COVALENT FIXATION OF PEPSIN TO AGAROSE DERIVATIVES

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1. Introduction

In recent years the covalent attachment of enzymes to water-insoluble carriers has become fairly routine [1, 2]. Proteolytic enzymes have received particular attention. Pepsin, however, has presented some difficulty since it is rapidly and irreversibly denatured at the alkaline pH values where most fixation procedures proceed at optimal rate. The only polymer-linked pepsin preparations that have been reported [3–5] are characterized by low catalytic activity or by rather summary washing after the fixation to remove non-covalent bound enzyme molecules.

We shall report here the preparation of catalytically active, water-insoluble pepsin conjugates. The enzyme has been attached to amino derivatives of Sepharose^R by reaction with an isocyanide and an aldehyde [6]. The insolubilized pepsin is able to digest haemoglobin and bovine serum albumin.

2. Materials and methods

Pepsin (2X crystallized) was purchased from Worthington Biochem. Corp., Freehold, N.J., USA, and haemoglobin (type II) from Sigma Chem. Co., St. Louis, Mo., USA. Cyclohexyl isocyanide was from EGA-Chemie, Steinheim/Albuch, West Germany, *p*-phenylenediamine (PDA) from May and Baker Ltd., Dagenham, England, and 4,4′-methylene dianiline (MDA) from Aldrich Chem. Co., Milwaukee, Wisc., USA. 3-Dimethylaminopropyl isocyanide was synthesized according to Ugi [7].

2.1. *Preparation of the carrier*

As starting material we used Sepharose 4B and Sepharose 2B, the latter being cross-linked and reduced according to Porath et al. [8]. The gel (200 mg dry weight) was activated with CNBr as described by Axén et al. [9] and allowed to react with 100 mg PDA or 25 mg MDA in 10 ml 0.1 M $NaHCO_3$. Nitrogen was bubbled through the solution for 15 min and the coupling was then allowed to proceed in a closed vessel for 16 hr in the dark at 23° with slow rotation. The polymer then was washed in a column with the following buffers: 0.1 M Na borate buffer (1 M in NaCl), pH 8.5 (24 hr); 0.1 M Na acetate buffer (1 M in NaCl), pH 4.5 (24 hr); 0.01 M Na acetate buffer, pH 4.5 (24 hr) and finally, distilled water (4 hr). The polymer was stored in the 0.01 M acetate buffer, containing 0.02% NaN_3.

2.2. *Coupling of pepsin*

The carrier polymer (50 mg) was suspended in 4 ml distilled water in a small pH-stat vessel; 25 μl acetaldehyde was added, followed by 10 or 25 mg pepsin and 25 μl isocyanide. The pH was kept at 5.8 by means of a pH-stat (Radiometer, Copenhagen) for 6 hr. The product was washed on a G3 glass filter with water and then in a small column with the following buffers: 0.1 M Na citrate buffer (1 M in NaCl), pH 4.0 (48 hr); 0.1 M Na acetate buffer (1 M in NaCl), pH 5.4 (24 hr); and finally 0.01 M Na citrate buffer, pH 4.0. The conjugates were stored in the last buffer, containing 0.02% NaN_3, at 4°. Their protein content was determined by amino acid analysis [10].

2.3. *Activity measurements*

The insolubilized pepsin was assayed at pH 4.0 towards denatured haemoglobin. Haemoglobin (2.5 g) was dissolved in 100 ml distilled water and filtered twice through glass wool; 80 ml of the filtrate was mixed with 20 ml 0.3 M HCl. The assay mixture consisted of 0.5 ml of the haemoglobin solution, 1 ml 0.1 M Na citrate buffer, pH 4.6, and 0.5 ml enzyme suspension in 0.01 M Na citrate buffer, pH 4.0. Seven enzyme concentrations and two controls were used for each determination. The mixtures were incubated at 37° for 20 min with slow stirring. The reaction was stopped by the addition of 3 ml 5% (w/v) trichloroacetic acid (TCA). The absorbancies of the supernatants at 280 nm were plotted vs. enzyme content of the samples. The slope of the tangent to the initial part of the curve was taken as a measure of enzymatic activity. The free enzyme was assayed in the same manner and the relative activities are expressed on a percent basis. Activity determinations at pH 3.2 were performed in the same system except that the buffer of pH 4.6 was replaced by 1 ml 0.1 M Na citrate buffer, pH 3.9.

2.4. *Leakage tests*

For these experiments we used a reaction mixture of the same composition as in the activity measurements but with a total volume of 20 ml. Incubation was performed at 37° with slow stirring. 0.02% NaN_3 was added to prevent bacterial growth. At regular intervals, 1 ml samples were withdrawn and pipetted into 4 ml 5% TCA. After 30 min the precipitates were removed by filtering and the absorbancies of the supernatants were measured at 280 nm. 30 min after the start of the incubation the catalyst was removed from the incubation mixture by filtration on a G3 glass filter. The filtrate was incubated again and further samples taken out. A control without enzyme was incubated as a reference. No net production of UV-absorbing material takes place after removal of the catalyst (fig. 1).

2.5. *Digestion of bovine serum albumin (BSA)*

Dimer-free BSA was obtained by chromatography of the commercial product on Sephadex G-150; 10 mg BSA and 10 mg of conjugate 3 (see table 1) were allowed to react in 1 ml 0.1 M Na acetate buffer (pH 4.0) at 37° for 16 hr. The supernatant was then chromatographed in the same buffer on a 14 X 400 mm Sephadex G-75 column. Another sample of BSA was incubated in the absence of enzyme and chromatographed in the same system. The chromatograms are shown in fig. 2.

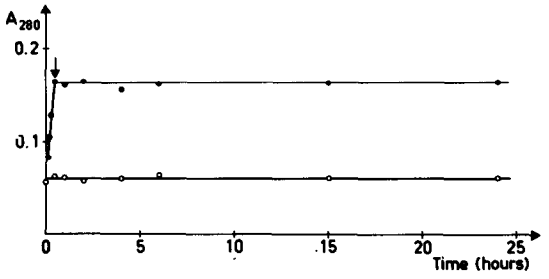

Fig. 1. Test for leakage at pH 3.2. Upper line: test solution. The catalyst (conjugate 4) is removed at the arrow. Lower line: control. If the catalyst is not removed, the absorbancy at time = 24 hr will be 0.5. For further details, see text.

3. Results and discussion

Enzyme contents and catalytic activities of the pepsin conjugates are shown in table 1. If isocyanide and aldehyde are absent from the coupling mixture, the enzyme content of the product will be 2–3 mg/g conjugate.

It is important that free enzyme is not released from the conjugate under the conditions of use or measure-

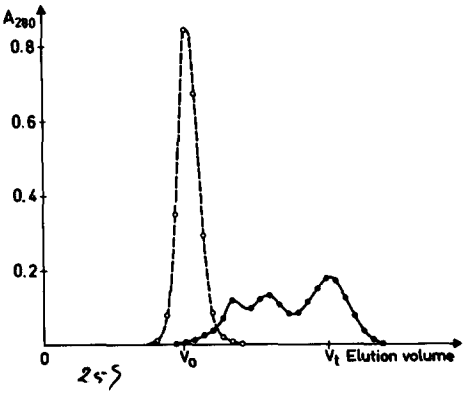

Fig. 2. Chromatography on Sephadex G-75 in 0.1 M Na acetate buffer, pH 4.0, of BSA digested by insoluble pepsin (solid line). Column: 14 X 400 mm, flow rate: 6 ml/hr. The broken line is untreated BSA.

Table 1
Chemical fixation of pepsin to 50 mg carrier polymer.

Conjugate no.	Carrier	Isocyanide	Amount of enzyme added (mg)	Amount of enzyme fixed (mg/g conjugate)	Relative activity*	
					pH 4.0	pH 3.2
					%	
1	PDA-Sepharose	3-dimethyl-aminopropyl-isocyanide	25	185	18	–
2	MDA-Sepharose	3-dimethyl-aminopropyl-isocyanide	25	150	26	–
3	Cross-linked PDA-Sepharose	Cyclohexyl isocyanide	10	105	13	5
4	Cross-linked PDA-Sepharose	Cyclohexyl isocyanide	25	165	13	4

* The absolute activity of free pepsin at pH 4.0 and 3.2 was 1200 and 4300 units/mg, respectively. One unit of activity corresponds to an increase of 0.001 per min in the absorbancy at 280 nm of the TCA-soluble hydrolysis products.

ment. The leakage test described above showed that leakage does not occur at pH 4.0 for any of the conjugates. Conjugates 1 and 2, prepared from Sepharose which has not been crosslinked, release activity into the solution when assayed at lower pH. Conjugates 3 and 4, on the other hand, are perfectly stable at pH 3.2 (fig. 1) but start to leak below pH 3. However, our results show that the activity of the insolubilized pepsin at pH 4.0 is sufficient for degradation studies.

Conjugates 3 and 4 have been assayed for activity also at pH 3.2. The relative activity at this pH is lower because the activity of the free enzyme increases more rapidly when the pH is lowered (table 1). The fixed enzyme does not lose its activity even after long periods of storage. After one month essentially all activity is left. Conjugate 4 showed no siginificant decrease in activity after four months.

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