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Note

Active, water-insoluble derivatives of D-glucose oxidase and alginic acid, chitin, and Celite

J. F. KENNEDY AND C. E. DOYLE

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

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The insolubilisation of enzymes, with retention of activity, by attaching them to water-insoluble, polymeric matrices is now well known to provide a type of enzyme which has considerable advantages over the soluble forms, and such advantages have been listed in a previous paper[1]. Although no one matrix is ideal for enzyme insolubilisation, it is clear that covalent attachment is preferable to physical adsorption if undesirable leakage is to be avoided. As the search for suitable matrices and new methods of coupling continues, we have turned our attention to coupling to a titanium chelate of poly(*N*-acryloyl-4- and -5-aminosalicylic acids)[1], but such a matrix, although fulfilling the aims of a multipurpose reagent for the insolubilisation of enzymes[1] and antibiotics[2] and for metal-ion extraction[3], involves synthesis of the polymeric matrix. However, it has been shown[4,5] that chelation of titanium and other metals to nylon and cellulose, for example, yields a complex which can be used as an insoluble matrix for the insolubilisation of glucoamylase.

D-Glucose oxidase is used extensively, for example in hospital laboratories, in the estimation of D-glucose, but the use of the enzyme in insolubilised form would facilitate automated analyses. However, the covalent derivatives of D-glucose oxidase thus far reported all involve extensive chemical manipulation of the matrices employed. In view of the simple nature of the titanium chelation and coupling reactions, we have investigated them with a view to providing a facile means of insolubilising D-glucose oxidase in a stable form. Alginic acid, chitin, and Celite are all readily available; the polysaccharides were selected for their hydrophilic nature which would be expected to help stabilise the insolubilised enzyme on storage, whereas Celite has the advantage of being non-biodegradable.

From the results (Table I), it is clear that an active enzyme-derivative can be prepared by using titanium chelates of all three supports but that higher activities are obtained when the treated support is not dried in the presence of titanous chloride. Since the titanium ion is octahedrally co-ordinated with six water molecules, we envisage that the chelation process involves replacement of one or more of these ligands, for example with polysaccharide hydroxyl groups, the remaining water ligands subsequently being displaced by the free amino groups of the incoming enzyme. As

drying of the activated matrix proceeds, water may be driven off, inducing cross-linking chelation and thereby decreasing the ability of the titanium to chelate enzyme. Variation of the coupling time showed that maximum coupling was achieved within one hour and that longer times resulted in products of lower activity. The values for physical adsorption of the enzyme to the supports were generally low (< 16 units/g), and the chelated supports did not give any significant, non-specific contribution to the D-glucose oxidase assay.

TABLE I

ACTIVITIES OF D-GLUCOSE OXIDASE COUPLED TO TITANIUM-CHELATED ALGINIC ACID, CHITIN, AND CELITE

	Ti-alginic acid		*Ti-chitin*		*Ti-Celite*	
Weight of enzyme used in coupling (mg)	4	40	4	40	4	40
Activity of enzyme used in coupling (units)	120	1200	120	1200	120	1200
Ratio of enzyme to support (units/g)	1200	12000	1200	12000	1200	12000
Enzyme activity coupled to support (*A* type)						
(units/g dry wt.)	143	227	241	196	88	328
(units/g wet wt.)	49	79	96	81	47	175
Enzyme activity coupled to support (*B* type)						
(units/g dry wt.)	37	19	58	153	37	152

A ten-fold increase in the enzyme concentration employed in the coupling to the activated supports did not increase proportionally the activity of the product; this is presumably due either to overcrowding of the enzyme molecules or to saturation of the chelating sites on the support. The highest efficiency of coupling was achieved with chitin where, for the lower enzyme concentration, the enzyme activity as coupled enzyme represents 20% of the activity of the amount of free enzyme employed. This is a very acceptable economic factor, and supplementation of the supernatant from the coupling would permit re-use of uncoupled enzyme.

Although insoluble enzymes can be stored in the damp form at 4°, in our experience it is best to store them in the freeze-dried state if the activity is to be retained for some time. Thus far, no report has been made of the stability to freeze-drying of enzymes insolubilised by titanium chelation. Freeze-drying of the D-glucose oxidase attached to activated chitin and Celite caused extensive loss of activity (Table II), whereas the carboxyl groups of alginic acid appeared to confer some stability. However, in each case, greater activities were retained when freeze-drying was carried out in the presence of D-glucitol. Presumably, the small D-glucitol molecules are able to pack around the enzyme molecules and thereby provide a hydrophilic environment which resists denaturation of the enzyme.

In conclusion, the methods of insolubilisation and freeze-drying appear to be suitable for the easy preparation of water-insoluble D-glucose oxidase and can be expected to be applicable to other enzymes. For D-glucose oxidase, alginic acid appears to be the best of the three supports.

TABLE II

STABILITIES OF SOLID-PHASE PREPARATIONS OF D-GLUCOSE OXIDASE ON FREEZE-DRYING

	Activity (%) of D-glucose oxidase derivatives retained		
	Ti-alginic acid	*Ti-chitin*	*Ti-Celite*
Suspension freeze-dried directly	85	39	11
Suspension freeze-dried in presence of D-glucitol	100	76	88

EXPERIMENTAL

Determination of D-glucose oxidase activity. — D-Glucose oxidase activity was determined by a procedure developed from a new method of determination of D-glucose[6] which employs the ammonium salt of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as a redox indicator for the spectrophotometric determination of D-glucose with D-glucose oxidase and peroxidase. The method was adapted to enable determination of D-glucose oxidase rather than D-glucose, and the following is the modified version.

A mixture of ABTS (Boehringer Mannheim GmbH) solution in 0.1M sodium phosphate buffer (pH 7.0; 0.5 g/l, 25 ml) and aqueous D-glucose (10% w/v; 5 ml) was equilibrated at 25°. Peroxidase (E.C. 1.11.1.7, Boehringer, Grade II) solution in the same buffer (2 mg/ml, 100 μl) was added and the absorbance at 415 nm determined. Aliquots of free enzyme solutions or of solid-phase enzyme suspensions were added and, after stirring for 5 min at 25°, the reaction was arrested by rapid centrifugation. Absorbances were immediately read at 415 nm. The rate of change of absorbance (ΔE/min) was calculated, and the activity of the enzyme unknown was obtained from the equation

$$\alpha = \frac{(\Delta E/\text{min})\, a}{bc} . 10^6$$

where a = total volume of solution in the assay (in l), b = molar extinction coefficient of oxidised ABTS (approx 38.10^3 at pH 7.0), c = weight of enzyme or insolubilised enzyme (in g), and α = specific activity of enzyme or activity of insoluble enzyme preparation in units/g. One unit of D-glucose oxidase activity is that which under the defined conditions transforms 1 μmole of D-glucose in 1 min.

Activation by chelation of alginic acid, chitin, and Celite. — Alginic acid (Koch–Light), chitin, or Celite (British Drug Houses, 80–120 mesh) (5 g) were stirred for 15 min at 20° with 12.5% aqueous titanous chloride (50 ml) previously filtered to remove any oxidised reagent. The solids were filtered off, and one half of each was stored at 20° until used (*A* samples). The remainders were dried at 45–60° in an oven for 16 h, ground to a powder, and stored under desiccating conditions at 20° until used (*B* samples).

Derivatisation of chelated alginic acid, chitin, and Celite with D-*glucose oxidase.* — Duplicate samples of the activated materials (*A* and *B*, 100 mg) were washed twice with 20mM sodium phosphate buffer (pH 5.1). The samples were then stirred with a solution of D-glucose oxidase (E.C. 1.1.3.4; Boehringer, Grade II, 30 units/mg) in 0.1M sodium phosphate buffer (pH 4.5) (2 and 20 mg/ml, 2 ml) for 1 h at 4°. The solids were then washed five times by agitation with 0.1M sodium phosphate buffer (pH 5.1, 2 ml) followed by centrifugation, five times with 0.5M sodium chloride in 0.1M sodium phosphate buffer (pH 5.1, 2 ml), and finally twice with the pH 5.1 buffer (2 ml). Alginic acid, chitin, and Celite (100 mg) were also subjected to the above coupling procedure (enzyme concentration, 2 mg/ml) to provide controls for physically adsorbed enzyme. Activated materials were also subjected to the coupling procedure, but with omission of D-glucose oxidase from the buffer, to provide controls for the non-specific contribution of the materials to the ABTS assay. The solid-phase, coupled enzymes and other samples were all finally suspended in sodium phosphate buffer (pH 5.1, 2 ml), and aliquots were assayed for D-glucose oxidase activity (Table I).

Stability and storage of insoluble D-*glucose oxidase derivatives.* — Suspensions of the D-glucose oxidase derivatives (*A* samples) were (*a*) freeze-dried directly or (*b*) treated with D-glucitol (to give a concentration of 20% w/v) and then freeze-dried. The freeze-dried materials were re-suspended in the original buffer and their enzyme activities determined (Table II).

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