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PEPSIN IMMOBILIZED BY COVALENT FIXATION TO HYDROXYALKYL METHACRYLATE GELS: PREPARATION AND CHARACTERIZATION

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Summary

Insoluble active derivatives of pepsin (EC 3.4.23.1) were prepared by covalent binding of this enzyme to hydroxyalkyl methacrylate gels modified with 1,6-diaminohexane or ε-aminocaproic acid in an acid medium by means of water-soluble carbodiimide. The amount of attached enzyme, its proteolytic activity, pH activity curves of the preparations obtained and the time and pH dependence of their stability were determined.

Introduction

One of the most frequent procedures for the attachment of enzymes is the cyanogen bromide method [1] involving binding of the enzyme on a carrier in an alkaline medium. Since at a pH higher than 6.0 pepsin is practically completely inactivated, its attachment must be conducted in an acid medium. In the initial stage of the investigation of the preparation of insoluble pepsin the enzyme was bound directly onto polymers containing reactive groups, such as polydiazonium salt of polyaminostyrene [2,3] or a nitrated copolymer of methacrylic acid and methacrylic acid *m*-fluoroanilide [4]. Later, pepsin was immobilized after the preceding activation of the carrier with various compounds capable of reacting with functional groups of the carrier and of the enzyme. Thus, isocyanates were used for binding pepsin onto Sepharose by a modified reaction with *p*-phenylenediamine or 4,4'-methylenedianiline [5], or glutaric aldehyde was used for attachment of pepsin onto AE-cellulose [6]. Water-soluble carbodiimides were used for coupling of pepsin to glass [7] and to the copolymer of ethylene and maleic anhydride [8]; before this, both carriers were modified by a reaction with 1,6-diaminohexane. The insoluble pepsin derivatives mentioned above are predominantly characterized by a low relative proteolytic activity. The only exception was pepsin bound to glass

whose relative proteolytic activity amounted to 65% of the original activity of free enzyme; at the same time the sample exhibited high storage stability.

With respect to the facts which have been outlined above, we used the reaction of carbodiimides with a free -NH_2 or -COOH group of the carrier on the one hand and its reaction with the carboxylic or amine group of the enzyme on the other in order to achieve attachment of pepsin to hydroxyalkyl methacrylate gels [9] with 1,6-diaminohexane or ϵ-aminocaproic acid bound to them after the activation with cyanogen bromide. Hydroxyalkyl methacrylate gels Spheron P-300, Spheron 300 and more hydrophilic Spheron 1000 BTD which contains roughly 2.5 times more hydroxyl groups than the first gel were chosen as carriers. This work had as its objective a verification of the feasibility of pepsin fixation coupling onto hydroxyalkyl methacrylate gels in an acid medium, characterization of the immobilized enzyme compared to a soluble sample and determination of the effect of different hydrophilicity of the carriers on the properties of the bound enzyme.

Material and Methods

Pepsin, crystalline, lyophilized, proteolytic activity 4.34 A_{280nm}/min/mg was a product of Léčiva n.p. Prague. Spheron P-300 was produced by Lachema n.p. Brno, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride and 1,6-diaminohexane were products of Fluka, Switzerland. The carriers Spheron 300 and Spheron 1000 BTD are prototype carriers developed at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences in Prague [9].

Preparation of NH_2-Spheron and COOH-Spheron

After activation with cyanogen bromide [1,10,11], either 1,6-diaminohexane or ϵ-aminocaproic acid were bound to hydroxyalkyl methacrylate gels of the Spheron type by a modified procedure after Cuatrecasas [12]. After their characterization the derivatives thus obtained were denoted NH_2-Spheron and COOH-Spheron.

Preparation of insoluble pepsin derivatives

4.8 ml of NH_2-Spheron or COOH-Spheron were suspended in 15 ml of 0.1 M sodium acetate, pH 4.0, containing 300 mg of pepsin. After stirring for 5 min, 100 mg *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride was added to each suspension and stirring was continued with a magnetic stirrer at 4°C. After 22 h both samples were decanted several times with 0.1 M sodium acetate, pH 4.1, containing 1 M sodium chloride and washed on the column alternately with the above buffer and 3 M urea, pH 3.0. The gels were finally washed with 0.01 M sodium acetate, pH 4.1, and with 0.1 M acetic acid and stored in the wet cake form at 4°C. The amount of fixed enzyme was determined from that of acid and neutral amino acids present in the hydrolyzate after acid hydrolysis of dried gels [13].

Determination of proteolytic activity of free and bound pepsin

The determinations were performed by a modified method according to

Anson [14]. Each time, to 4 ml of hemoglobin solution pH 2.0, used as substrate, 200 μl of pepsin solution with increasing amount of enzyme ($5 \cdot 10^{-3}$—$5 \cdot 10^{-2}$ mg in 0.01 M sodium acetate, pH 4.1) was added; in the case of bound enzyme, 5—20 mg of gel filtered by suction containing the immobilized enzyme was used. After incubation at 37°C for 5 min the cleavage was completed by adding 10 ml of 5% trichloroacetic acid. For the determination of the pH activity curves of free and bound enzyme, hemoglobin solutions pH 1 to 5 and free pepsin concentration 0.25 mg/ml or 10 mg pepsin bound to NH_2-Spheron P-300, 5 mg pepsin bound to NH_2-Spheron 1000 BTD and 1 mg pepsin bound to COOH-Spheron 300 were used. The absolute proteolytic activity of pepsin is given in the units A_{280nm}/min/mg of free or bound enzyme. The relative proteolytic activity of bound enzyme was then determined as the ratio of the absolute activities of bound and free enzyme expressed as a percentage.

Determination of stability of free and bound enzyme

The determination was conducted in Britton-Robinson buffers pH 1.86 to 7.04. To 4 ml of a buffer having the respective pH, 50 μl of pepsin solution was pipetted each time (20 mg in 1.0 ml 0.1 M sodium acetate pH 4.0), or 30 mg of gel with bound pepsin was added, and the solution was left to stand at 4°C 5 h. The proteolytic activities of all samples were then determined as described above.

Results and Discussion

The insoluble derivatives of pepsin were prepared by attachment to Spheron with bound 1,6-diaminohexane (NH_2-Spheron) and ε-aminocaproic acid (COOH-Spheron). In the preparation of NH_2-Spheron two types of hydroxyalkyl methacrylate gels were used differing by the contents of hydroxyl groups. Spheron 1000 BTD contains about 2.5 times more OH-groups than Spheron P-300, it is more hydrophilic as a result and has also a higher capacity for enzyme immobilization. Pepsin was bound to all the gels mentioned above by means of carbodiimide in mild acid medium.

The highly active insoluble derivatives of pepsin thus prepared differed predominantly by the amount of bound enzyme (Table I). As expected, a larger quantity of pepsin was bound to this gel which has a higher number of reactive groups. Table I also gives a value for a preparation of immobilized

TABLE I

PROPERTIES OF PEPSIN IMMOBILIZED ON HYDROXYALKYL METHACRYLATE GELS

Support	Quantity of pepsin bound (mg/g)	Bed volume (ml/g)	Proteol. activity of bound pepsin (A_{280}/min/mg bound enzyme)	Proteol. activity of bound pepsin (A_{280}/min/g conjugate)	Relative proteol. activity of bound pepsin (%)
NH_2-Spheron P-300	13	3.2	4.0	52.3	92.8
NH_2-Spheron 300	46.8	3.3	2.8	133.5	65.7
COOH-Spheron 300	50.8	3.3	2.0	100	45.3
NH_2-Spheron 1000 BTD	65.0	4.0	1.6	106.6	37.8

pepsin with a lower protein content, obtained under slightly modified conditions of coupling. This value is given in order to demonstrate explicitly how the relative proteolytic activity of immobilized pepsin preparations decreases with the increasing quantity of enzyme bound. We have observed the same phenomenon earlier with immobilized chymotrypsins; it could perhaps be ascribed to sterical hindrances.

In order to exclude the possibility of non-covalent adsorption of pepsin to Spheron we investigated the dependence of the activity and the total amount attached to the gel on the ionic strength of the washing solutions. Even at 2.0 M solution of NaCl and 3.0 M solution of urea we did not observe any change in either value.

The dependence of proteolytic activity on pH for free and bound enzyme is shown in Fig. 1. In both cases the pH optimum of bound enzyme is insignificantly shifted towards the more acid region.

The stability of bound pepsin as a function of pH is somewhat lower (Fig. 2). While free enzyme is virtually stable within a range of pH 2.0 to pH 6.0, the bound enzyme exhibits a certain optimum of stability the value of which for pepsin bound to NH_2-Spheron 1000 BTD lies around pH 3.0.

We also studied the stability of bound pepsin if stored for a longer time in the wet cake form at 4°C (cf. Fig. 2). After thirty days no drop in activity could be observed, neither with pepsin bound to the more hydrophilic NH_2-Spheron 1000 BTD nor for that bound to NH_2-Spheron P-300.

The methods described above prove the feasibility of preparation of highly active derivatives of pepsin immobilized by covalent bonds to hydroxyalkyl methacrylate gels; under the conditions used, the increased content of hydrophilic groups did not show any influence upon the increase in the stability of basic Spheron materials carrying active pepsin molecules.

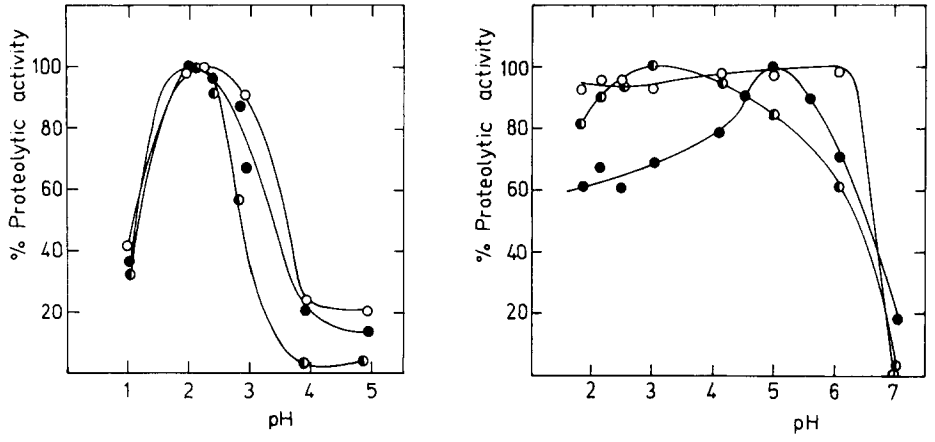

Fig. 1. Dependence of proteolytic activity on pH in respect to hemoglobin for free and bound pepsin. ●——●, pepsin bound to NH_2-Spheron P-300; ◐——◐, pepsin bound to NH_2-Spheron 1000 BTD; ○——○, free pepsin (100% = proteolytic activity at optimum pH).

Fig. 2. Dependence of stability of free and bound pepsin on pH. ●——●, pepsin bound to NH_2-Spheron P-300; ◐——◐, pepsin bound to NH_2-Spheron 1000 BTD; ○——○, free pepsin (100% = proteolytic activity at optimum pH).

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