A NEW METHOD OF COUPLING PROTEINS TO INSOLUBLE POLYMERS

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SUMMARY

1) A new mild method for the covalent coupling of enzymes to COOH group bearing insoluble polymers such as CM-cellulose and CM-Sephadex is described in this paper. N-ethoxycarbonyl-2-ethoxy 1,2-dihydroquinoline (EEDQ) is used to activate the polymer before reacting with the enzyme.
2) Optimal amounts of enzyme are coupled when the polymer is activated at pH 4.
3) Large amounts of protein retaining appreciably high specific activity may be immobilized by this procedure.

INTRODUCTION

In recent years more and more methods have been developed for the attachment of proteins, nucleotides and cofactors (1,2,3) to insoluble polymers. The need for such a vast number of methods has been created by the large variety of polymers, enzymes and ligands used notwithstanding how the immobilized products are subsequently used. The method described in this paper is facile and produces products with high coupling yields and specific activities.

MATERIALS AND METHODS

EEDQ used in the experiments with trypsin was a generous gift by Dr. V. DiTullio. Further samples were obtained from Aldrich Company, U.S.A. Trypsin was the 3 x crystalline product from Worthington Biochemicals Ltd. (5.92 I.U./mg with BAEE as substrate). Urease V (3500 Sigma units/mg or 244.75 I.U./mg) was obtained from Sigma Chemical Company, U.S.A.

The coupling method consists of a two step procedure the first consisting of activation of the polymer with EEDQ to form a mixed carbonic anhydride derivative of the insoluble polymer which in the second step reacts with an amino group of an enzyme to form a peptide bond.

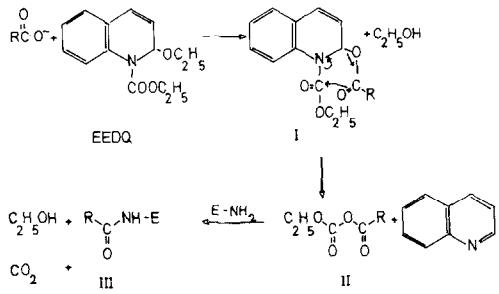

Figure 1

The reaction scheme is shown in Fig. 1 where $R-\overset{O}{\overset{\|}{C}}-O^-$ denotes the polymer and $E-NH_2$ the enzyme. The carboxyl group activation by EEDQ proceeds through a six-membered transition state (I) which presumably breaks down to form the mixed carbonic anhydride (II) eliminating a molecule of quinoline (4). The covalent binding of the enzyme results in (III) with the elimination of a molecule each of C_2H_5OH and CO_2.

The activation of the polymer and the subsequent enzyme coupling steps are achieved by a subtle but quick manipulation of the pH of the system which relies on the fact that the pH dependent stability of EEDQ and the mixed carbonic anhydride (II) are distinctly different. EEDQ is unstable and hydrolyzes at low pH values whereas the mixed carbonic anhydride becomes increasingly unstable above pH 7.

A typical coupling method was designed as follows: 1 g of the polymer (CM-cellulose, CM 50 Sephadex) was equilibrated in the solvent kept in ice. 0.6 to 1.0 g of EEDQ dissolved in 10 ml of 10 % acetone was added slowly in drops and the pH maintained at a selected value by a pH stat. This first step was completed in 20 - 30 minutes. The pH of the solution was then taken down quickly to pH 1.0 with conc. HCl and maintained for 90 seconds to destroy any excess EEDQ. The polymer was washed with ice cold 0.1 M HCl and added to a solution of the protein made up in pH 8.0 phosphate buffer (I = 0.05) kept in ice. The enzyme was allowed to react for about half an hour. It was then

washed once with the coupling buffer followed by a liberal washing with 1 M NaCl to remove any adsorbed protein. The salt was removed by extensive washing with ice cold deionized water. The product was filtered in a Buchner funnel and stored in the coupling buffer. Slight modifications were introduced into this procedure to improve the method as discussed later (see also Table 2).

In one experiment ATP was bound to CM-cellulose in pH 7.0 borate buffer (I = 0.1) the concentration of the amount of nucleotide bound being determined by OD measurements at 259 nm of the initial and final solutions.

Measurements of Enzymic Activity

The enzymic activity of CMC-trypsin was measured in the stirred reactor vessel of a titrator, the pH being maintained constant at pH 8.0 by automatic titration. BAEE was added as a concentrated solution to the CMC-trypsin suspension in the reactor vessel to give a final concentration of 3 mM containing 0.015 M $CaCl_2$. Hydrolysis of the same substrate in pH 8.0 Tris buffer containing 0.015 M $CaCl_2$ was also assayed, by passing it through a column packed with the immobilized enzyme at a specified flow rate and 22°C. The extinction at 253 nm was measured (5) (Table 1).

Immobilized urease was assayed with the enzyme packed in column. The effluent was assayed for NH_3 by the previously described method (6).

Table 1: The pH dependence of the activation of COOH groups by EEDQ as revealed by final coupling yield.

pH	% Nitrogen	% Protein (w/w)	μmoles BAEE/min/mg CMC-Trypsin
3	0.4	2.7	0.15
4	0.8	5.4	0.305
5	0.5	3.3	0.180
6	0.3	2.0	0.115

Activities measured in a pH stat at pH 8.0 in 0.015 M $CaCl_2$. An extinction value of 1.15 OD units at 253 nm for a mM solution of benzoyl arginine was used (5) in the calculation of enzymic activity. CM-cellulose with an average exchange capacity of 0.8 m eq/gram was used. Trypsin activity specified at 180 units/mg of tosyl arginine methylester corresponds to 5.92 I.U./mg with benzoyl arginine ethylester.
The same preparations assayed in a column gave activities within $\pm$ 5 % shown above.

Protein Estimation

The amount of enzyme bound to the polymer was estimated by the Lowry method (7) after total hydrolysis of the products with 6 M HCl for 120 hours. The values are expressed on a w/w basis. In the case of the trypsin experiment total nitrogen content was also measured in the conventional manner.

RESULTS AND DISCUSSION

Table 1 shows the results of the pH dependence of the activation step of the polymer (step I). These experiments were carried out with trypsin and CM-cellulose. It is clear that optimal activation takes place at pH 4. In all the further immobilization experiments, that is urease and ATP, the polymer was activated at this pH. The activities of the CMC-trypsin were measured at pH 8.0 using a saturating concentration of 3 mM BAEE. It may be seen that the four different trypsin preparations show the same specific activity of 93 % even though the percentage protein bound differs in each case. The pH 4 and 6 preparations packed in columns gave activities of 0.32 and 0.12 μmoles/min/mg preparation, when assayed in pH 8 Tris buffer containing 0.015 M $CaCl_2$ at $22^{\circ}C$.

By a similar procedure an average of 1.04 μmoles of ATP coupled to a gram of CM-cellulose, coupling about 3.7 % of the amount of nucleotide started with. This is an alternate method to several other methods discussed elsewhere (3).

CM-Sephadex-urease prepared by essentially the same procedure, maintained 50 % of its specific activity after 2 weeks storage at $4^{\circ}C$. It was suspected that perhaps any quinoline adsorbed to the gel from the activation step may have inhibited the enzyme. Thus elaborate washing of the gel after the first step with cold acetone and 0.1 M HCl improved the stability with only a 26 % loss in 2 weeks. However, the gel does not stand up to this vigorous treatment and becomes sticky with a concomitant drop in flow rate. As shown in Table 2, CM-cellulose stands up to this washing procedure and gives a considerably more

stable preparation. The specific activity of this preparation is also noticeably higher than the CM-Sephadex-urease.

Activating CM-cellulose by mild refluxing for 2 hours in anhydrous THF containing not more than 1 % water and coupling enzyme overnight results in about 3.46 % (w/w) of urease being bound having a specific activity of 18.2 %. A rational explanation for the variation in specific activities (Table 2) and storage stabilities should be forthcoming after more detailed investigation.

In experiments with urease, no attempt was made to maximise the amount of enzyme coupled to the polymer. This was probably the reason for the variation in percentage protein bound to the polymer. In fact, in most cases most of the enzyme protein initially supplied was coupled. The coupling capacity also depends upon the exchange capacity of the polymer. Thus, CM 50 Sephadex with an average 3 - 4 m eq of COOH groups per gram couples more enzyme than CM-cellulose which has only 0.8 m eq per gram.

The immobilized urease showed optimal activity at pH 7.6 as against pH 7 for native urease. The activities at pH 7.6 were only about 2 % higher than at pH 7.

Table 2: Results of coupling urease to CM-cellulose and CM 50 Sephadex

Number	Polymer	Coupling Conditions	% Protein (w/w)	% Specific Activity
1	CM 50 Sephadex	As in text	19.75	49.0
2	"	EEDQ in DMF: acetone 1:1 (20 %,v/v)	1.17	89.0
3	"	EEDQ in acetone washed with acetone + 0.1 M HCl	7.32	47.75
4	CM-cellulose	EEDQ in 50 % acetone	2.0	74.0
5	"	anhydrous THF	3.46	18.2

All activities measured at pH 7.0 in phosphate buffer at 0.05 M urea containing mM EDTA.

In conclusion it may be said that activation of the polymer at pH 4 in 10 to 20 % organic solvent followed by extensive washing gives the most stable preparation. Completely anhydrous medium did not give better results in my experience and besides, not many polymers will stand up to these conditions.

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