*Carbohydrate Research*, 29 (1973) 497–501


## Note

# The use of cellulose carbonate for the insolubilisation of enzymes

J. F. KENNEDY AND A. ZAMIR

*Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)*

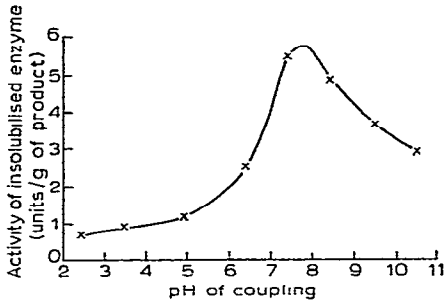
(Received December 11th, 1972; accepted for publication, January 8th, 1973)

The use and importance of enzymes in active, insolubilised forms is well established. Previously attempted attachment of β-D-glucosidase to cellulose *trans*-2,3-carbonate, *via* a nucleophilic attack of the amino groups of the enzyme on the strained carbonate ring[1], has demonstrated the potential of the cellulose derivative as a matrix for enzyme insolubilisation. Although the coupled product from β-D-glucosidase exhibited a number of the properties required for a successful insolubilised enzyme, the somewhat low activity and the difficulty in measuring protein contents were disadvantages. Ways in which the activity of the product can be improved and the dependence of the amount of enzymic protein coupled upon the coupling conditions are now reported. With a view to demonstrating a wider applicability of cellulose carbonate, the insolubilisation of a protease is also reported.

It was possible that the enzymic activity of the insoluble product could be increased by using a cellulose carbonate having a higher content of cyclic carbonate than that used previously (d.s. 0.12). This point required investigation since it was possible that, above a certain degree of substitution, overcrowding of the active sites of the enzyme could occur on attachment, as has been experienced for other matrices. We have now found it best to produce cellulose carbonates having different contents of cyclic carbonate by treating the product of highest content, prepared as described previously[2], with *N*,*N*-dimethylformamide for a controlled period. This treatment causes opening of the carbonate rings to give products having lower degrees of substitution. The use of a range of such cellulose carbonates, with the optimal[2] pH of 7.8 for the coupling, showed that the highest activities are obtained by using cellulose carbonate having the highest content of cyclic carbonate.

The use of an enzyme concentration higher than that previously employed, as might be expected, effected an increase in the activity of the insoluble product. In the interests of economy, it was also possible to decrease the overall volume of enzyme solution from that originally used (10 ml) to 2.5 ml per 200 mg of cellulose carbonate without decreasing the activity of the product. By using the higher concentration of enzyme for the coupling and a more highly substituted cellulose carbonate, an activity of 5.4 units β-D-glucosidase/g of product was achieved, *i.e.* an eight-fold improvement upon that previously reported.

By using the same concentration of enzyme in the coupling and the same cellulose carbonate, the coupling of trypsin to cellulose carbonate at various pH values showed the maximum of activity (5.4 units/g of product) for coupling at pH 7.8 (Fig. 1). This optimal pH for the coupling is identical to that for β-D-glucosidase and further demonstrates that, although higher pH values might be anticipated as optima for the nucleophilic reaction, cellulose carbonate is suitable for the insolubilisation of sensitive, biologically active molecules. As with β-D-glucosidase, the pH–activity profile of the bound trypsin was similar to that of the free enzyme (Fig. 2), although greater activity was exhibited at lower pH.



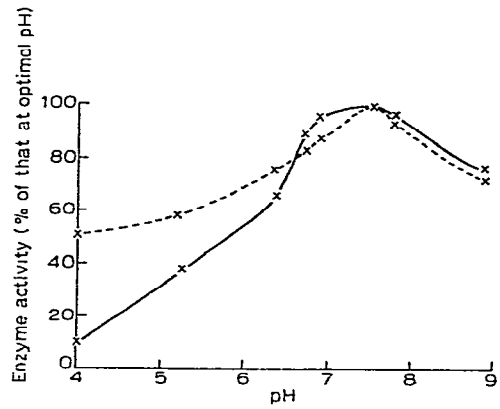




Fig. 1. Enzyme activities of trypsin insolubilised by coupling to cellulose carbonate at various pH values.

Fig. 2. pH–Activity profiles for soluble (———) and insolubilised (— — —) trypsin.

The coupling reactions were carried out at 4° to avoid any degradation of the enzyme, although relatively short coupling times are quite adequate[1]. When the effect of raised temperatures on the coupling reaction was investigated, no increase in activity could be achieved (Table I). The decrease in activity of the product (Table I) with increase in coupling temperature presumably arises from a number of effects, including degradation/denaturation of the enzyme, autodigestion (trypsin), and destruction of cyclic carbonate groups.

TABLE I

EFFECT OF TEMPERATURE ON THE COUPLING REACTION OF ENZYMES TO CELLULOSE *trans*-2,3-CARBONATE

| | *Activity of coupled enzyme product* (%) | | | | |
|---|---|---|---|---|---|
| *Temperature (degrees)*: | *4* | *10* | *14* | *22* | *37* |
| Trypsin | 100 | 98 | 96 | 91 | 52 |
| β-D-Glucosidase | 100 | — | — | 59 | 44 |

Under the conditions presently employed, sufficient enzymic protein became bound to the cellulose carbonate to enable quantitative determination without the problems experienced previously[1]. For both enzymes, the pH at which the maximal amount of protein was bound coincided with the pH at which maximal activity of the insoluble enzyme product was achieved. Thus, the maximal specific activity of the coupled enzyme protein (trypsin illustrated in Fig. 3) was also retained on coupling at this pH. This suggests that the reaction which brings about attachment of the enzyme to the matrix is of a much simpler nature and involves fewer parameters than the coupling of these two enzymes to poly(allyl cyclic carbonate)[3] where, for example, maximal specific activity of coupled enzyme does not arise from coupling at the pH which gives maximal binding of protein.

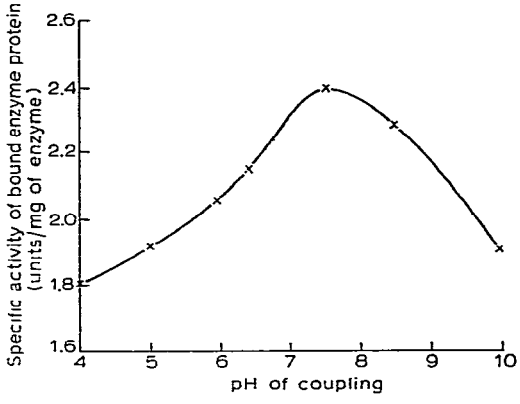



Fig. 3. Dependence of the specific activity of trypsin coupled to cellulose carbonate on the pH of coupling.

In conclusion, it is recommended that, in any approach to the use of cellulose carbonate for enzyme insolubilisation, a cellulose carbonate having a high content of cyclic carbonate is used as the matrix and that, in the coupling, a high concentration of enzyme, a pH of 7.8, and a temperature of 4° are employed.

## EXPERIMENTAL

*Cellulose carbonate.* — Cellulose *trans*-2,3-carbonate was prepared from cellulose (Sigmacell type 38) as described previously[2], using the optimal reaction conditions. Quantitative infrared spectroscopy[2] indicated that the product had a d.s. of 0.31. Cellulose carbonates of lower d.s. were obtained by treatment of batches (300 mg) of the material having d.s. 0.31 with a mixture of *N*,*N*-dimethylformamide (10 ml), water (10 ml), and triethylamine (10 ml) for 0–4 h. The samples were finally washed with water, ethanol, and ether.

*Coupling of β-D-glucosidase and trypsin to cellulose carbonate.* — To suspensions of cellulose carbonate (d.s. 0.31, 200 mg) in the appropriate buffer, solutions of β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21; *ex* sweet almonds,

Koch–Light) or trypsin (EC 3.4.4.4; *ex* bovine pancreas, twice crystallised, Koch–Light) in the same buffer (2.0 mg/ml, 2.5 ml) were added, and the mixtures were stirred for 4 h. The coupling was carried out at temperatures in the range 0–40°, and the pH of coupling was varied by using 0.1M citric acid-0.2M disodium phosphate (pH range 2.5–5.5), 0.1M sodium phosphate (pH range 5.5–8.3), and 0.05M sodium borate-0.2M boric acid (pH range 8.3–10.0) buffers. Controls for the couplings were carried out, under the same conditions, using cellulose. In the case of β-D-glucosidase, couplings were also carried out at pH 7.0, using cellulose carbonates having lower contents of cyclic carbonate.

After coupling, the solids were recovered by centrifugation and subjected to five cycles of alternate washing with 0.2M sodium acetate buffer (pH 5.0, 10 ml) and a solution of M sodium chloride and M sucrose in the same buffer (10 ml). Finally, the solids were washed twice with the acetate buffer (10 ml) and stored at 5° as suspensions in this buffer.

*Determination of β-D-glucosidase activity.* — To suspensions of the coupled-enzyme products (10 mg) in 5mM sodium acetate buffer (pH 5.0, 1.0 ml) were added solutions of *o*-nitrophenyl β-D-glucopyranoside (2.0 mg/ml, 5.0 ml) in the same buffer. The mixtures were stirred at 37° for 40 min, after which any reaction was terminated by the addition of 0.2M sodium carbonate (5.0 ml). After centrifugation, the absorbances of the supernatants at 420 nm were determined, and the amounts of *o*-nitrophenol liberated were calculated by reference to a calibration curve. One unit of β-D-glucosidase activity is defined as that amount of enzyme which, under the described conditions, releases 1 μmole of *o*-nitrophenol in 1 min.

*Determination of trypsin activity.* — The method adopted was a modification of that of Bergmeyer[4]. To suspensions of the coupled-enzyme products (10 mg) in 0.1M sodium phosphate buffer (pH 7.6, 1.0 ml), solutions of casein (Hammarsten, B.D.H.) in the same buffer (1 g/100 ml, 1 ml) were added, and the mixtures were stirred at 37° for 20 min. The reaction was then terminated by the addition of 5% aqueous trichloroacetic acid (3.0 ml) and, after a further 30 min, the mixtures were centrifuged and the absorbances of the supernatants at 280 nm determined. One unit of trypsin activity is defined as that amount of enzyme which, under the described conditions, liberates sufficient hydrolysis products (soluble in trichloroacetic acid) to increase the absorbance at 280 nm by 1.0 in 1 min.

For determination of the pH–activity profiles of the soluble and insolubilised trypsin, the above procedure was repeated by using the buffers listed under the method of coupling for dissolution/suspension of the enzyme.

*Determination of bound protein.* — Dried samples of the soluble enzymes and solid-phase preparations (10 mg) were hydrolysed with 6M hydrochloric acid (2.0 ml) for 18 h in sealed tubes in a boiling, saturated salt bath. The hydrolysates were removed from the tubes which were then washed with 8M sodium hydroxide (1.5 ml), and the pH of the combined hydrolysates and washings was adjusted to 7.0 with alkali. The total contents of amino acid in the hydrolysates were determined by an automated ninhydrin method[5].

ACKNOWLEDGMENTS

The authors thank Professors M. Stacey, C.B.E., F.R.S., and S. A. Barker for their interest in this work, and Ranks Hovis McDougal Research Ltd. for a research scholarship to A.Z.

REFERENCES

1 S. A. BARKER, S. H. DOSS, C. J. GRAY, J. F. KENNEDY, M. STACEY, AND T. YEO, *Carbohyd. Res.*, 20 (1971) 1.
2 S. A. BARKER, H. CHO TUN, S. H. DOSS, C. J. GRAY, AND J. F. KENNEDY, *Carbohyd. Res.*, 17 (1971) 471.
3 J. F. KENNEDY, S. A. BARKER, AND A. ROSEVEAR, *J. Chem. Soc. Perkin I*, (1972) 2568.
4 H. V. BERGMEYER, *Methods of Enzymic Analysis*, Verlag Chemie, Weinheim, 1965, p. 811.
5 D. H. SPACKMAN, W. H. STEIN, AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.