all the immunoglobulin molecules took place and only 3-5% of whole molecules remained in the mixture (Table 1).

The proportion of the total protein going into the $F(ab')_2$ fragments formed in an acid medium at pH 3.0 under the action of the microbial proteinase was 55%, and the fraction corresponding to the Fc fragment was 43%. Theoretically, knowing the structure of the immunoglobulin molecule, it may be assumed that on the limited proteolysis of all the molecules of this protein 66% should go to the $F(ab')_2$ fragments and approximately 34% of the total protein to the Fc fragment. The increased amount of fraction corresponding to the Fc fragment on the hydrolysis of immunoglobulin by aspergillopepsin A in acid solutions may probably be explained by the inclusion in this fraction of univalent Fab fragments having a molecular weight close to that of the Fc fragment. On the hydrolysis of immunoglobulin at pH 4.0 by the carboxylic proteinase of mold fungi (37°C, 30 min, enzyme: substrate ratio 1:100), about 40% of the initial molecules underwent cleavage, and at pH 5.0 only 25%.

Thus, in the pH ranges studied, the farthest-reaching hydrolysis of immunoglobulin by aspergillopepsin A takes place at pH 3.0. In a pepsin hydrolyzate under the same conditions (37°C, pH 3.0, enzyme:substrate ratio 1:100, 30 min) there was a larger amount of uncleaved molecules (60%), and the proportion of $F(ab')_2$ fragments was 33% and of the Fc fragment only 6% (see Table 1). It is known that the Fc fragment is comparatively readily cleaved into smaller peptides which, because of diffusion and perhaps difficulty in staining, are not revealed under the conditions of the given experiment. This may explain the low content of the Fc fragment.

To determine more accurately the ratios between the fragments in hydrolyzates of immunoglobulin obtained under the action of porcine pepsin and aspergillopepsin A, we chromatographed them on polyacrylamide-agarose gel (Ultrogel AcA-44). This material has been used previously for the separation of a plasmin hydrolyzate of immunoglobulin [9]. Figure 3 shows a chromatogram of a hydrolyzate obtained by the action of aspergillopepsin A on immuno-

globulin in solution (37°C, enzyme:substrate ratio 1:100, 30 min) at pH 3.0. Chromatography on Ultrogel AcA-44 permits the separation of the high- and low-molecular-weight components of the hydrolyzate, and also the isolation of fractions corresponding to short peptides. The latter is yet one more proof of the further decomposition of the unstable Fc fragment.

As can be seen from Fig. 3, the immunoglobulin hydrolyzate separates into five peaks, and these were analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate. It was established that the first peak contained whole molecules of gamma-globulin (6% of the total amount of protein). gamma-Globulin molecules were also present in the second peak. Its right-hand slope contained practically pure $F(ab')_2$ fragments, making up about 22% of the total protein. Peak IV, corresponding to the Fc fragment, contained 12% of the total protein and, thus, its amount determined from the results of electrophoresis (43%) is actually too high. In addition to this there was peak V, corresponding to the low-molecular-weight hydrolysis products (15% of the total amount of protein).

The possibility of separating the Fc fragment and the short peptides from the other components of the mixture is the advantage of polyacrylamide-agarose gel (Ultrogel AcA-44) as compared with Sephadexes G-150 and G-200, upon which it is impossible to bring about this separation and thereby to establish the actual amount of Fc fragment in the mixture.

With respect to the formation of comparatively short peptides — hydrolyzates soluble in TCA — it was established that the pH optimum for the hydrolysis of immunoglobulin by aspergillopepsin A in solution is pH 3.5 (Fig. 4). It can be seen from Fig. 4 that soluble aspergillopepsin A cleaves another serum protein — bovine serum albumin — best at pH 3.3. The optimum pH value for the nonspecific far-reaching cleavage of a mixture of serum proteins obtained after the elimination of the gamma-globulin by precipitation with ammonium sulfate is also at pH 3.3.

We also studied the action on immunoglobulin of another proteinase of mold fungi — aspergillopepsin F. It was found that aspergillopepsin F, like pepsin and aspergillopepsin A, specifically cleaves the gamma-globulin molecule to $F(ab')_2$ and Fc fragments (see Fig. 2E, F).

The possibility of the use of immobilized carboxylic proteinases for the limited proteolysis of immunoglobulin is of interest. Under the action of porcine pepsin and aspergillopepsin A and F immobilized on amino-Silochrome in the pH range from 2.0 to 6.0 the same characteristic fragments $F(ab')_2$ and Fc as under the action of these enzymes in solution were formed. In the given pH range during 1-2 h the most intensive cleavage of immunoglobulin, according to electrophoresis, is observed in the case of immobilized porcine pepsin at pH 4.0, while in more acidic and more alkaline media the appearance of $F(ab')_2$ and Fc fragments is detected 6-10 h from the beginning of proteolysis. In the action on immunoglobulin of immobilized aspergillopepsin A for 1-2 h, the most intensive cleavage is ob-

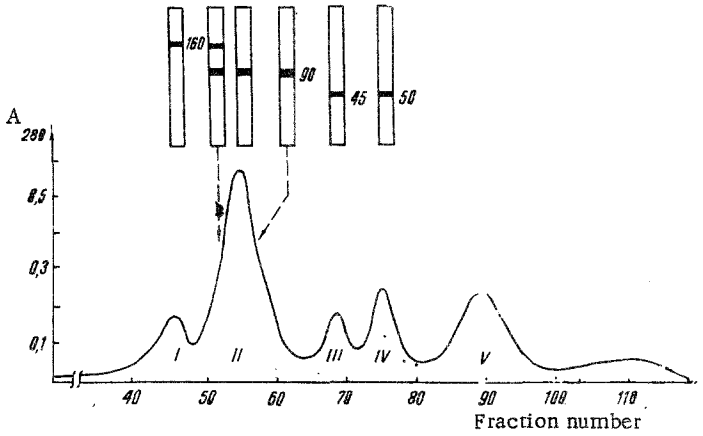

Fig. 3. Chromatography of a hydrolyzate of immunoglobulin obtained under the action of aspergillopepsin A in solution at pH 3.0. The molecular weights on the electrophoretograms of the corresponding peaks are given in thousands of daltons.

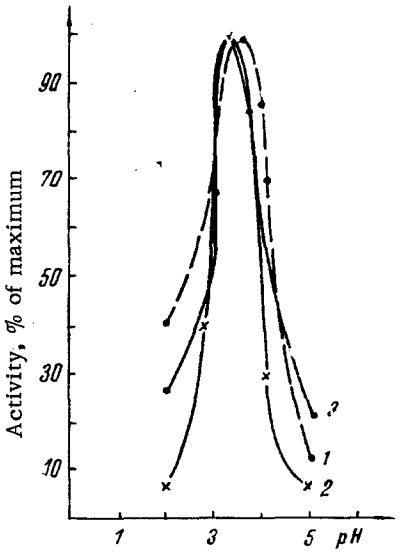

Fig. 4

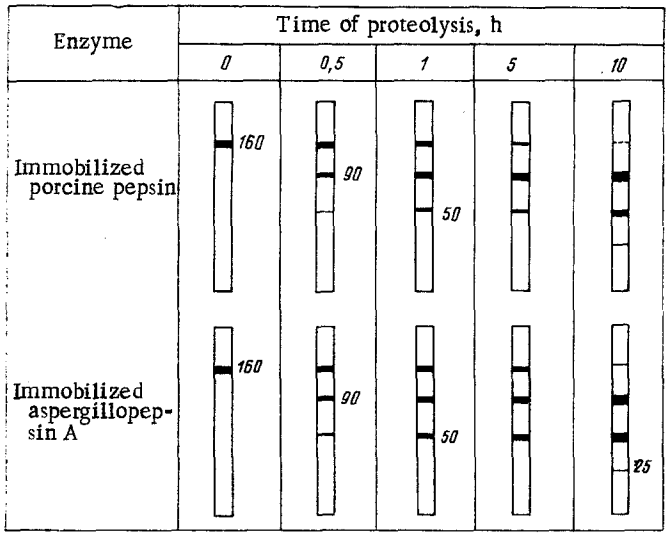

Fig. 5

Fig. 4. Dependence of the activity of aspergillopepsin A in solution on the pH in its action on various substrates: 1) horse antitetanic immunoglobulin; 2) bovine serum albumin; 3) a mixture of serum proteins excluding immunoglobulin.

Fig. 5. Electrophoresis of hydrolyzates of immunoglobulin obtained under the action of immobilized porcine pepsin and aspergillopepsin A at pH 4.0 as functions of the time. The molecular weights are shown in thousands of daltons.

served at pH 3.0-3.5. At pH 4.0 after the hydrolysis of immunoglobulin with immobilized porcine pepsin for 10 h or with immobolized aspergillopepsin A for 15 h whole molecules of immunoglobulin have disappeared from the reaction mixture.

Thus, carboxylic proteinases immobilized on amino-Silochrome perform the limited proteolysis of the immunoglobulin molecule like the analogous enzymes in solutions, although this process takes place more slowly, which can be explained by the difficult access of the high-molecular-weight substrate (molecular weight about 160,000) to the enzyme rigidly fixed to macroporous silica. The advantage of the use of immobilized proteolytic enzymes is, however, the possibility of stopping the hydrolysis reaction at any desired intermediate stage, which is done simply by removing the enzyme from the mixture.

The results obtained once more confirm the fact that the sites of action of different proteolytic enzymes on immunoglobulin are similar [10]. In the limited proteolysis of immunoglobulin by aspergillopepsins A and F, these enzymes, like animal proteinases, probably attack and cleave the so-called hinge region of the immunoglobulin molecule. The more effective hydrolysis of immunoglobulin by the carboxylic proteinases of microscopic fungi in solutions at pH 3.0-3.5 can be explained by the assumption that these pH values are close to the pH optimum of the action of these proteinases. At the same time, the pH optimum of porcine pepsin shifts into the more acid region (pH 1.8-2.0). Consequently, the carboxylic proteinases of microscopic fungi can be used for the limited proteolysis of immunoglobulin in addition to the proteolytic enzymes of animal and plant origin employed previously.

EXPERIMENTAL

The work was performed with highly purified preparations of porcine pepsin [11] and aspergillopepsins A and F isolated in our laboratory. All the preparations were homogeneous according to disk electrophoresis.

Isolation of gamma-Globulin. Horse antitetanic gamma-globulin was isolated from native plasma [12]. gamma-Globulin was isolated similarly from human donor blood plasma. According to the results of electrophoresis and immuno-electrophoresis, both preparations were immunochemically pure gamma-globulin.

Preparation of Immobilized Carboxylic Proteinases. To 15 mg (0.45 μmole) of highly purified aspergillopepsin A were added 6.6 mg (15.6 μmole) of the water-soluble N-cyclohexyl-N'-[2-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate and 500 mg of amino-Silo-

chrome (150 μeq of amino groups) suspended in 2 ml of water. The resulting mixture was stirred at +22-24°C for 1 h, after which 6.6 mg (15.6 μmole) of carbodiimide was added and the reaction was continued for 1 h, the pH being maintained at 4.5-5.0 by the addition of 1 N HCl. After the end of the reaction, the aqueous solution of unchanged aspergillopepsin A was removed by decantation. In order to eliminate the enzyme adsorbed on the spot by ion-exchange, the immobilized aspergillopepsin A was washed successively with 0.1 N HCl and 0.1 N acetate buffer, pH 5.0 and was subsequently stored at 4°C. The yield of immobilized aspergillopepsin A was 30%.

Immobilized porcine pepsin and immobilized aspergillopepsin F were obtained similarly. In all cases, the amount of enzyme added to the amino-Silochrome was determined from the results of amino acid analysis. In the case of the immobilization of aspergillopepsin A, to 1 g of support was added 20-25 mg (0.6-0.75 μmole), and in the immobilization of porcine pepsin and aspergillopepsin F 30 mg (0.85 μmole) and 6 mg (0.25 μmole) of enzyme, respectively. The smaller amount of aspergillopepsin F in the immobilized sample is explained by the fact that a smaller amount of soluble aspergillopepsin F (0.56 μmole) per gram of support was used in the reaction [13].

Limited Proteolysis of gamma-Globulin. To 1 ml of a 1% solution of gamma-globulin in 0.1 M acetate buffer, pH 3.0, was added 100 μl of a 1% solution of highly purified aspergillopepsin A in the same buffer. The enzyme:substrate ratio was 1:100. Hydrolysis was carried out for 30 min in a thermostat at 37°C with stirring. The reaction was stopped by bringing the pH to 7.0-8.0 with 2.5 N NaOH. Then the hydrolyzate was dialyzed against 0.01 M phosphate buffer, pH 7.0, and was analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate. The limited proteolysis of gamma-globulin by porcine pepsin and by aspergillopepsin F, and also at different enzyme:substrate ratios and pH values of the medium was carried out in a similar manner to that described above.

Chromatography on Ultrogel AcA-44. Chromatography was performed on a column of Ultrogel AcA-44. The immunoglobulin hydrolyzates obtained under the action of aspergillopepsin A at pH 3.0 (5 ml, total optical units 50) were deposited on a column (2.5 × 100 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 7.5, in the presence of 10^{-3} M EDTA. Then the column was washed with the initial buffer at 4°C at the rate of 15 ml/h. The protein was determined spectrophotometrically at 280 nm. The proteins eluted in the corresponding peaks were analyzed in polyacrylamide gel.

Electrophoresis in Polyacrylamide Gel. Electrophoresis was performed in 5% polyacrylamide gel in the presence of 0.1% of sodium dodecyl sulfate in a "Savant" instrument at a current strength of 14 mA per tube. The gels were stained with 0.25% Coomassie R-250 in 7% acetic acid and were washed free from the dye with the same solvent. The molecular weights of the components of the hydrolyzates were determined by the method of Weber and Osborn [14], using the calibration curve described in our previous paper [12]. Before deposition on the gel, the pH of the samples under investigation were brought to 7.0 with 0.1 M phosphate buffer, pH 7.0. The samples were incubated with a 1% solution of sodium dodecyl sulfate at 100°C for 2 min. In the analysis of samples with a high content of salts, the latter were eliminated by dialysis against the pH 7.0 electrode buffer for 5-10 h.

The densitometry of the colored protein zones obtained was performed on a High Soft Laser laser densitometer (Serva, GFR). The ratio of the densities of the protein bands in the gel was calculated from the areas of the peaks measured with the aid of a planimeter. The area of each peak was referred to sum of all the areas.

SUMMARY

1. It has been shown that the carboxylic proteinases of microscopic fungi perform the limited proteolysis of immunoglobulin molecules.

2. Like porcine pepsin, aspergillopepsins A and F specifically cleave immunoglobulin molecules to $F(ab')_2$ and Fc fragments, the ratio of which was determined accurately by chromatography on Ultrogel AcA-44. More effective hydrolysis of immunoglobulin molecules in acid solutions by these enzymes than by porcine pepsin was observed.

3. Immobilized carboxylic proteinases also performed the limited proteolysis of immunoglubulin similarly to the soluble enzymes.

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DETERMINATION OF N-ACETYLGLUCOSAMINE IN HUMAN IMMUNOGLOBULIN M AND ITS FRAGMENTS

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The determination of N-acetylglucosamine (AGA) is an essential component of the investigation of glycoproteins. For this purpose a method is frequently used which includes the hydrolysis of the glycoprotein with hydrochloric acid followed by the determination of the glucosamine hydrochloride formed on an automatic amino acid analyzer. The concentration of hydrochloric acid and the temperature and time of hydrolysis vary in the work of different authors, but the majority of workers adhere to averaged conditions of hydrolysis: 3-4 N HCl, 100°C (more rarely 110°C), 4 h [1]. Strictly speaking, the use of such averaged conditions for the accurate determination of AGA in different glycoproteins is illegitimate and the conditions and temperature of hydrolysis should be selected for each concrete case.

We have carried out such work in the course of the isolation and determination of the characteristics of human immunoglobulin M (IgM) (Waldenström's disease) [2] and have found that the maximum splitting out of glucosamine is achieved in the hydrolysis of IgM with 3 N HCl at 110°C for 4 h. By using these conditions for the determination of AGA in the $(Fc)_5$ and Fab fragments of the same IgM we found that it is impossible to draw up a balance: the total amount of AGA found in the $(Fc)_5$ and Fab fragments was substantially lower than in the initial IgM. This induced us to consider in more detail in the present work the dependence of the amount of AGA found in these three preparations on the temperature and time in the hydrolysis of the samples with 3 N HCl.

As can be seen from Table 1 and Fig. 1, the amount of AGA found in IgM and its $(Fc)_5$ and Fab fragments during hydrolysis at 110°C reach a maximum with time for all three preparations, after which they fall again. However, the positions of these maxima are different, being 4, 6, and 8 h, respectively, for IgM and the $(Fc)_5$ and Fab fragments, respectively. During the hydrolysis of the samples at 100°C, a similar situation is observed, but the process takes place considerably more slowly and at the same time it does not reach those maximum values that were obtained at 110°C (see Table 1). Apparently, the length of the hydrolysis process at 110°C leads to the predominance of reactions involving the secondary destruction of AGA in the process of its liberation.

The results obtained suggest the possibility of achieving still higher yields of hydrolyzed glucosamine with a rise in the temperature of hydrolysis (above 110°C). However,

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