Biochimica et Biophysica Acta, 327 (1973) 132–137

BBA 67049

A NEW POLYAMINE CARRIER FOR THE IMMOBILIZATION OF PROTEINS

WATER-INSOLUBLE DERIVATIVES OF PEPSIN AND TRYPSIN

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(Received June 4th, 1973)

SUMMARY

A new polymeric carrier, containing primary amino groups was prepared by coupling an ethylene–maleic anhydride (1:1) copolymer (EMA), with 1,6-diaminohexane (HMD) in the presence of dicyclohexylcarbodiimide. The resin (designated EMA–HMD) was used for the immobilization of pepsin, through its carboxyl groups and following activation with glutaraldehyde for the immobilization of trypsin, through the enzyme's amino groups.

INTRODUCTION

Enzymes have been bound covalently to a wide variety of water-insoluble polymeric carriers. In most cases, the polymeric reagents employed were divised to react with primary amino groups (*e.g.* the ε-amines of lysine) or with tyrosyl residues on the enzymic protein[1–3]. In the case of highly acidic proteins, these methods are often unapplicable[4,5]. This communication describes the preparation of an easily synthesized, water-insoluble polymeric carrier containing primary amino groups, which can be utilized for the immobilization of enzymes *via* their carboxyl groups.

The starting material is a commercially available (1:1) copolymer of ethylene and maleic anhydride (EMA). By coupling EMA with 1,6-diaminohexane (hexamethylenediamine, (HMD) in the presence of dicyclohexylcarbodiimide (DCC), a high capacity polyamine resin, copoly-ethylene–maleic acid-bis-(*N*-ε-aminohexylamide) (EMA–HMD) is obtained (Fig. 1). This resin was used for the immobilization of pepsin, by activating the enzyme carboxyl groups with a water-soluble

Abbreviations: HMD, 1,6-diaminohexane (hexamethylenediamine); EMA, ethylene–maleic anhydride (1:1) copolymer; DCC, *N.N'*-dicyclohexylcarbodiimide; EMA–HMD, copoly-ethylene–maleic acid-bis-(*N*,ε-aminohexylamide) (see Fig. 1).

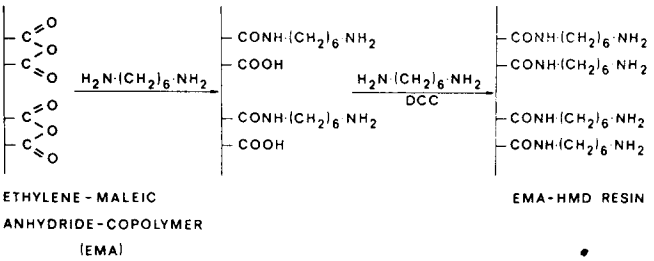


Fig. 1. Synthesis of EMA–HMD resin.

carbodiimide[6] at pH values low enough to prevent the irreversible denaturation of the protein. Following activation with glutaraldehyde, the EMA–HMD resins could also be used for the immobilization of trypsin.

MATERIALS AND METHODS

Pepsin, porcine (three times crystallized, B grade), and trypsin (three times crystallized and lyophilized) were bought from Calbiochem, Los Angeles, Calif., and Worthington Biochemical Corporation, Freehold, N.J., respectively. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl, was a product of Ott Chemical Company, Muskegon, Mich. Glutaraldehyde, 25% aqueous solution, was obtained from Fluka, Switzerland. EMA (mol. wt about 20 000) was obtained from Monsanto Company, St. Louis, Mo. The EMA samples were dried at 110 °C over P_2O_5 *in vacuo* for 24–28 h before use.

Preparation of carrier

Oven-dried EMA (1.26 g; 0.01 base mole anhydride) was dissolved in redistilled dimethylsulfoxide (20 ml). A solution of HMD (3.5 g, 0.03 mole) in dimethylsulfoxide (20 ml) was added slowly to the vigorously stirred EMA solution. The reaction mixture solidified almost immediately. The hard gel was broken with a glass rod and left overnight at toom remperature in a covered vessel. The glassy material was dispersed in a DCC solution (3 g; 0.0145 mole, in 20 ml of dimethylsulfoxide) and stirred magnetically for 3–4 h. The swollen resin was centrifuged down, resuspended in dimethylsulfoxide, and centrifuged down again. The precipitate was washed twice with hot ethanol (to remove traces of reagents and dicyclohexyl urea); it was then suspended in water, ground in a blender and lyophilized. Net weight of lyophilized powder 3.1 g.

The nitrogen content of the EMA–HMD resin was determined by the Dumas combustion method[7]. The amount of free amino groups was determined by the Van Slyke method[8].

Coupling of pepsin

EMA–HMD resin (100 mg) was suspended in 1 M HCl (2 ml) and stirred at room temperature for about 10 min. The swollen resin was separated on a suction filter washed on the filter with 100 ml water brought to pH 4, resuspended in 2 ml distilled water, and the suspension brought to pH 4.0. A freshly prepared aqueous

solution of pepsin (10 mg dissolved in 1 ml was added to the magnetically stirred suspension and the pH adjusted again to 4.0; 1-ethyl-3-(3-dimethylaminoprophyl)-carbodiimide·HCl (20 mg in 1 ml water)[6] was then added and the pH maintained at 4.0 with 0.1 M HCl. After 30–60 min when no measurable changes in pH could be observed, the reaction mixture was transferred to the cold room and left stirring overnight. The immobilized enzym was separated by centrifugation or filtration washed with 1 M KCl acidified to pH 5.0 (100 ml) and then with 0.01 M HCl; it was then suspended in 0.01 M HCl (5 ml) and stored 4 °C.

Coupling of trypsin

EMA–HMD resin (100 mg) was suspended in 0.1 M NaOH stirred for about 5 min washed with water and resuspended in 4 ml of a 12% glutaraldehyde solution at pH 9.0 (this solution was prepared by diluting commercial 25% aqueous glutaraldehyde with an equal volume of 0.2 M borate, pH 9.0 and readjusting the pH). The reaction mixture was stirred for 1 h at room temperature, washed exhaustively with water and finally with 0.1 M phosphate, pH 8.0. The resin was resuspended in cold 0.1 M phosphate, pH 8.0 (2 ml). Trypsin (10 mg dissolved in 1 ml of the same buffer) was then added to the magnetically-stirred suspension and the reaction allowed to proceed overnight in the cold room. The immobilized enzyme was separated by centrifugation or filtration, washed with 1 M KCl (100 ml) and then with water, and resuspended in water.

Assay methods

The concentrations of the native enzymes were estimated from the 280-nm absorbance using $E_{280\ nm}$ values of 14.7 (ref. 9) and 14.4 (ref. 10) for pepsin and trypsin, respectively.

The enzymic activity of crystalline pepsin and the immobilized pepsin derivatives was determined at 37 °C by the hemoglobin method, essentially as described by Herriott[11] and Anson[12]. The reaction mixtures containing immobilized enzyme were stirred magnetically to ensure effective mixing of the reagents. One unit of enzymic activity was defined following Anson[12] as that amount of pepsin or immobilized pepsin derivative which led to a change in absorbance at 280 nm of the trichloroacetic acid-soluble fraction of 0.001 per min, under the conditions of the assay. The specific activity of the crystalline pepsin sample used was 3150 units per mg.

The enzyme activities of native and immobilized trypsin were determined by the pH-stat method, at pH 8.2 and 9.0, respectively, using $1.5 \cdot 10^{-2}$ M benzoyl-L-arginine ethyl ester, 0.005 M KCl as substrate (5 ml) as described by Goldstein[2]. One unit of esterase activity was defined as that amount of enzyme which catalyzed the hydrolysis of 1 μmole of substrate per min under the specified conditions. The specific activity of the crystalline trypsin sample used was 35 esterase units per mg.

Milk-clotting experiments were carried out routinely at 30 °C by the addition of about 20 μg crystallised pepsin (about 80 units) or the equivalent amount of immobilized pepsin to 10 ml of reconstituted skim milk (12 g skim milk powder dissolved in 100 ml 0.01 M $CaCl_2$, pH 5.6 (ref. 13).

RESULTS AND DISCUSSION

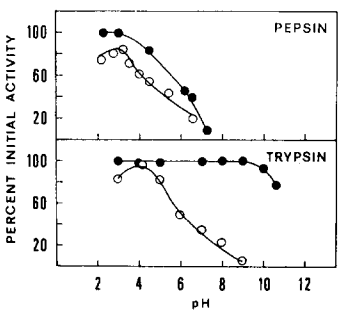
Characterization of resin

The HMD content of a standard EMA–HMD preparation, estimated from its nitrogen content (11.17% N by the Dumas method) was 3.99 ± 0.02 mmole HMD per g resin. The amount of free amino groups on the resin, estimated from its amine nitrogen (2.57% N by the Van Slyke method) was 0.92 ± 0.04 mequiv/g. These two values indicate that about 20% of the HMD molecules were incorporated in the resin by one-point attachment and available for coupling to proteins. The maximal binding capacity of EMA–HMD for pepsin or trypsin was determined by coupling the resin with varying amounts of enzyme and plotting the data as the amount of active bound protein (estimated by a rate assay) *vs* the amount of protein in the coupling mixture[14]. The maximal capacity of EMA–HMD estimated from the region where the binding curve levelled off was 100 mg protein per g resin. This value was confirmed by amino analysis[15]. The recovery of immobilized enzymic activity was about 20% for pepsin and about 40% for trypsin.

Properties of the immobilized derivatives of pepsin and trypsin

Aqueous suspension of immobilized trypsin or pepsin (at pH 7.0 and 2.5, respectively) could be stored at 4 °C for 3–4 months without significant loss of activity. On lyophilization the immobilized pepsin preparations retained 70–80% of their activity; immobilized trypsin samples retained only about 25% of their activity.

Incubation of immobilized trypsin at 37 °C for 30 min at various pH values caused no significant inactivation in the pH range 2–10, in contrast to native trypsin which rapidly loses activity above pH 6.0 (Fig. 2). The immobilized pepsin derivatives exhibited improved stability in the pH range 4–6 (Fig. 2). The immobilized derivatives of both enzymes showed higher temperature stability (Fig. 3). The



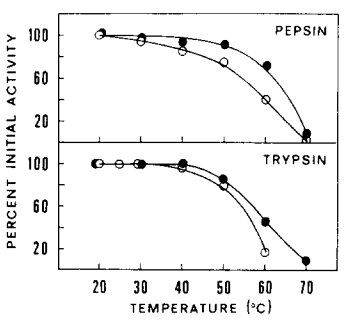


Fig. 2. Effect of pH on the stability of immobilized derivatives pepsin and trypsin. ○, native enzyme; ●, immobilized enzyme. A solution or suspension of enzyme (containing 40 pepsin units/ml or 15 trypsin units/ml) was brought to the desired pH by addition of dilute acid or alkali and incubated at 37 °C for 30 min. Aliquots were withdrawn and the residual activity determined as described in the experimental section.

Fig. 3. Effect of temperature on the stability of immobilized pepsin and trypsin. ○, native enzyme; ●, immobilized enzyme. The test samples containing native or immobilized enzyme (40 pepsin units/ml or 15 trypsin units/ml) at the pH of optimal stability (see Fig. 2), were incubated at the specified temperature for 15 min. Aliquots were withdrawn and the residual activity determined by the standard procedures.

enhanced stability of the immobilized enzymes is probable duy, mainly to the prevention of autodigestion.

The pH–activity profile of immobilized pepsin with hemoglobin as substrate was broader than that of the native enzyme. The pH–activity profile of immobilized trypsin acting on benzoyl-L-arginine ethyl ester was rather diffuse and displaced by more than two pH units towards more alkaline pH values relative to the native enzyme (Fig. 4). With both enzymes these effects were independent of ionic strength. The perturbed pH–rate profiles could be attributed to pH changes in the domain of the immobilized enzyme particles resulting from the generation of local product-concentration gradients as the enzymic reaction proceeds[3]. In the case of immobilized trypsin, benzoyl-L-arginine, ethanol and H^+ are produced in the enzymic hydrolysis of benzoyl-L-arginine ethyl ester at neutral and slightly alkaline pH values, and a large shift in the pH–activity profile is observed. With immobilized pepsin, hydrolysis of hemoglobin at acidic pH values produced partially ionized carboxyl groups ($pK \approx 3.7$; ref. 16) and amino groups in the NH_3^+ form; the observed pH shifts are consequently small. The pH shifts increase, however, with increasing pH, in the pH range of ionization of the carboxyl group (pH 3–5; Fig. 4).

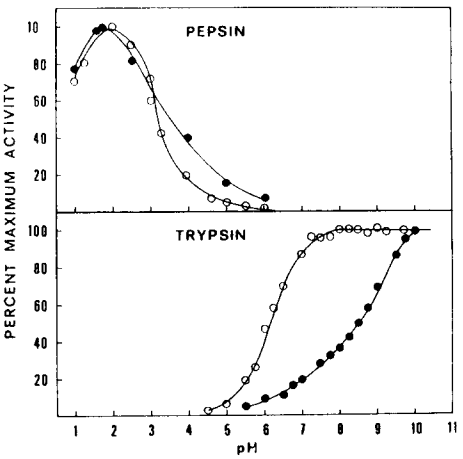


Fig. 4. pH–activity profiles of immobilized pepsin and trypsin. ○, native enzyme; ●, immobilized enzyme. For pepsin and immobilized pepsin, the substrate solution (2% hemoglobin, 2.5 ml) was brought to the desired pH and the enzyme (20–40 pepsin units in 0.5 ml) added. The reaction was allowed to proceed for 10 min at 37 °C and terminated by the addition of 5% trichloroacetic acid[11,12]. For trypsin and immobilized trypsin (1–1.5 trypsin units per assay), the rate of hydrolysis of $1.5 \cdot 10^{-2}$ M benzoyl-L-arginine ethyl ester, 0.05 M KCl (5 ml), at various pH values was determined by the pH-stat method as described in the experimental section.

Immobilized pepsin clotted reconstituted milk (0.01 M in $CaCl_2$) similarly to the native enzyme. Clotting by immobilized pepsin was, however, slower by a factor of about 2.5 as compared to the native enzyme.

The two stages of the clotting process, the enzymatic stage and the secondary coagulation stage[17], could be separated when clotting was effected by immobilized pepsin. The primary enzymatic stage was carried out at 4 °C in the absence of Ca^{2+}. The immobilized enzyme was then removed by filtration and the coagulation stage

carried out at 30 °C following the addition of $CaCl_2$ (to 0.01 M), in the absence of enzyme.

Repeated use of the immobilized enzyme led to gradual loss of its ability to clot milk. This may be due to the absorption of casein or to the slow formation of a milk clot around the enzyme particles.

In conclusion, the EMA–HMD resins offer a high-capacity polymeric carrier of considerable versatility. By proper choice of activation procedure it can be coupled to proteins through their amino as well as through their carboxyl groups.

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