IMMOBILIZATION OF TRYPSIN ON PERIODATE-OXIDIZED GLUCOSEHYDROXYALKYL METHACRYLATE GEL SEPARON-H 1000-GLC

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The dependence of the amount of bound enzyme on the oxidation time, molarity of the oxidizing solution, pH, time, temperature and enzyme concentration in the reaction mixture were investigated for the case where trypsin is covalently bound on the hydroxyalkal methacrylate gel containing glucose on its surface (Separon-H 1000-glc) after the gel had been periodate-oxidized. The immobilized derivatives of trypsin were examined with respect to their catalytic activity and stability in continuous hydrolysis.

The study of immobilized enzymes is related to the development of solid carriers. The utilization of immobilized enzymes for practical application and for theoretical studies is greatly dependent on the general knowledge of the effect of the matrix and of the mode of attachment on the catalytic properties of the enzyme and on its stability. Goldstein[1] has demonstrated how the stability of trypsin, chymotrypsin and subtilisin can be affected by coupling on modified ethylene–maleic anhydride copolymer. It should be pointed out, however, that it is not only the character of the solid carrier that influences the properties of the immobilized enzyme, but also the concentration of active groups on the carrier, conditions of coupling, the amount of the protein bound, the number of attached amino acid residues on the surface of the coupled protein, their character and distance from the active site of the enzyme. With various enzymes these effects are operative to a various extent[2].

Advantages of the immobilization of enzyme *via* reductive alkylation on solid carriers containing diols have been demonstrated by Royer and coworkers[3], who coupled trypsin and carboxypeptidase A and B on dextran coated porus glass, Sepharose and glass coated with glyceryl silane after their oxidation with $NaIO_4$. Similarly to the procedure employed by them, we bound trypsin on the hydroxyalkyl methacrylate gel containing glucose on its surface[4].

The glycosylated copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate Separon-H 1 000-glc (saccharide content 15–20% w/w) preserves good

mechanical properties of the original carrier. Porosity is reduced by c. 15% by the saccharide, so that the exclusion limit of molecular weight amounts to 850 000 daltons in the determination by means of a standard polydextrane series. Hydrophylization of the internal pore surface has considerably reduced the hydrophobic adsorption effects of Separon, as has been demonstrated by experiments involving application of these materials in the high-efficiency gel permeation chromatography of proteins[5].

EXPERIMENTAL

Material: The hydroxyalkyl methacrylate gel, particle size 100—200 μm containing 18·4% D-glucose and called Separon-H 1000-glc, was prepared by a method described earlier[4]. The specific surface of the gel determined by the method according to Kljachko–Gurvich[6] was 22 m^2/g. The contents of C (52·2%), H (7·17%) and N (0%) were determined by elemental analysis.

Lyophilized trypsin was produced by Léčiva, Prague, its activity determined by using N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) as substrate according to Erlanger and coworkers[7] was 1·22 μmol/min per mg at pH 8.

Oxidation of Separon-H 1000-glc: Separon-H 1000-glc (1 g) was oxidized with $NaIO_4$ according to Royer and coworkers[3]. The gel was left to swell in water overnight. After that it was filtered by suction, suspended in 30 ml 0·1M (or 0·5M) $NaIO_4$ solution and stirred at room temperature for one hour (also 2, 8, or 24 h). The oxidized gel was transferred into a column and washed with water until equilibrium was reached between the conductivity of eluent and water used for washing. The oxidized gel was kept in suspension in water at 4°C.

Coupling of trypsin on the oxidized Separon-H 1000-glc *as a function of* pH: The following buffer solutions were used: 0·05M sodium acetate pH 4·0 and 5·6; 0·05M borate buffer pH 7·6; 8·4 and 9·2 and 0·05M borax buffer pH 10, all with 0·001M benzamidine. Buffers pH 4—7·6 contained 0·02M-$CaCl_2$, the remaining buffers contained 0·005M-$CaCl_2$. 1 g of gel filtered by suction (c. 1 ml) and oxidized with 0·1M-$NaIO_4$ for one hour was suspended after equilibration in 10 ml of trypsin solution (2·5 mg) having the respective pH. The suspension was stirred at room temperature. The decrease in tryptic activity was determined in the supernatant using the BAPA substrate[7] in 10 min intervals. The activity of control solutions of trypsin containing corresponding amounts of unoxidized gel were taken as 100% (Fig. 1). The quantity of immobilized trypsin as function of pH was determined by using amino acid analysis[8] (Fig. 2).

Coupling of trypsin on oxidized Separon-H 1000-glc *as a function of the oxidation time*: 1 ml of the gel oxidized for 1, 2, 4, and 8 h with 0·1M-$NaIO_4$ and with 0·5M-$NaIO_4$ solution was suspended after equilibrium in 10 ml of trypsin solution (10 mg) in 0·05M borax buffer, pH 10, containing 0·001M benzamidine and 0·005M-$CaCl_2$. The process of coupling occurred with continuous stirring at room temperature for 15 h (Fig. 3*A*).

Coupling of trypsin on the oxidized Separon-H 1000-glc *as a function of the duration of coupling and temperature*: The coupling on the gel oxidized for one hour (0·1M-$NaIO_4$) occurred similarly to the procedure described above at room temperature (25°C ± 1) and 4°C for 1, 2, 4, 8 and 22 h (Fig. 3*b*).

Coupling of trypsin on the oxidized Separon-H 1000-glc *as a function of trypsin concentration in the solution*: 3 g of wet gel filtered by suction and equilibrated (corresponds to 1 g of dry gel) and oxidized for 1 h with 0·1M-$NaIO_4$ was suspended in 50 ml 0·05M borax buffer, pH 10, containing 0·001M benzamidine and 0.005M-$CaCl_2$, and also containing 10, 50, 100, 250, or 500 mg

trypsin (Fig. 4). The suspension was stirred at room temperature 8 h and then 2·5 mg of solid $NaBH_4$ was added. The same amount of $NaBH_4$ was added once again after 20 min. After another 20 min the suspensions were transferred into columns, in which they were alternately washed with 0·1 M Tris-HCl buffer pH 8 containing 0·02M $CaCl_2$ and 2M-NaCl, and with 0·1M sodium acetate containing 2M-NaCl, pH 4. The gels were eventually washed with the 0·1M-Tris-HCl buffer containing only 0·02M-$CaCl_2$. The samples were stored in the latter buffer at 4°C.

Determination of the catalytic activity of free and immobilized trypsin: The activity was determined using a modified[9] method of Erlanger and coworkers[7] with BAPA as substrate. The activity of trypsin as a function of pH was determined using $1 . 10^{-3}$M-BAPA solutions in Britton–Robinson buffers, pH 4—11 (Fig. 5).

Evaluation of binding capacity: The amount of bound enzyme was determined using amino acid analysis[8]. Before analysis, the gel samples were washed with water, ethanol, n-butanol, ethanol, and dried to constant weight at 105°C. The hydrolysis in 6M-HCl at 110°C took 20 h.

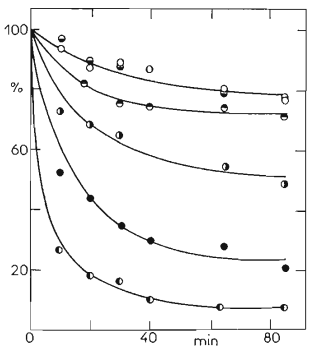

FIG. 1

Time Course of Trypsin Attachment of Periodate-Oxidized Separon-H 1000-glc in Dependence of pH

The coupling was performed at pH 10·0 (◐); 9·2 (●); 8·4 (◑); 7·6 (◓); 5·6 (○); pH 4·0 (◒) and room temperature. The activity (%) in supernatant is given on the *y* axis. In each case the activity of control solution of trypsin in the same buffer with the addition of the same amount of unoxidized Separon-H 1000-glc is taken as 100%.

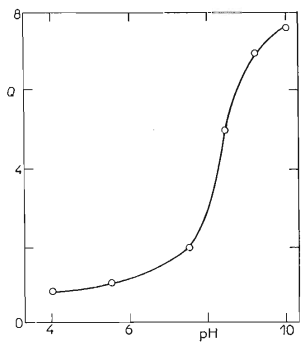

FIG. 2

Quantity of Immobilized Trypsin (*Q*, in mg Protein per g of Dry Conjugate) as Function of pH

For pH 4·0—5·6: acetate buffers; pH 7·6—10 borate buffers. The coupling was performed at room temperature for 2 h.

Determination of the stability of immobilized trypsin by means of the continuous hydrolysis of N^{α}*-benzoyl-*L*-arginine ethyl ester solution*: The assays were carried out continuously with immobilized trypsin (activity determined by means of BAPA was 19·6 μmol/min per g of dry gel at pH 8) packed in a jacketed column by passing the substrate solution through the column (5 ml) at

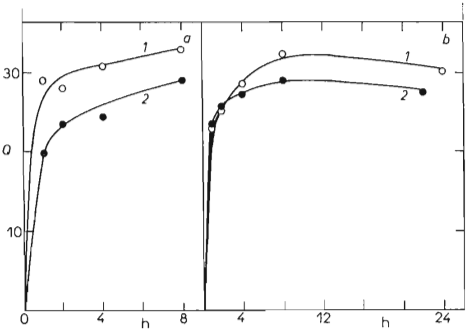

FIG. 3

Quantity of Immobilized Trypsin (Q, in mg Protein per g of dry conjugate) as Function of (*a*) Time of Periodate-Oxidation and Molarity of $NaIO_4$, (*b*) Time and Temperature of Coupling

Reaction conditions: 1 ml periodate-oxidized Separon-H 1 000-glc (in Fig. 3*b* oxidized 1 h) in 10 ml solution of trypsin (10 mg) in 0·05M borate buffer pH 10, containing 0·005M-$CaCl_2$ and 0·001M benzamidine; agitation: in Fig. 3*a* at room temperature for 15 h, (0·1 M, *1*; 0·5M, 2), in Fig. 3*b* at 25°C and 4°C (25°C, *1*; 4°C, 2).

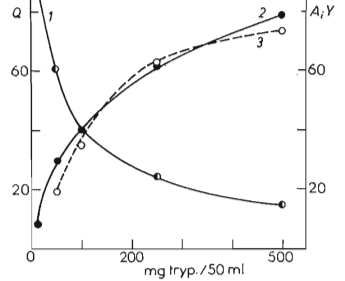

FIG. 4

Quantity of Immobilized Trypsin (Q, in mg of Protein per g of Dry Conjugate, 2) and Its Activity (A, in μmol/min per g Determined with BAPA, 3) as Function of the Content of the Enzyme

Yield of bound protein (Y, in %) determined from mass balance is also shown in the figure (*1*). Reaction conditions: 3 g wet gel (approx. 1 g dry gel) oxidized 1 h with 0·1M-$NaIO_4$ in 50 ml solution of trypsin (10 to 500 mg) in 0·05M borate buffer pH 10, containing 0·005M-$CaCl_2$ and 0·001M bezamidine; agitation at room temperature for 8 h.

constant flow rate (0·5 ml/min) and at constant temperature (37°C). The BAEE concentration was $5 . 10^{-5}$M. The product stream was sampled periodically and analyzed for benzoyl-L-arginine (followed by an increase in absorbance at 254 nm).

RESULTS

The attachment of proteins to solid carriers containing aldehyde groups *via* reductive alkylation is based on the formation of Schiff bases formed between an aldehyde and the amino group which may be reduced with sodium borohydride[3]. The time courses for the immobilization of trypsin on oxidized Separon-H 1000-glc in dependence on pH are shown in Fig. 1. Using control tests, in which similarly to the binding process solutions of trypsin were mixed with non-oxidized gel and in which no loss in tryptic activity could be observed virtually within the whole range of pH under investigation, the rate of formation of Schiff bases between the carrier and enzyme may be estimated from the curves obtained. Fig. 2 shows the amount of trypsin bound on periodate-oxidized Separon-H 1000-glc depending on pH. With increasing oxidation time the amount of bound trypsin increases, but it decreases with increasing molarity of the solution of sodium periodate used in the oxidation (Fig. 3*a*). A decrease in the amount of bound protein at a higher concentration of the oxidizing solution has also been observed by Wilson and Nakane[10], if the concentration of the periodate solution was higher than the determined $NaIO_4$ concentration optimum. Results presented in Fig. 3*a* seem to indicate treatment with 0·1M-$NaIO_4$ lasting one hour as the best oxidation conditions.

With increasing time of coupling the amount of protein bound rather decreases (Fig. 3*b*). A much more pronounced decrease in the amount of immobilized chymo-

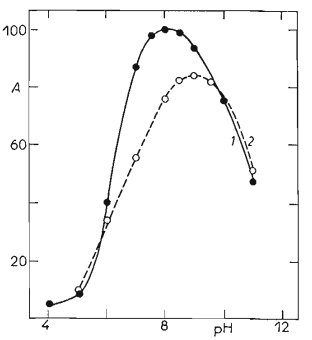

FIG. 5

Catalytic Activity *A* (with respect to BAPA) of Trypsin (*2*) and Trypsin Covalently Bonded (*1*) as Function of pH

The quantity of immobilized enzyme 61·5 mg per g of dry gel. Activity of free trypsin 1·22 μmol/min per mg at pH 8, determined with BAPA. Activity ratio of bound to free enzyme 84·1% (activity determined with BAPA).

trypsin with extension of the time of binding beyond the optimum value has been observed by Torchilin and coworkers[11] in the coupling of the enzyme on periodate-oxidized Sephadexes. These authors explain the decrease by the instability of the polysaccharide carrier.

The dependence of the protein bound to the carrier and of the activity of immobilized trypsin derivatives on the amount of protein added to the reaction mixture is shown in Fig. 4. The relative activity values for immobilized trypsin determined using a trypsin sample with the activity 1·22 μmol/min per mg (ascertained by BAPA) were as follows: 54·8% with 30·2 mg of protein per g of dry conjugate, 71·3% with 40·5 mg/g, 84·1% with 61·5 mg/g and 76·3% with 79·0 mg/g. Fig. 4 also shows that the yield of bound protein determined from mass balance steeply decreases with increasing concentration of the enzyme in the reaction mixture.

It can be seen from Fig. 5 that immobilization shifted as usual the pH optimum to a more alkaline region, probably due to the accumulation of the product on the surface of the solid carrier.

Using continuous hydrolysis of the BAEE solution at 37°C by studied immobilized trypsin, a decrease to half the original activity occurring within almost eight days could be recorded, in complete agreement with an analogous test carried out with a column of trypsin bound on periodate-oxidized cellulose[12].

All that has been observed allows us to conclude that the periodate-oxidized Separon-H 1000-glc is suitable for the preparation of immobilized enzymes.

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