IMMOBILIZATION OF CARBOXYLIC PROTEINASES ON AMINO-SILOCHROME

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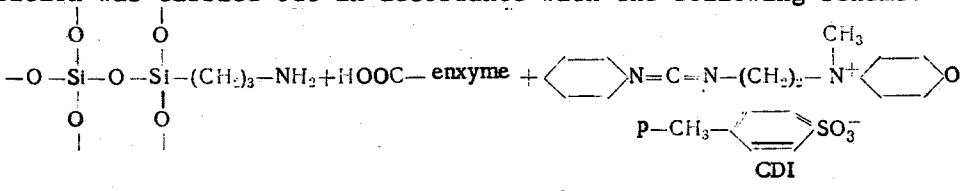
Carboxylic proteinases are widely used in a number of technological processes. The production of immobilized enzymes of this class would increase their stability, ensure the possibility of the repeated use of the enzymes, and broaden the range of their application.

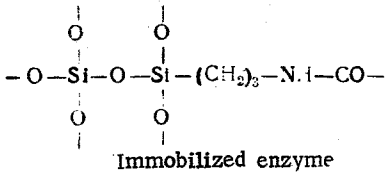
The immobilization of pepsin on aminoethylcellulose using glutaraldehyde [1] and on hydroxyalkyl methacrylate gels by means of N-(dimethylaminopropyl)-N'-ethylcarbodiimide [2] has been described. Water-soluble carbodiimides have also been used for the attachment of pepsin to modified glass [3]. The first experiments on the immobilization of pepsin on amino-Silochrome were carried out in our laboratory in 1974.

In the present paper we consider in more detail the immobilization of two carboxylic proteinases — porcine pepsin and the carboxylic proteinase of the microscopic fungus *Aspergillus awamori*, asperfillopepsin A [4] — on modified Silochrome (macroporous silica) and their properties.

Immobilization was carried out in accordance with the following scheme:



Amino-silochrome



Immobilized enzyme

To select the conditions for the immobilization reaction we used porcine pepsin. The optimum molar ratio of pepsin to carbodiimide proved to be of the order of 70-80 (Fig. 1A).

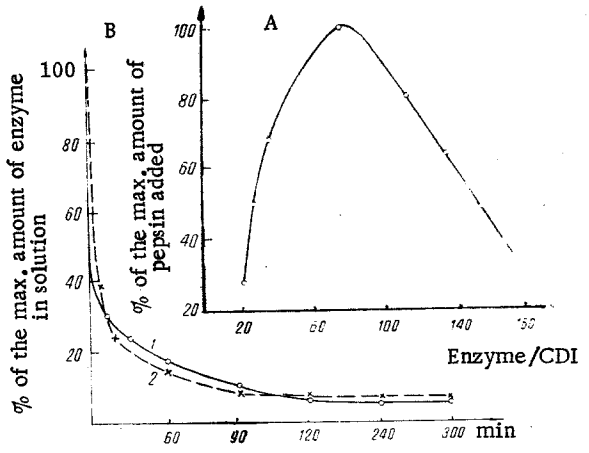


Fig. 1. Dependence of the amount of added porcine pepsin on the molar ratio of enzyme to carbodiimide (A) and dependence of the immobilization process on the time of the reaction (B): 1) pepsin; 2) aspergillopepsin A.

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We compared the various samples of amino-Silochrome as supports for immobilization. Silochromes differing in pore size and specific surface were modified with γ-aminopropyltriethoxysilane under similar conditions and were used in the reaction with pepsin. Below we give the results of amino-acid analysis (in all experiments, to modify 1 g of support we used 204 μmole of water-soluble carbodiimide and 2.8 μmole of porcine pepsin):

Support (Silochrome)	Pore size, Å	Specific surface, m^2/g	Amino groups introduced, μeq/g	Amount of immobilized pepsin, μmole/g
CX-1.5	2000	20—30	280	0.74
CX-2	1000	50	260	0.62
CX-3	500—700	80—100	300	0.80
C-80	50—100	80—130	300	0.85
C-120	500—700	130	280	0,85

They show that in all samples of amino-Silochrome, differing in pore size (50-2000 Å) and in specific surface (20-130 m^2/g) immobilization of approximately the same amount of enzyme, of 0.6-0.8 μmole per gram of support, takes place.

Thus, within the limits considered saturation with the enzyme does not depend on the specific surface of the support. For this reason, the immobilization of porcine pepsin and aspergillopepsin A was subsequently carried out on Silochrome C-80 containing 300 μeq of amino groups per gram. The addition of the enzyme to the support was monitored by determining the amount of protein in solution spectrophotometrically at 280 nm. After only 10 min from the beginning of the reaction, not more than 30% of unchanged enzyme remained in the solution, and then the process of addition slowed down (Fig. 1B), which was due to the rapid consumption of the enzyme. After the end of the reaction, the amount of porcine pepsin added to the amino-Silochrome was, according to the results of amino-acid analysis, 30 mg per gram of support, and for the immobilized aspergillopepsin A it was 20-25 mg per gram of support.

The proteolytic activity of the immobilized pepsin in relation to hemoglobin amounted to 40-50 a.u./mg of enzyme (this activity corresponds to 90-100% of the activity of the same amount of porcine pepsin in solution) and the proteolytic activity of the immobilized aspergillopepsin A for hemoglobin at the same pH of 1.8 was 0.6-0.7 a.u./mg of enzyme (60-70% of the activity of the soluble enzyme). When it was stored at +4°C in 0.1 M acetate buffer at pH 5.0, the immobilized pepsin did not lose its activity for at least 20 months, and the immobilized aspergillopepsin A for 10 months. When dried under a fan, simply in air, and in a desiccator over P_2O_5, the immobilized carboxylic proteinases lose from 60 to 100% of their activity, and it is therefore desirable to store them under a solution.

We studied the dependence of the degree of cleavage of a standard solution of hemoglobin at pH 1.8 on the time of contact with the immobilized pepsin. The dependence has a linear nature. The more slowly the solution of hemoglobin is passed through a thermostated column of immobilized enzyme, the more far reaching is the cleavage of the substrate. It is obviously possible to select proteolysis conditions at which final products with different degrees of degradation can be obtained. It must be mentioned that in all cases a considerable reduction in the activity of the preparation of immobilized pepsin was found in the first ten minutes of the reaction, with its subsequent stabilization. This can be explained by inhibition of the immobilized pepsin by cleavage products of the hemoglobin.

We studied the pH-dependence of the catalytic activities of the preparation of immobilized pepsin and aspergillopepsin obtained. The immobilized pepsin showed its greatest activity in the hydrolysis of hemoglobin at pH 1.6-1.8, which corresponds to the optimum for the action of the enzyme in the dissolved form (Fig. 2A). The immobilized aspergillopepsin A split hemoglobin best at pH 2.4-2.7 (Fig. 2B). This figure also corresponds to the pH optimum of the action of soluble aspergillopepsin A.

We considered the dependence of the stability of the immobilized enzymes on the pH (Fig. 3A). After incubation for 6 h in a strongly acid medium (pH 1-2), 90 to 100% of the proteolytic activity of the immobilized aspergillopepsin A was lost. At pH 3.5-6.0, the immobilized fungal carboxylic proteinase was stable for a long time. As compared with this, the immobilized porcine pepsin was considerably more stable in an acid pH range, and after being kept at pH 1-3 for 6 h it retained no less than 70% of its initial activity. In neutral and alkaline pH ranges, both immobilized carboxylic proteinases were inactivated.

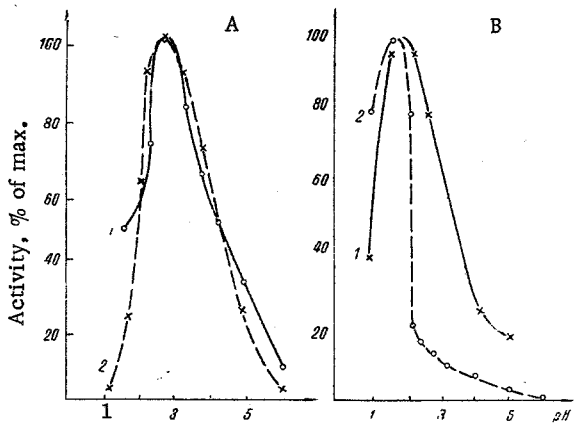

Fig. 2. Dependence of the proteolytic activity of soluble (1) and immobilized (2) pepsin (A) and aspergillopepsin A (B) on the pH.

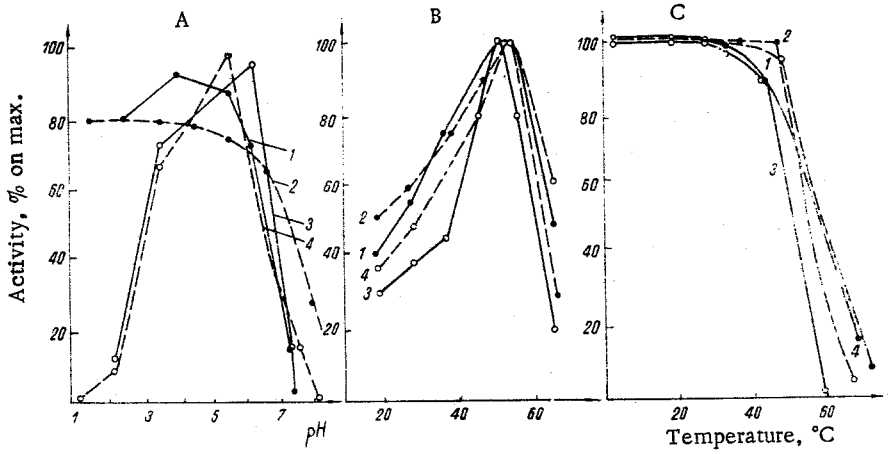

Fig. 3. Dependence of the stability of soluble and immobilized pepsin and aspergillopepsin A on the pH (A) and the dependence of their proteolytic activity (B) and stability (C) on the temperature: 1, 2) soluble and immobilized pepsin; 3, 4) soluble and immobilized aspergillopepsin A, respectively.

The temperature optimum of the action of the immobilized porcine pepsin and aspergillopepsin A is found at 60°C. This agrees with the temperature optimum for the action of the analogous soluble enzymes (Fig. 3B). We also studied the stability of the immobilized enzymes at various temperatures (Fig. 3C). Thus, the results of an investigation of the carboxylic proteinases immobilized on amino-Silochrome that have been obtained show that in a number of properties they are close to the soluble enzyme. Immobilized porcine pepsin exhibits considerable stability in a strongly acid medium, which may find practical application.

EXPERIMENTAL

Preparations of highly purified porcine pepsin obtained by a method described previously [5] and of aspergillopepsin A obtained in our laboratory were used. According to the results of disk electrophoresis, both enzymes were homogeneous. The proteolytic activity of the porcine pepsin was 70 a.u./o.u., and that of the carboxylic proteinase from fungi was 13 a.u./o.u.

Preparation of the Amino-Silochrome. A mixture of 200 g of Silochrome and 20 g of γ-aminopropyltriethoxysilane in 600 ml of ethanol was heated at 37°C for 72 h. The amino-Silochrome obtained was washed with ethanol (3 × 1.5 liters) and dried at 120°C for 24 h. A de-

termination of the total nitrogen by a modified Kjeldahl method [6] showed that 1 gram of amino-Silochrome contained 300 μmole of nitrogen and, consequently, 300 μeq of amino groups.

Preparation of the Immobilized Pepsin. To 50 mg (1.4 μmole) of highly purified porcine pepsin was added 22 mg (51 μmole) of the water-soluble N-cyclohexyl-N'-[2-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate and 0.5 g (150 μeq of amino goups) of amino-Silochrome suspended in 5 ml of water. The suspension so obtained was stirred at 22-24°C for 1 h, after which another 22 g (51 μmole) of the CDI was added and the reaction was continued for another 1.5 h. The pH of the reaction mixture was kept between 4.5 and 5.5 by the addition of 1 M HCl. After the end of the reaction, the aqueous solution of unchanged pepsin was removed by decantation. To eliminate the enzyme adsorbed by ion exchange, the immobilized pepsin obtained was washed successively with 0.1 M HCl and 0.1 M acetate buffer, pH 5.0, and it was stored in the latter at 4°C. The yield of immobilized pepsin was 30%.

Preparation of Immobilized Aspergillopepsin A. To 10 g (0.3 μmole) of highly purified aspergillopepsin A was added 4.4 mg (10.4 μmole) of water-soluble carbodiimide and 300 mg of amino-Silochrome (90 μeq of amino groups), suspended in 2 ml of water. The resulting mixture was stirred at 22-24°C for 1 h, after which 4.4 mg (10.4 μmole) of the CDI was added and the reaction was continued for another 1 h, the pH being maintained at 4.5-5.5. The subsequent operations were carried out in the same way as in the case of the preparation of immobilized pepsin.

Determination of the Amount of Added Enzyme. A tube was charged with 100 mg of the enzyme immobilized on amino-Silochrome that had been dried to constant weight in a desiccator over P_2O_5, 2 ml of 5.7 M HCl was added, the tube was sealed in vacuum, and hydrolysis was carried out at 120°C for 24 h. After this, the support was separated off on a glass filter and was carefully washed with water. The amount of amino acids in an aliquot was determined on a Bio-Cal BC-200 amino-acid analyzer and, knowing the amino-acid composition of the enzyme, its amount on 1 g of support was calculated. In the case of the immobilization of porcine pepsin, 30 mg (0.85 μmole) of enzyme had been added to 1 g of support, and in the case of the immobilization of the aspergillopepsin A on amino-Silochrome, 20-25 mg (0.6-0.75 μmole).

Proteolytic Activity of the Immobilized Enzyme Determined in a Flow. A 1-mg sample of immobilized enzyme was placed in a thermostated flow-through column at 37°C into which a standard solution of hemoglobin at pH 1.8 was fed by means of a peristaltic pump. With different times of contact of the immobilized enzyme with the hemoglobin, 1-ml samples were taken, to each of them was added 5 ml of a 5% solution of TCA.

After the formation of a precipitate, the mixture was filtered through dense paper filters. The degree of cleavage of the hemoglobin by the immobilized enzyme was determined in the filtrates obtained from the adsorption at 280 nm against the corresponding control. In the control samples, to 1 ml standard solution of hemoglobin incubated for a time corresponding to the length of time of contact of the immobilized enzyme with the hemoglobin in the column was added 5 ml of the 5% TCA solution and it was filtered as described above. The filtrate obtained was used as the control.

Proteolytic Activity of the Immobilized Enzyme Determined under Static Conditions. To 1 ml of standard solution of hemoglobin with pH 1.8 previously incubated at 37°C for 5 min was added 10 mg of immobilized enzyme by means of a calibrated sampler. The sampler consisted of a 1-ml syringe the end of which was connected by means of flexible tubing with a glass tube. Between it and the tubing there was a sieve preventing the immobilized enzyme from passing into the tubing. The internal volume of the glass tube was 25 μl — the volume occupied by 10 mg of the dry immobilized enzyme. The mixture was kept at 37°C for 10 min. The reaction was stopped by the addition of 5 ml of 5% TCA solution. After the formation of the precipitate, the mixture was filtered through dense paper filters. The absorption in the filtrates was measured at 280 nm against a control. As control was used 1 ml of standard hemoglobin solution to which, after incubation at 37°C for 10 min, 5 ml of 5% TCA solution and then 10 mg of immobilized enzyme had been added, followed by the filtration of the mixture. The A_{280} value obtained under standard conditions referred to 1 mg of enzyme was used as the activity of the immobilized enzymes.

Dependence of the Stability of the Immobilized Enzymes on the pH. To 2 ml of 0.1 M acetate buffer with pH 2.0-6.0, 1 M HCl with pH 1.0, and 0.1 M phosphate buffer with pH 6.0-8.0 was added 10 mg of insoluble enzyme derivative. The mixture was kept at 37°C for 6 h. After the removal of the supernatant, the proteolytic activity was determined under static conditions.

Dependence of the Stability of the Immobilized Enzymes on the Temperature. To 1 ml of 0.1 M acetate buffer with pH 4.5 was added 10 mg of immobilized enzyme. The mixture obtained was kept at 10-70°C for 1 h. Then the buffer was decanted off at 20°C and the proteolytic activity was determined under static conditions.

Dependence of the Proteolytic Activity on the pH. To 1 ml of a 2% solution of hemoglobin with pH 1.0-6.0 (1 M HCl and 0.1 M acetate buffer) was added 10 mg of immobilized enzyme, and the proteolytic activity was determined under the static conditions.

Dependence of the Proteolytic Activity on the Temperature. To 1 ml of a 2% solution of hemoglobin with pH 1.8 was added 10 mg of immobilized enzyme, and the proteolytic activity was determined under static conditions at temperatures of 10-70°C.

The preparation of aspergillopepsin A was kindly given to us by V. I. Ostaslavskaya and E. K. Kotlova.

SUMMARY

Highly purified porcine pepsin and aspergillopepsin A immobilized on amino-Silochrome have been obtained. The enzymatic properties of these insoluble derivatives have been studied.

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ISOLATION AND ANTIBIOTIC PROPERTIES OF cis-HEXADECA-4,7,10,13-TETRAENOIC ACID FROM THE ALGA *Scenedesmus obliquus* UA-2-6

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There is information on the manifestation by extracts from the cells of green [1] and blue-green [2] algae of antibiotic properties and of the therepeutic effectiveness of these preparations [3]. Sarganin and chonalgin isolated from marine forms of brown algae have proved to be more active than penicillin and nystatin [4].

The assumption exists that the antibiotic properties of preparations from the greenalgae *Chlorella* and *Scenedesmus* are due to the presence in their cells of chlorophylls [5] and fatty acids [6], but there is no information on the isolation and identification of substances possessing such activity.

The capacity of local strains of the organisms *Scenedesmus acuminatus* UA-2-7 and *Scen. obliquus* UA-2-6 for producing antibiotic substances having a lipid nature has been detected previously [7, 8]. In this communication we give information on the isolation from the biomass of *Scen. obliquus* UA-2-6 of cis-hexadeca-4,7,10,13-tetraenoic acid and its antibiotic properties.

A lipid extract was obtained from the air-dry biomass of the *Scenedesmus*. The extract consisted of a dark green oily liquid with a peculiar odor. The compositions of the classes

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