# COVALENT ATTACHMENT OF PROTEINS TO SPHERON BY MEANS OF BENZOQUINONE

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The dependence of the amount and the activity of coupled chymotrypsin and trypsin on the activation time and pH, coupling time and pH, enzyme concentration in the reaction mixture and the molar ratio $CaCl_2$/trypsin concentration were investigated for case where enzymes are covalently bound to the hydroxyalkyl methacrylate gel (Spheron) by means of benzoquinone.

A large variety of carriers for immobilization of biologically important moleculues have been reported[1,2]. Synthetic chemistry put as its aim to prepare materials possessing the distinct advantages of agarose as carrier (hydrophilic character, considerable swelling capacity, high percent of retained enzyme activity) as well as better mechanical strength and better resistance to microbial attack then agarose. Hydroxyalkyl methacrylate gels (Spherons)[3] could be considered an achievement in this respect. Their applicability for immobilization of enzymes has been shown[4,5]. In this paper we examine the activation of Spheron by means of benzoquinone, described previously for Sepharose[6].

## EXPERIMENTAL

### Materials and Methods

Spheron 300 was a product of Lachema, Brno. Chymotrypsin (proteolytic activity 0·45 unit $A_{280nm}$ per min and mg and esterase activity determined by means of N-acetyl-L-tyrosine ethyl ester 135 μmol $min^{-1}$ $mg^{-1}$); trypsin (proteolytic activity 0·535 unit $A_{280nm}$ per min and mg) were products of Léčiva, Prague. The activation of Spheron 300 as well as the coupling of proteins were carried out in analogy of the procedures, described for Sepharose[6].

*Activation of Spheron* 300. An appropriate volume of swollen gel in phosphate buffer, 0·1M, pH 8 was mixed with equal volume of a solution of benzoquinone (40% ethanol, 0·1M benzoquinone). The activation was allowed to proceed at room temperature for 2 h. Activated gel samples were washed on a glass filter with 20% ethanol, water, 1M-NaCl and water. For the study of the effect of pH on the activation step 3 ml of swollen Spheron 300 were treated at various pH in interval 4—10, and activation time 1 h.

*Coupling of proteins to benzoquinone-activated Spheron* 300. An appropriate volume of activated washed gel was mixed with equal volume of buffer of corresponding pH (usually 0·1M hydrogen carbonate buffer), containing the protein in concentration 10 mg $ml^{-1}$ (bovine serum albumin 20 mg $ml^{-1}$). The shaked for 24 h at 7°C products were washed with water on a glass filter, then on small columns with 1M-KCl/0·1M sodium acetate buffer pH 4·0 (24 h), 1M-KCl/0·1M sodium hydrogen carbonate buffer pH 8·5 (24 h) and finally with water. Gel samples for determination of protein content were washed with acetone and dried by heating at 100°C for 24 h. The amount of immobilized protein was determined from the amino acid analysis[7] after acid hydrolysis of a sample obtained according to Axen and Ernback[8].

*Activity measurements.* The proteolytic activity of free and immobilized enzymes was determined according to[4]. The proteolytic activity is expressed in $A_{280nm}$ per min and per mg units of free enzyme or $A_{280nm}$ per min and per g damp gel.

## RESULTS

The activation step was studied using bovine serum albumin. Maximum of fixed protein has been attained at activation pH around 8 (Fig. 1). Effect of activation time at pH 8 on the amount of fixed serum albumin is ilustrated in Fig. 2. The activation was completed in 2–2 1/2 h. The pH profile of the activation step (Fig. 1) is very similar to that displayed by Sepharose, but the rate of activation in the case of Spheron is slower.

The amount of fixed chymotrypsin and trypsin in dependence on coupling time is in Fig. 3. The coupling process is slow and an appropriate time is 24 h. Effect of coupling pH on the amount of fixed protein and activity of the immobilized preparations was studied by use of serum albumin, chymotrypsin and trypsin. The immobilized preparations were characterized in terms of amount of covalently fixed enzyme, proteolytic activity and activity ratio bound to free enzyme (%). These results are summarized in Table I and in Fig. 4. The amount of fixed chymotrypsin and the activity of the resulted immobilized preparations as a function of the concentration of enzyme is summarized in Table II. Effect of the molar ratio $[CaCl_2]/[E]$ on the activity of the immobilized trypsin preparations is summarized in Table III. Considering all the effects the conditions for obtaining high active immobilized chymotrypsin and trypsin preparations were derived: for chymotrypsin – 30 hours coupling reaction at pH 9·5, chymotrypsin solution 50 mg $ml^{-1}$; for trypsin – 30 h coupling reaction at pH 9·5, trypsin solution 44 mg $ml^{-1}$, 0·02M in respect to $CaCl_2$. The characteristics of the preparations so obtained are shown on Table IV. Comparatively small amount of fixed protein was obtained, but the activity ratio bound to free enzyme in respect to proteolytic activity was very high. The conjugates formed are very stable and leakage of the protein seems to be low.

The pH dependence of the amount of fixed chymotrypsin and bovine serum albumin are virtually similar to those revealed in the case of Sepharose[6]. Trypsin and chymotrypsin were coupled to Spheron in rather low amounts at low pH values and only above pH 8·0 the coupling is effective. Such kind of pH dependence imply participa-

tion of only one type enzyme functional groups in the covalent attachment of the protein. In the case of bovine serum albumin probably various enzyme functional groups participate in the reaction with the matrix.

The activity of immobilized chymotrypsin preparations as a function of coupling pH steadily increases, while the activity ratio exhibits a maximum at pH 8·0 (Table I). It is evident that at coupling pH above 8·0, the increasing amount of fixed enzyme leads to a lower specific activity. This could be ascribed as in other cases[5] to inappropriate orientation of enzyme molecules caused by steric hindrance. The more complex character of the dependence of relative proteolytic activity of trypsin immobilized preparations on coupling pH is probably determined by the contribution of several pH dependent factors: rate of the coupling reaction, accessibility and activity of enzy-

TABLE I

Characteristics of the Immobilized Chymotrypsin and Trypsin Preparations

| pH of coupling step[a] | Amount of enzyme mg/g dry conjug. | Activity of damp conjugate $A_{280}$/min g | Activity of dry conjugate $A_{280}$/min g | Relative activity % |
|---|---|---|---|---|
| | | Chymotrypsin | | |
| 4 | 0·40 | 0·050 | 0·105 | 58·33 |
| 5 | 0·55 | 0·080 | 0·168 | 68·01 |
| 6 | 0·80 | 0·150 | 0·315 | 87·50 |
| 7 | 1·00 | 0·200 | 0·420 | 93·33 |
| 8 | 1·50 | 0·310 | 0·651 | 94·44 |
| 9 | 6·00 | 1·170 | 2·457 | 91·00 |
| 10 | 8·00 | 1·300 | 2·730 | 75·83 |
| | | Trypsin | | |
| 4 | 1·2 | 0·020 | 0·042 | 6·54 |
| 5 | 1·4 | 0·100 | 0·210 | 28·03 |
| 6 | 1·4 | 0·115 | 0·291 | 38·58 |
| 7 | 1·7 | 0·300 | 0·630 | 69·25 |
| 8 | 2·0 | 0·250 | 0·525 | 49·06 |
| 9 | 3·8 | 0·460 | 0·966 | 47·51 |
| 10 | 4·3 | 0·650 | 1·365 | 59·34 |

[a] Conditions of coupling: 3 ml activated gel + 3 ml trypsin solution (10 mg/ml$^{-1}$) in a buffer of appropriate pH, 7°C, 24 h shaking. Proteolytic activity of 100 mg samples of the preparations was determined by a modified Anson's procedure[4]; weight ratio damp gel/dry gel = 2·1. Activity of free chymotrypsin $A_{280}$ per mg and per min = 0·45 and of free trypsin $A_{280}$ per min and per mg = 0·535.

me functional groups involved in the coupling reaction and enzyme conformation. Immobilization of trypsin in the presence of $CaCl_2$ at varying molar ratio $[CaCl_2]/[E]$ results in significant change of the relative proteolytic activity (Table III). This implies the possibility to trap a more active and temperature stable enzyme conformer

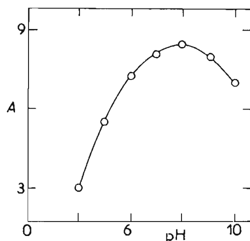


FIG. 1

Effect of Activation pH on the Amount of Fixed Bovine Serum Albumin *A* (mg per g of dry conjugate) on Spheron 300

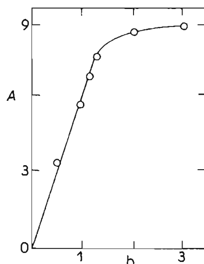


FIG. 2

Effect of the Activation Time on the Amount of Fixed Bovine Serum Albumin *A* (mg per g of dry conjugate) on Spheron 300

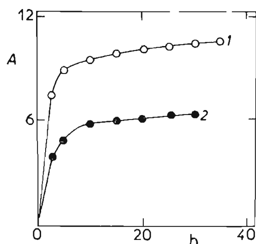


FIG. 3

Effect of Coupling Time on the Amount of Fixed Chymotrypsin *1* and Trypsin *2*: *A* (mg per g of dry conjugate) on Spheron 300

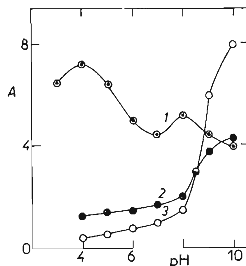


FIG. 4

Effect of Coupling pH on the Amount of Fixed Proteins *A* (mg/g dry conjugate) on Spheron 300

*1* Bovine serum albumin; *2* trypsin; *3* chymotrypsin.

by means of immobilization technique. Such $Ca^{2+}$-induced conformer is assumed in the case of trypsin[9].

Table IV reveals marked difference in the amount of fixed protein: 6 fold higher amount (in terms of mg per g of dry conjugate) was found in the case of benzoquinone-activated Sepharose 4B, but the amount of the bound protein calculated in terms of mg per ml conjugate for both carrier is similar (2–3 mg per ml conjugate). This could be explained by the difference in the porosity and the swelling capacity

TABLE II

Characteristics of the Immobilized Chymotrypsin Preparations in Dependence on the Concentration of the Enzyme Solution at the Coupling Step

| Concentration of the chymotrypsin solution[a] | | Amount of enzyme mg/ml dry conjugate | Activity of damp conjugate $A_{280}$/min g | Activity of dry conjugate $A_{280}$/min g | Relative activity % |
|---|---|---|---|---|---|
| mg/ml | M | | | | |
| 10 | $0·4 . 10^{-3}$ | 8·00 | 1·300 | 2·73 | 75·83 |
| 50 | $2·0 . 10^{-3}$ | 9·67 | 1·700 | 3·57 | 82·06 |
| 100 | $4·0 . 10^{-3}$ | 10·50 | 1·500 | 3·15 | 67·02 |

[a] Conditions of coupling as described in Table I, only the concentration of chymotrypsin solution being varied, pH 10.

TABLE III

Characteristics of Immobilized Trypsin Preparations in Dependence on the Presence of $CaCl_2$ (20 mM)

| Concentration of trypsin solution[a] | | Molar ratio $CaCl_2$/E | Amount of enzyme mg/g dry conjugate | Activity of damp conjugate $A_{280}$/min g | Activity of dry conjugate $A_{280}$/min g | Relative activity % |
|---|---|---|---|---|---|---|
| mg/ml | mM | | | | | |
| 10 | 0·428 | 46·7 | 2·1 | 1·43 | 3·00 | 267·1 |
| 22 | 0·940 | 21·2 | 3·4 | 1·20 | 2·52 | 138·5 |
| 44 | 1·880 | 10·6 | 4·6 | 1·33 | 2·79 | 113·4 |

[a] Conditions of coupling as described in Table II only $CaCl_2$ (20 mM) being present and concentration of the trypsin solution being varied, pH 10.

of both gels. The amount of chymotrypsin bound to Spheron activated with cyanogen bromide[4] is 5fold higher compared with the amount of chymotrypsin bound to Spheron activated by means of benzoquinone, but the relative proteolytic activity is considerably higher (2 fold) in the case of activation with benzoquinone. The immobilized chymotrypsin and trypsin preparations coupled on Spheron 300 with benzoquinone display no shift of pH optimum of proteolytic activity, compared with the corresponding free enzymes. Both preparations were stored at 5°C for 1 month in 0·01M sodium acetate buffer pH 5 without any decrease in enzymatic activity or protein content. Treatment of the immobilized preparations with 6M guanidine hydrochloride solution did not result in change of protein content.

The advantage of the immobilization procedure by means of benzoquinone in its simplicity and avoiding of the cyanogen bromide. In contrary to the differences in the pH dependence of proteins coupled to epoxy derivatives of Sepharose and Spheron[10], the behaviour of both gels after the benzoquinone activation is similar, as described also for the cyanogen bromide activation[4].

TABLE IV

Comparison of the Characteristics of the Immobilized Enzymes on Sepharose and Spheron 300

| Carrier | Procedure for activation | Enzyme mg/g dry conjugate | Enzyme mg/ml conjugate | Relative activity % |
|---|---|---|---|---|
| | | Chymotrypsin | | |
| Sepharose | cyanogen bromide | 67·0 | 2·23 | 32 |
| | benzoquinone | 72·0 | 2·40 | 68 |
| Spheron | cyanogen bromide | 55·6 | 17·70 | 44 |
| | benzoquinone | 9·6 | 3·00 | 78 |
| | | Trypsin | | |
| Sepharose | cyanogen bromide | 30 | 1 | 25 |
| Spheron | benzoquinone | 4·3 | 1·34 | 110[a] |

[a] In the presence of $Ca^{2+}$.

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