IMMOBILIZATION ON CELLULOSE IN BEAD FORM AFTER PERIODATE OXIDATION AND REDUCTIVE ALKYLATION

Jaroslava TURKOVÁ[a], Jiří VAJČNER[a], Drahomíra VANČUROVÁ[a] and Jiří ŠTAMBERG[b]

[a]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, and*
[b]*Institute of Macromolecular Chemistry, Academy of Sciences, 162 06 Prague 6*

Received May 18th, 1979

The covalent binding of trypsin to cellulose beads after periodate oxidation was examined. The degree of cellulose derivatives solubilization is directly related to cellulose oxidation and increases with the increasing pH of the reaction mixture. The quantity of trypsin immobilized was examined as a function of pH, reaction time, and concentration of native trypsin in the reaction mixture during the coupling reaction. The catalytic activity and the stability of the preparations of immobilized trypsin during continuous hydrolysis of low-molecular-weight and high-molecular-weight substrates was assayed.

In an effort to obtain a solid support suitable for routine applications we decided to investigate the coupling of enzymes to cellulose prepared in bead form[1-3] which is in many respects superior to the conventional cellulose preparations. As can be derived from literature data[4-7] the optimal coupling conditions will depend both on the characteristics of the cellulose used and on the properties of the protein attached. In order to be able to compare the properties of various cellulose derivatives we used in this study trypsin, one of the enzymes most often immobilized on periodate-oxidized cellulose[5,6].

EXPERIMENTAL

Material: Cellulose in bead form was prepared in the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, by dispersion of a cellulose (viscose) xanthogenate solution in chlorobenzene, solidifying the suspension by heating at 90°C for 30 min, and completion of the cellulose regeneration in the absence of organic solvents. Viscose was made of a mixture of spruce cellulose sulfite (65%) and beech cellulose sulfate (35%), polymerization degree 750 and 600, respectively. The viscose contained 8·0 to 8·3% of cellulose, 6·0—6·2% of NaOH, and 2·3—2·4% of S. Cellulose beads were freed of traces of chlorobenzene after dispersion by distillation with water vapor. Excess water was centrifuged off. The beads contained 85% of water. A fraction of particle size 0·17—0·31 mm was separated by sieving of the cellulose in wet state.

Lyophilized trypsin was a product of Léčiva (Prague, Czechoslovakia) and its activity determined with N^{α}-benzoyl-D,L-arginine *p*-nitroanilide (BAPA) as substrate at pH 8 according to Erlanger and coworkers[8] was 1·99 μmol/min per mg.

Oxidation of cellulose: Swolen cellulose beads were washed on the filter with water and filtered off. The wet cellulose beads (10 g) were treated with 100 ml of 0·1M-$NaIO_4$ and the suspension was mildly shaken for 1, 2, 4, and 24 h at room temperature. The oxidized gel was placed in a column and washed with water until the conductivity of the eluant was the same as the conductivity of water. The oxidized cellulose was stored as a suspension in water at 4°C.

Coupling of trypsin to oxidized cellulose as function of pH: was examined in the following buffers: 0·05M sodium acetate, pH 4·0 and 5·6, 0·05M borate, pH 7·6, 8·4, 9·0, and 10·0, all containing 0·001M benzamidine. The buffers at pH 4—7·6 contained 0·02M-$CaCl_2$, the remaining buffers 0·005M-$CaCl_2$. Wet, filtered-off cellulose beads (0·5 g, corresponding to 67 mg of dry cellulose) oxidized for 1 h and equilibrated at the required pH were mixed with 6 ml of the trypsin solution (3 mg/12 ml) at the corresponding pH, and placed in a centrifugation tube. The suspension was stirred at room temperature. The residual activity of uncoupled trypsin was determined at time intervals with BAPA as substrate[8] in the supernatant after centrifugation of the suspension. The trypsin solution (6 ml) to which 0·5 g of wet, unoxidized cellulose (equilibrated at the required pH) had been added served as a control; this activity measured at the corresponding time intervals was taken to represent 100% (Fig. 1).

Solubility of aldehyde-cellulose as function of pH, *buffer molarity, and time of oxidation*: The wet cellulose (0·5 g) oxidized 1 or 4 h was suspended in 50 ml of each of the buffers given in Table I. The suspensions were shaken 4, 8, or 24 h at room temperature. The samples of cellulose were then transferred to centrifugation tubes, washed five times with 10 ml of water, ethanol solutions of increasing concentration, and dried to constant weight. A 0·5 g sample of wet oxidized cellulose washed with water only was treated in the same manner. The weight of this sample was taken to represent 100%. The weight loss (% w/w) as a function of pH, ionic strength, and time of oxidation is shown in Table I and Fig. 2.

Coupling of trypsin to aldehyde-cellulose as function of coupling time: Aldehyde-cellulose (1 ml), oxidized 1 h in 0·1M-$NaIO_4$, was suspended in 10 ml of a solution of 10 mg of trypsin in 0·1M borate buffer, pH 10, containing 0·005M-$CaCl_2$ and 0·001M benzamidine. The coupling was allowed to proceed 1, 2, 4, 9, 26, or 46 h at room temperature with constant stirring (Fig. 3).

TABLE I

Dependence of Solubilization (% w/w) of Aldehyde-Cellulose on the Time of Oxidation and on the Time of Solubilization

Solvent	Oxidation for 1 h			Oxidation for 4 h		
	4 h	8 h	24 h	4 h	8 h	24 h
0·1M Phosphate, pH 7	2·4	4·7	7·4	8·8	16·9	23·3
0·1M Phosphate, pH 8	11·7	14·0	15·7	22·0	33.7	41·7
0·1M Borate, pH 9	14·3	16·0	17·9	37·4	44·0	50·9
0·05M Borate, pH 10	21·1	22·2	22·8	45·8	48·5	53·1
0·1M Borate, pH 10	19·2	20·4	22·6	45·2	48·7	54·5
0·05M Carbonate, pH 10	20·1	20·9	21·8	43·0	47·0	78·5
0·1M Carbonate, pH 10	20·9	21·9	23·5	44·5	46·5	74·3

The stabilization of the bond and the elimination of the remaining aldehyde groups were effected by two additions of $NaBH_4$, as described by Royer and coworkers[7]. The suspension was treated first with 5 mg of $NaBH_4$ and the same quantity of the reagent was added 20 min afterwards. The mixture was set aside for 20 min, immobilized trypsin was transferred to a column and washed alternatively with 0·1M Tris-HCl buffer, pH 8·0, containing 2M-NaCl and 0·02M-$CaCl_2$, and with 0·1M acetate buffer, pH 4·0, containing 2M-NaCl and 0·02M-$CaCl_2$. Immobilized trypsin was washed with 0·1M Tris-HCl buffer containing 0·02M-$CaCl_2$ and stored as a suspension in this buffer at 4°C.

Coupling of trypsin to aldehyde cellulose as function of trypsin concentration of coupling mixture was effected as described above except that the coupling time was 4 h and the trypsin concentration 10, 20, 50, 75, or 100 mg/10 ml (Fig. 4).

Activity of free and immobilized trypsin was determined with BAPA as substrate using the modified[9] method of Erlanger and coworkers[8]. Solutions of BAPA ($1 . 10^{-3}$M) in Britton–Ro-

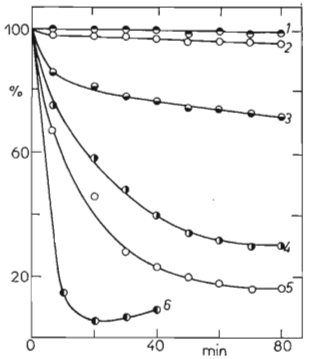

FIG. 1

Time Course of Trypsin Attachment to Periodate-Oxidized Cellulose as Function of pH

The coupling was carried out at pH 10·0 (*6*), 9·2 (*5*), 8·4 (*4*), 7·6 (*3*), 5·6 (*2*), pH 4·0 (*1*) and at room temperature. The activity (%) in supernatant is given on the *y* axis. In each case the activity of a control solution of trypsin in the same buffer to which the same amount of unoxidized cellulose was added is taken to represent 100%.

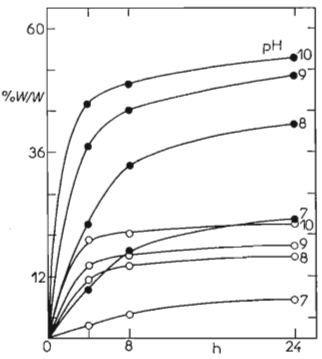

FIG. 2

Time Course of Solubilization of Periodate-Oxidized Cellulose Samples (% w/w) as Function of Oxidation Degree and pH of Buffers.

For pH 7 and 8: 0·1M phosphate buffers; for pH 9 and 10: 0·1M borate buffers. Cellulose after oxidation with 0·1M-$NaIO_4$ for 1 h (○), and 4 h (●); at room temperature.

binson buffers, pH 4—11, were employed for the determination of trypsin activity as function of pH.

Binding capacity: The quantity of enzyme attached was determined by amino acid analysis[10]. The gel samples for analysis were washed with water, ethanol, 1-butanol and ethanol, and dried to constant weight at 105°C; subsequently the samples were hydrolyzed in 6M-HCl, 20 h at 110°C.

Determination of stability of immobilized trypsin: The assays were carried out continuously with immobilized trypsin (activity determined with BAPA as substrate 150·5 μmol/min per g of dry cellulose at pH 8) packed in a jacketed column; the substrate was passed through the column (5 ml) at a flow rate of 0·5 ml/min and at temperature of 37°C. The substrate concentrations in 0·1M-Tris-HCl buffer, pH 8, were BAEE $5 . 10^{-5}$M and denatured haemoglobin $2 . 10^{-5}$M. The effluent was periodically removed and analyzed for benzoylarginine (measurement of absorbance increase at 254 nm) or presence of soluble peptides in the trichloroacetic filtrate after haemoglobin precipitation (measurement of absorbance at 280 nm).

RESULTS

In order to be able to compare our polyaldehyde cellulose derivative with analogous derivatives of cellulose used earlier we examined the attachment of trypsin as function of pH, Fig. 1. Even though all experiments were carried out at room temperature the activity of control trypsin solutions (100%) in the individual buffers containing benzamidine, calcium ions, and an analogous quantity of unoxidized cellulose, did not decrease over the period examined. The rate of Schiff base formation by the reaction of the aldehyde groups of the polysaccharide carrier with the amino groups of the enzyme[11] increases with the increasing pH. The activity increase in suspension at pH 10 can be ascribed to the solubilization of the derivative of immobilized trypsin. The quantity of trypsin attached, determined by amino acid analysis, was 12·5 mg of protein per g of dry material at pH 10 and 6·6 mg per g at pH 8·4.

The yield of the immobilized preparation decreases with the increasing oxidation period and pH. As can be seen in Table I the quantity of aldehyde-cellulose in solution increases with the increasing pH and with the prolongation of the oxidation period. Analogous results were reported by Torchilin and coworkers[12] who investigated the dependence of solubility of aldehyde-Sephadexes on varying oxidation degree over the pH-range 6—8·2. These authors prepared homogeneous samples of immobilized α-chymotrypsin in solubilized state which had high enzymatic activity, unaltered kinetic characteristics, and increased thermal stability. According to Torchilin and coworkers[12] the preparations of immobilized enzymes capable of slowing down and easily controlling solubilization could have a wide range of medical application.

Solubilization is most undesirable if immobilized enzymes are prepared. Fig. 2 shows the time profile of the release of soluble fragments of oxidized cellulose as a function of pH; preparations oxidized 1 and 4 h are shown. As obvious, the oxidation time should not exceed 1 h. The data given in Table I show that there is practically no difference between the 0·05M and 0·1M buffer at pH 10. There were differences

between the borate and the carbonate buffer, which were observed after 24 h only. But when trypsin was coupled to oxidized cellulose at pH 10 the results obtained with the carbonate buffer were always worse than with the borate buffer.

The quantity of trypsin attached to aldehyde cellulose and the activity of the preparations obtained as a function of coupling time are shown in Fig. 3. The curve shows a maximum at 4 h. A decrease of the quantity of protein attached with the increasing coupling time has been observed by Torchilin and coworkers[12] studying the attachment of α-chymotrypsin to periodate-oxidized Sephadex; they showed that the position of the maximum can be shifted either toward longer intervals by a reduction of the degree of aldehyde-Sephadex oxidation or to shorter intervals by raising the enzyme concentration or temperature of the reaction. As shown in Fig. 3 the decrease of the activity of immobilized trypsin is faster than the decrease of the quantity of protein attached.

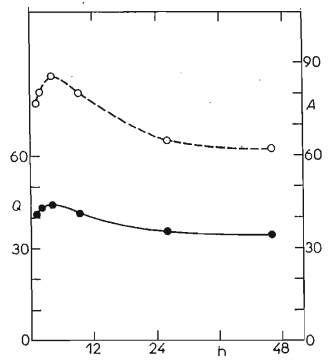

FIG. 3

Quantity of Immobilized Trypsin (Q, in mg of protein per g of dry conjugate, ●) and Its Activity (A, in μmol/min, g determined with BAPA, ○) as Function of Coupling Time

Reaction conditions: 1 ml of aldehyde-cellulose (oxidized 1 h) in 10 ml solution of trypsin (10 mg) in 0·1M borate buffer, pH 10, containing 0·005M-$CaCl_2$ and 0·001M benzamidine; stirred at room temperature.

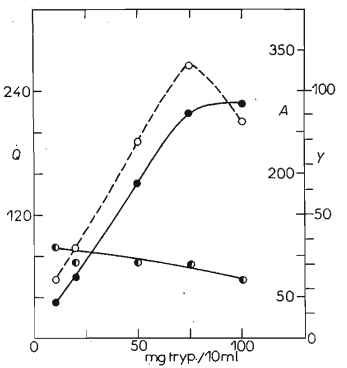

FIG. 4

Quantity of Immobilized Trypsin (Q, in mg of protein per g of dry conjugate, ●) and Its Activity (A, in μmol/min, g determined with BAPA, ○) as Function of Enzyme Content of the Reaction Mixture

The yield of the protein bound (Y, in %) was determined from mass balance and is also shown in the figure (◐). Reaction conditions: The same as given in legend to Fig. 3, coupling time 4 h.

The quantity of protein bonded to the carrier and the activity of immobilized trypsin derivatives as a function of the quantity of protein present in the reaction mixture are shown in Fig. 4. The quantity of immobilized protein levels off at high trypsin concentrations in the coupling mixture while its activity, determined with BAPA, decreases. The preparations of low trypsin content show a high relative activity (98·8% with respect to BAPA); with the increasing quantity of trypsin attached the activity decreases and the preparation containing 262·5 mg per g of dry conjugate shows a relative activity of 58·3%. Fig. 4 also shows the yields of protein bound in % determined from mass balance; they are relatively low and almost the same for all the preparations. There is practically no shift of the curve which shows the activity of immobilized trypsin, determined with BAPA, as function of pH, no matter whether preparations of the lowest or the highest activity are assayed, Fig. 5.

When the stability of trypsin attached to periodate-oxidized cellulose was assayed by continuous hydrolysis of the BAPA solution at 37°C, the activity dropped to 50% in 8 days. When the continuous hydrolysis of a solution of denatured haemoglobin examined at 37°C, this decrease was observed after 24 days. The columns of trypsin-oxidized cellulose did not loose their good flow characteristics during the whole experiment with continuous hydrolysis.

Van Leemputten and Horisberger[6] were the first to show the necessity of stabilization of the bond of trypsin to oxidized cellulose by $NaBH_4$ when these immobilized preparations are to be used for the hydrolysis of protein substrates. We have examined the loss of binding capacity of oxidized cellulose brought about by the reduction of aldehyde groups by $NaBH_4$ under the conditions described. Since the quantity

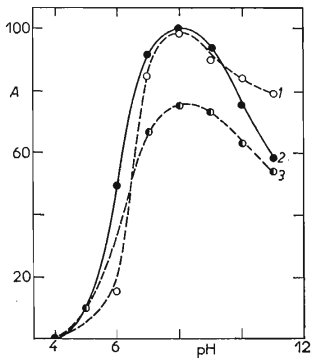

FIG. 5

Catalytical Activity *A* (with respect to BAPA) of Trypsin (———) and Trypsin Covalently Bonded to Aldehyde-Cellulose (– – –) as Function of pH

The quantity of immobllized enzyme 36 mg (○) and 218·5 mg (◐) per g of dry gel. Activity of free trypsin 1·99 μmol/min per mg at pH 8, determined with BAPA. Activity ratio of bound to free enzyme 98·8% (*1*) and 75·7% (*3*) (activity determined with BAPA).

of $NaBH_4$ required depends on the oxidation degree it is advantageous to determine the minimum quantity for each preparation; high doses of $NaBH_4$ could unfavorably affect the activity of the immobilized enzyme.

It may be concluded from our results that periodate-oxidized cellulose beads are a suitable polysaccharide carrier for the attachment of enzymes; it should be born in mind though that losses may occur because of solubilization of the preparations oxidized for longer periods, especially in solutions at high pH.

We thank Miss J. Chundelová for skilful technical assistance, Mr J. Zbrožek, Mrs V. Gelnarová, and Mrs E. Držková for the amino acid analyses.

REFERENCES

1. Peška J., Štamberg J., Hradil J., Illavský M.: J. Chromatogr. *125*, 455 (1976).
2. Baldrián J., Pleštil J., Štamberg J.: This Journal *41*, 3555 (1976).
3. Peška J., Štamberg J., Hradil J.: Angew. Makromol. Chem. *53*, 73 (1976).
4. Habeeb A.F.S.A., Hiramoto R.: Arch. Biochem. Biophys. *126*, 16 (1968).
5. Flemming C., Gabert A., Roth P.: Acta Biol. Med. Ger. *30*, 177 (1973).
6. Van Leemputten E., Horisberger M.: Biotechnol. Bioeng. *16*, 997 (1974).
7. Royer G. P., Liberatore F. A., Green G. M.: Biochem. Biophys. Res. Commun. *64*, 478 (1975).
8. Erlanger B. F., Kokowsky N., Cohen W.: Arch. Biochem Biophys. *95*, 271 (1961).
9. Turková J., Bláha K., Malaníková M., Vančurová D., Švec F., Kálal J.: Biochim. Biophys. Acta *524*, 162 (1978).
10. Spackman D., Stein W. H., Moore S.: Anal. Chem. *30*, 1190 (1958).
11. Wilson M. B., Nakane P. K.: J. Immunol. Method. *12*, 171 (1976).
12. Torchilin V. P., Tischenko E. G., Smirnov V. N., Chazov E. I.: J. Biomed. Mater. Res. *11*, 223 (1977).