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PURIFICATION OF CARBOXYLIC PROTEINASES ON AMINOSILOCHROME

T. A. Solov'eva, S. V. Belyaev, and V. M. Stepanov

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Carboxylic proteinases, especially the pepsins of various animals and rennin or chymosin find wide practical use, but to obtain specimens of these proteinases with a high degree of purity in preparative amounts remains a difficult task.

Anion-exchange resins mainly based on cellulose are usually used for the chromatographic fractionation of proteins with a low isoelectric point. Sorbents based on inorganic materials (silica – Silochrome) have a number of advantages over organic matrices. The inorganic matrix of Silochrome with amino groups immobilized on the surface of the support does not contract or swell in solvents and is stable to the action of acids and organic solvents. The macroporous support (250-700 Å) has no denaturing action on the labile molecule of an enzyme. The particle size of Silochrome (0.25-0.5 mm) ensures a high rate of flow of the solution. Such an ion-exchange material can be used repeatedly. We have shown the possibility of obtaining analytical amounts of porcine pepsin by chromatography on a column of Aminosilochrome C-80 [1]. The present communication is devoted to a description of the preparative separation and purification of a number of carboxylic proteinases: the separation of calf pepsin and chymosin and the purification of bovine and porcine pepsins on Aminosilochrome.

The presence of a large number of carboxy groups on the surface of an enzyme when the number of residues of basic amino acids is low shifts the isoelectric points of the carboxylic proteinases into the acid region (pI of pepsin atout 2.0) or the weakly acid region (pI of the chymosins about 4.5). Knowing the values of pI enabled us to select the conditions [1] for the sorption and desorption of proteinases on aminated Silochrome. Sorption of the enzymes was carried out at pH 5.5. At this pH the possibility of autolysis is reduced to a minimum and at the same time the enzymes possess a charge opposite in sign to that of the anion-exchange material. The conversion of the enzymes into the isoelectric state ensures the suppression of these ionic interactions and their desorption.

All-Union Scientific-Research Institute of the Genetics and Breeding of Industrial Microorganisms, Moscow.

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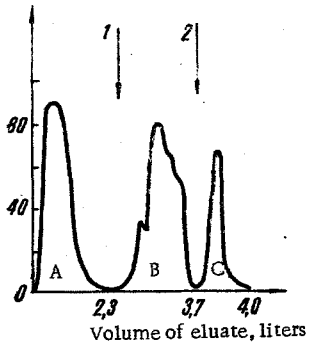

Fig. 1

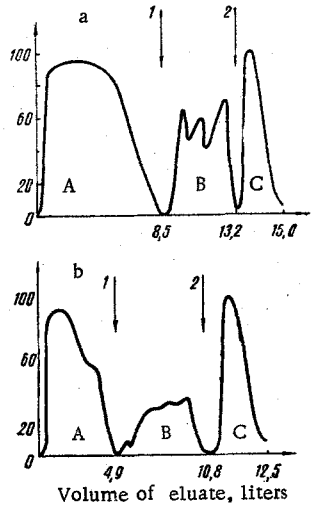

Fig. 2

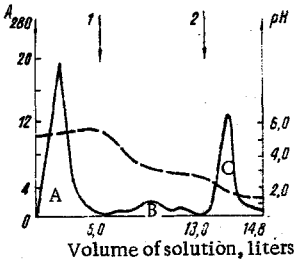

Fig. 3

Fig. 1. Chromatography of a preparation of pepsin and chymosin from calf stomach on Aminosilochrome C-80 (along the axis of ordinates readings of a Uvicord 11 instrument, LKB, Sweden. The arrows show the beginnings of elution): 1) 0.005 M HCl, pH 2.5; 2) 0.05 M HCl, pH 1.6. Peak A) an impurity possessing no enzymatic activity; B) chymosin; c) calf pepsin. For the experimental conditions, see text.

Fig. 2. Chromatography of a commercial preparation of bovine pepsin (a) and of porcine pepsin (b) on Aminosilochrome C-120: peak A) inactive impurity; B) minor components of the pepsin; C) bovine pepsin; D) porcine pepsin. Symbols and elution conditions as for Fig. 1.

Fig. 3. Purification of a commercial preparation of porcine pepsin on Aminosilochrome C-120 under static conditions: peak A) inactive impurity; B) minor components of the pepsin; C) porcine pepsin.

The pepsins were eluted from the column with 50 mM HCl, pH 1.6; the HCl was eliminated from the pepsin-containing eluates by means of an anion-exchange resin in the OH^- form (instead of dialysis) which considerably shortens the time of preparation of the enzyme for freeze-drying. However, on storage the activity of such a freeze-dried preparation gradually fell by approximately 30% and its solubility decreased. Apparently, dry preparations of pepsin in which practically all the carboxy groups are protonated are unstable. In view of this the enzyme was stabilized by adding 30 moles of sodium acetate per mole of pepsin, which approximately corresponds to the number of free COOH groups in its molecule. Under these conditions the pH of a solution of the pepsin remained at 4.8. The preparation obtained by freeze-drying of such a solution dissolves readily and retains a high activity for a long time – for at least six months (on storage in a desiccator at 4°C).

As can be seen from Figs. 1-3, the conditions of ion-exchange fractionation of "acidic" proteinases from various sources are similar and a single chromatographic process permits the clear separation of related enzymes such as calf pepsin and chymosin, the separation of the minor pepsins of the ox and the pig from the forms of the pepsin present in major amount, and the production of specimens with a high degree of purity in practically quantitative yield. The high degree of purification is shown by the high values of the specific activities of the enzymes.

The specimens of porcine and bovine pepsins obtained were homogeneous according to disk electrophoresis in polyacrylamide gel, and the chymosin preparation showed the presence of three bands of closely similar mobilities corresponding to isoforms of this enzyme. It must be emphasized that Aminosilochrome is particularly convenient as a sorbent for the isolation of proteins under static conditions. In this case, the sorption-desorption of the enzyme does not include lengthy operations which enables the time of purification to be substantially shortened. Thus, one working day is sufficient for the isolation of 10 g of pure pepsin by the method described.

EXPERIMENTAL

Preparation of the Aminosilochrome. To 200 g of Silochrome C-80 or C-120 in 600 ml of ethanol was added 30 ml of γ-aminopropyltriethoxydilane, the mixture was stirred and was kept at 37°C for 72 h, and then, with stirring, 10 ml of concentrated HCl was added dropwise and after 15-20 min the excess of γ-aminopropyltriethoxysilane was eliminated by washing with ethanol (3-4 × 1 liter) and with distilled water (twice). Where

necessary, the preparation was dried at 70°C for a day. The capacity [2] of the preparation of Aminosilochrome obtained amounted to 0.30-0.35 meq/g. The loading of the Aminosilochrome with protein amounted to 100 mg/g of sorbent.

The proteolytic activities of the specimens were determined by Anson's method [3, 4]. To 1 ml of a 2% solution of hemoglobin, pH 2.0, previously heated at 37°C for 3 min was added the number of microliters of the enzyme that corresponded, judging from the optical density of the enzyme solution at 280 nm, to approximately 10 μg of pure pepsin. The mixture was kept at 37°C for 10 min. The reaction was stopped by the addition of 5 ml of a 5% solution of trichloracetic acid (TCA). The reaction mixture was kept at 37°C for another 2 min and was then cooled to 20°C over 30 min and filtered through a filter paper for fine precipitates, and the optical density of the filtrate was measured at 280 nm against the control sample. In the control sample a solution of hemoglobin was incubated at 37°C for 10 min, and then 5 ml of TCA and, only after this, the enzyme solutions were added. The number of units of activity was calculated from the formula

$$\text{a.u./o.u.} = \Delta E_{280} \cdot 1000/E_{280} \cdot V,$$

where ΔE_{280} is the optical density of the TCA filtrate of the experimental sample (after deduction of the control), 1000 is a factor, E_{280} is the optical density of the solution of the enzyme under investigation, V is the volume of the enzyme sample, μl, and a.u./o.u. is activity units/optical units.

Determination of Milk-clotting Activity by Ginodman's Method [5]. To 3 ml of a 0.5% solution of dry defatted milk in 0.1 M acetate buffer, pH 5.6, and in 0.03 M $CaCl_2$ previously heated at 37°C for 3 min was added that number of microliters of enzyme that corresponded to approximately 1 μg of purified pepsin or chymosin, and the mixture was stirred and was kept in the water bath at 37°C with periodic stirring by means of a glass rod. The time of appearance of flocs was recorded by means of a stopwatch. The activity (a.u./o.u.) was calculated from the formula:

$$\text{a.u./o.u.} = 1000/E_{280} \cdot V \cdot t,$$

where E_{280} is the optical density of the solution of enzyme under investigation at 280 nm, V is the volume of the solution of enzyme investigated, ml, and t is the time of appearance of the first flocs of milk, sec.

Chromatography of a Preparation of Chymosin. To 100 g of a mixture of chymosin and pepsin from calf stomach was added 700 ml of 0.1 M acetate buffer, pH 5.6, and it was dialyzed against the same buffer overnight and was then centrifuged at 8000 rpm. The precipitate was discarded. The supernatant, the activity of which in the cleavage of hemoglobin was 2.8 a.u./o.u. and in the clotting of milk 2400 a.u./o.u was deposited at the rate of 3 ml/min on a column containing 100 g of Aminosilochrome C-80 equilibrated with 0.1 M acetate buffer, pH 5.6. Then the column was washed with 0.1 M acetate buffer, pH 5.6, to E_{280} = 0.1 in the eluate.

The chymosin was eluted with 0.005 M HCl, pH 2.5. The yield of chymosin was 16% (970 mg) of the total protein deposited on the column, or 80% of the total milk-clotting activity and 8% of the total hemoglobin activity; the activity of the specimen obtained in relation to the cleavage of hemoglobin was 4 a.u./o.u. and in relation to the clotting of milk 20,000 a.u./o.u. The pepsin was eluted with 0.05 M HCl, pH 1.6. The yield of calf pepsin was 5% (300 mg) of the total protein deposited on the column, and 20% of the total milk-clotting activity and 50% of the total hemoglobin activity. The activity of the specimen obtained was 34 a.u./o.u. with respect to the cleavage of hemoglobin and 11450 a.u./o.u. with respect to the clotting of milk. The column was regenerated with 1 M HCl and was then equilibrated with 0.1 M acetate buffer, pH 5.6. All the operations were carried out at 4°C.

Chromatography of Bovine Pepsin. To 115 g of a commercial preparation of bovine pepsin was added 2400 ml of 0.1 M acetate buffer, pH 5.5, and then the mixture was stirred and was centrifuged at 8000 rpm for 20 min. The precipitate was discarded. The supernatant, the activity of which in relation to the cleavage of hemoglobin was 2.4 a.u./o.u., was deposited at the rate of 4 ml/min on a column containing 180 g of Aminosilochrome C-120 equilibrated with 0.1 M acetate buffer, pH 5.5. The column was washed with the same buffer to E_{280} = 0.2 in the eluate, and then with 0.005 M HCl to E_{280} = 0.08. The pepsin was eluted with 0.05 M HCl, pH 1.6. The yield of bovine pepsin was 7.3% (1.5 g) of the total protein deposited on the column or 74% of the total proteolytic activity. The activity for the preparation of bovine pepsin obtained with respect to hemoglobin was 24.5 a.u./o.u.

Chromatography of Porcine Pepsin. To 40 g of a commercial preparation of porcine pepsin was added 1600 ml of 0.1 M acetate buffer, pH 5.5. The mixture was centrifuged at 8000 rpm for 20 min. The precipitate was discarded. The supernatant, the activity of which in relation to the cleavage of hemoglobin was 32.8 a.u./o.u. was deposited on a column containing 180 g of Aminosilochrome C-120 equilibrated with 0.1 M acetate buffer, pH 5.5. Then purification was carried out as in the chromatography of bovine pepsin. The yield of porcine

pepsin was 10 g or 47% of the total amount of protein deposited on the column, and 100% of the total proteolytic activity. The activity of the specimen obtained was 74.3 a.u./o.u.

Accelerated Method of Purifying a Commercial Preparation of Porcine Pepsin on Aminosilochrome. To 40 g of a commercial preparation of porcine pepsin was added 1800 ml of a 0.1 M acetate buffer, pH 5.5, and the mixture was stirred and centrifuged as described above. The supernatant, the activity of which was 24.2 a.u./o.u., was transferred to a 3-liter beaker with 180 g of Aminosilochrome C-120 equilibrated with 0.1 M acetate buffer, pH 5.5. The mixture was kept for 1 h with the periodic stirring up of the ion-exchange material. The solution was removed by decantation, after which the Aminosilochrome was washed with the initial buffer (3 × 1) liter and then with 0.005 M HCl (7 × 1 liter) until the pH of the eluate had fallen to 2.5 and $E_{280} = 0.1$. The pepsin was desorbed by washing the Aminosilochrome with 0.05 M HCl, pH 1.6, the ion-exchange material being carefully stirred up (3 × 800 ml). The pepsin solutions separated from the Aminosilochrome by decantation were combined and were neutralized with 270 ml of AV-16 GS anion-exchange resin in the OH^- form with a contact time of 20 min and periodic stirring. During neutralization, the solution gradually became turbid (isoelectric point of the pepsin), and then at pH 3.7 it became transparent. The anion-exchange resin was separated from the enzyme solution by filtration through a glass filter No. 1. Then the pepsin was stabilized by the addition of 30 μmole of sodium acetate per 1 μmole of enzyme. To the resulting solution of pepsin, containing 13,000 o.u., 6.5 ml of a 10% solution of sodium acetate (about pH 4.8) was added dropwise. After freeze-drying, 9.6 g of pure pepsin was obtained. The yield was 45% of the total protein and 94% of the total proteolytic activity deposited on the Aminosilochrome. The activity of the pepsin preparation obtained in the cleavage of hemoglobin was 61 a.u./o.u. After storage of this pepsin preparation at 4°C (in a desiccator) for six months, the activity in relation to hemoglobin was 59.4 a.u./o.u.

V. P. Starikov provided technical assistance in the work.

SUMMARY

1. The possibility has been shown of the preparative production of a number of carboxylic proteinases by the ion-exchange chromatography of commercial preparations of these enzymes on a column of Aminosilochrome C-80 or C-120. Preparations of calf pepsin and chymosin and of bovine and porcine pepsins with a high degree of purity have been obtained in practically quantitative yield.

2. An accelerated preparative method for purifying commercial preparations of porcine pepsin on Aminosilochrome C-120 by the sorption-desorption of the enzyme under static conditions has been proposed.

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