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Note

The insolubilisation of β-D-glucosidase by attachment to glass

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That enzymes can be insolubilised, with retention of their activity, by covalent attachment to water-insoluble matrices is now well known, and polysaccharides have been used more extensively than other materials as the matrix[1]. The derivatisation of the polysaccharide to render it suitable for covalent reaction with an enzyme frequently involves a number of modifications, but it has been shown that cellulose may be activated for enzyme coupling by a single step involving chelation of the polysaccharide with titanium[2,3]. The titanium chelate of the polysaccharide is then able to bind enzyme molecules as ligands and thereby insolubilise them, and in this way insoluble derivatives of alpha-amylase, glucoamylase, trypsin, D-glucose oxidase, invertase, and urease[2,3] have been produced. The initial activation of the cellulose involves a simple treatment with a titanium salt, and it has been found that other matrices, namely, nylon 66 and glass[2,3], and poly(*N*-acryloyl-4- and -5-aminosalicylic acids)[4], can be activated equally effectively with titanium.

The use of glass as the matrix for enzyme insolubilisation was of particular interest on account of its stability and inert nature, and its potential in titanium-chelated form for simple preparation of miniature enzyme reactors, for example, for clinical assays. However, the previous reports[2,3] were not concerned with a comparison of the amount of enzyme activity coupled to glass before and after titanium activation nor with the optimal conditions for attachment of enzyme to activated glass. We now report on the amount of β-D-glucosidase activity coupled to titanium-treated, acid-treated, and untreated glass, and on the optimal pH for coupling.

EXPERIMENTAL AND RESULTS

Materials. — Laboratory-reagent-grade glass wool was obtained from Hopkin and Williams Ltd. Pyrex-glass tubing (external diameter, 6.0 mm; wall thickness, 1 mm) and soda-glass tubing (external diameter, 6.7 mm; wall thickness, 1 mm) were fresh, unused, standard laboratory stock. Before use, these materials were thoroughly washed with water.

β-D-Glucosidase (β-D-glucoside glucohydrolase, E.C.3.2.1.21, *ex* sweet al-

monds) and *o*-nitrophenyl β-D-glucopyranoside were obtained from Koch–Light Labs. Ltd. All other materials were AnalaR grade, and glass-distilled water was used throughout.

Titanium activation of the glass solid-phases. — (*a*) *Glass wool.* The glass wool was cut into lengths of ~6 mm, and batches (1 g) were treated with one of the following reagents: 12.5% w/v titanium(III) chloride in hydrochloric acid (20 ml), 12.5% w/v titanium(IV) chloride in hydrochloric acid (20 ml), 11.5M hydrochloric acid (20 ml), or water (20 ml) with stirring for 20 min at 40°. The glass wool was then treated in one of two ways: (*i*) it was washed with water (6 × 20 ml) and then dried at 120° for 6 h, or (*ii*) the supernatant reagent was removed, and the damp solid was dried at 45° for 24 h, washed with water (6 × 20 ml), and dried at 45° for 6 h.

(*b*) *Glass tubing.* Lengths (10 cm) of Pyrex and soda-glass tubing were sealed at one end. Series of six tubes in an upright position were treated with one of the four reagents listed in (*a*) (1 ml of each), care being taken to introduce the liquid directly to the bottom of the tube. After standing at 40° for 20 min, the tubes were drained, dried at 45° for 24 h, and washed with water (3 times).

Coupling of β-D-glucosidase to the activated-glass solid-phases. — (*a*) *Glass wool–β-D-glucosidase.* Samples of activated glass wool (25 mg) were suspended in buffer (4 ml; using 0.1M sodium acetate buffers at pH 3.0, 4.0, and 5.0, and 0.1M potassium orthophosphate buffers at pH 6.0, 7.0, and 8.0. Solitions of β-D-glucosidase in 5mM sodium acetate buffer (pH 5.0; 1 mg/ml, 1 ml) were added to the suspensions, and the mixtures were stirred at 4° for 16 h. The solids were then washed with water (6 × 5 ml).

(*b*) *Glass tubing–β-D-glucosidase.* Using the solutions described in (*a*), buffer (800 μl) and β-D-glucosidase solution (200 μl) were carefully added to the activated tubes. After standing for 16 h at 4°, the tubes were emptied and thoroughly washed with water (3 times).

Enzymic assay of coupled β-D-glucosidase. — (*a*) *Glass wool–β-D-glucosidase.* Samples of the solids were suspended in 0.1M sodium acetate buffer (pH 5.0, 2 ml), and solutions of *o*-nitrophenyl β-D-glucopyranoside in 5mM acetate buffer (pH 5.0; 2.4 mg/ml, 5 ml) were added. The mixtures were incubated with stirring for 25 min at 37°, after which time aliquots (500 μl) of the supernatant solutions were added to 0.2M sodium carbonate (500 μl). The absorbances of the solutions were determined at 420 nm, and the amount of *o*-nitrophenol which had been liberated by the enzyme was calculated by reference to a calibration curve prepared by use of solutions of *o*-nitrophenol in 0.1M sodium carbonate. The activities of the glass wool–β-D-glucosidase complexes prepared from glass wool activated by the two techniques are shown in Figs. 1 and 2. One unit of β-D-glucosidase activity is defined as that which, under the standard conditions, released 1 mole of *o*-nitrophenol in 1 min.

(*b*) *Glass tubing–β-D-glucosidase.* Conditions similar to those described in (*a*) were employed. *o*-Nitrophenyl β-D-glucopyranoside solutions (1 ml) were added to the tubes and, after incubation, the reaction mixtures were treated with 0.2M sodium carbonate (1 ml). The amount of *o*-nitrophenol released by the enzyme was calculated

as before. The activities of the Pyrex and soda-glass tubing–β-D-glucosidase complexes are shown in Figs. 3 and 4, respectively.

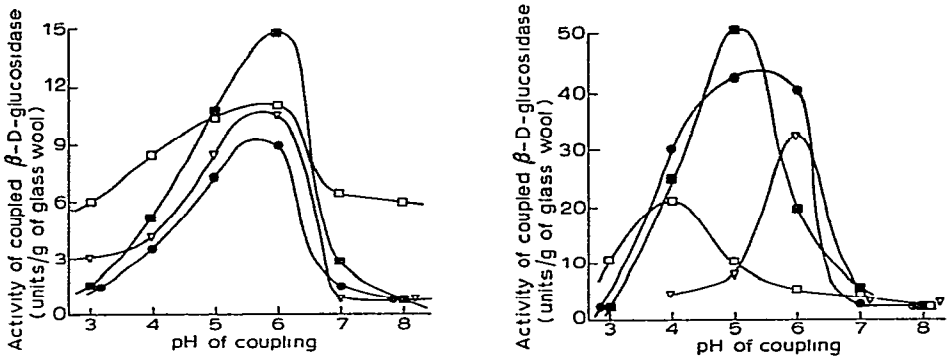

Fig. 1. Dependence of the enzyme activities of the insoluble products upon the pH of coupling of β-D-glucosidase to glass wool that had been dried at 120° after treatment with titanium(III) chloride (—■—), titanium(IV) chloride (—□—), hydrochloric acid (—●—), or water (—▽—), and after washing.

Fig. 2. Dependence of the enzyme activity of the insoluble product upon the pH of coupling of β-D-glucosidase to glass wool that had been dried at 45° after treatment as in legend to Fig. 1, but before washing.

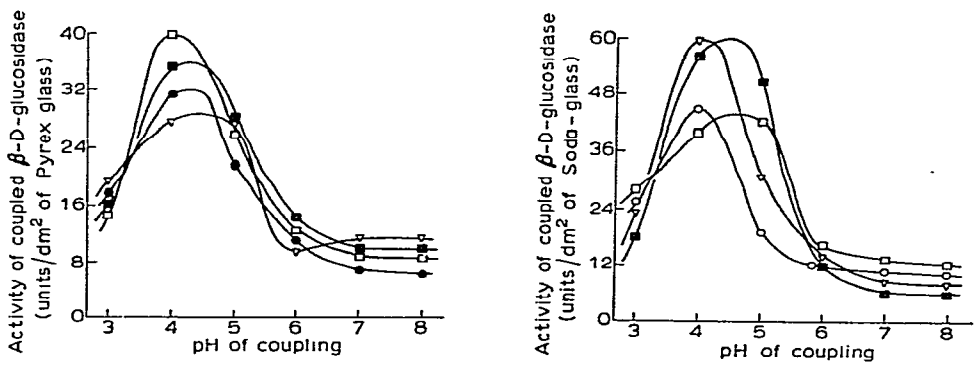

Fig. 3. Dependence of the enzyme activity of the insoluble product upon the pH of coupling of β-D-glucosidase to Pyrex-glass tubing that had been dried at 45° after treatment as in legend to Fig. 1, but before washing.

Fig. 4. Dependence of the enzyme activity of the insoluble product upon the pH of coupling of β-D-glucosidase to soda-glass tubing that had been dried at 45° after treatment as in legend to Fig. 1, but before washing.

DISCUSSION

From an examination of Figs. 1–4, it is clear that all the matrices, irrespective of the way in which they had been treated, coupled β-D-glucosidase with retention of

enzymic activity. The pH values at which maximal activities were attached, and the amounts of activities attached at those pH values, are summarised in Table I.

TABLE I

OPTIMAL pH FOR ATTACHMENT OF β-D-GLUCOSIDASE TO TREATED GLASSES, AND ACTIVITY OF THE PRODUCT FROM COUPLING AT OPTIMAL pH

Glass type	*Optimal coupling pH[a], and activity[b] coupled at that pH*			
	$TiCl_3$/HCl	*$TiCl_4$/HCl*	*HCl*	*H_2O*
Glass wool[c]	5.5–6.0[a] 14[b]	5.5–6.0 11	5.5–6.0 9	5.5–6.0 11
Glass wool[d]	5.0–5.5 51	4.0–4.5 21	5.5–6.0 44	5.5–6.0 34
Soda-glass tubing[d]	4.0–4.5 56[b]	4.5–5.0 43	3.5–4.0 45	3.7–4.2 58
Pyrex-glass tubing[d]	4.0–4.5 35	4.0–4.5 40	4.0–4.5 30	4.2–4.7 29

[a]pH in standard units. [b]Activity in units/g of glass, for glass wool, and units/dm^2 of surface, for glass tubing. [c]Activated, washed, and dried. [d]Activated, dried, and washed.

For glass wool that had been treated, but had not been dried until excess of reagent had been washed away, it will be seen (Fig. 1, Table I) that use of titanium(III) increased the activity of the coupled product (compared with that derived from glass treated with acid alone) only from 9 to 14 units/g, and that titanium(IV) gave only a marginal increase. It should be noted that titanium chlorides are supplied as solutions in hydrochloric acid, as they give the hydroxide in the absence of acid. Also of importance is the finding that glass which had been treated only with water yielded a product of activity comparable to that derived from acid-treated glass.

However, so far as activation of a matrix with titanium is concerned, the inclusion of a drying step in the presence of the titanium salt has been recommended as giving ultimately higher degrees of coupling of enzyme activity[5]. Whilst it is arguable that such a drying stage permits more extensive chelation of titanium to the matrix, and hence greater attachment of enzyme, it can be seen from Table I that, in fact, the drying in the presence of the titanium and acid reagents (before washing away of excess reagent) resulted in a 2–5-fold increase in activity of the coupled product in all cases. Thus, it appears that the increase in activity of the product effected by drying in the presence of titanium is not primarily due to increased chelation of titanium. Certainly, at the washing stage, all of the colour attributable to titanium was lost and the glass resumed its normal colour. It seems that the drying in the presence of acidic reagents induces sites, on the surface of the glass, which subsequently couple enzyme. It is, however, difficult to explain the lower activity of the product from untreated glass wool dried at 120°, compared with that dried at 40°,

other than to suggest that this observation is some function of the pre-existence of binding sites in the original glass. Generally, the optimal pH for coupling of the enzyme was 5.5–6.0.

It seemed possible that the high capacity for enzyme shown by glass wool that had not been chelated was some function of its mode of production and high ratio of surface area to volume. Thus, it was considered advisable to check if the phenomenon held for normal glass surfaces. Since higher activities had been obtained for glass wool before washing, glass tubing was tested in a similarly treated form. As will be seen from Figs. 3 and 4 and Table I, no great differences were observed in the amount of enzyme activity attached to the differently treated tubes, confirming the generality of the enzyme binding observed for glass wool. On the whole, use of soda glass resulted in a more active product and this may be attributed to the greater resistance of borosilicate glass to acid. Maximal coupling generally occurred at pH 4.0–4.5.

For each glass sample, the coupling of the enzyme occurred within a fairly narrow pH range (3–7). The limits of this range suggest that acidic groups on the surface of the glass are involved and that they associate very firmly with reactive groups of the protein chain of the enzyme. It may be that more than a simple ionic bond is involved, and certainly the firm binding of protein by porous glass has been observed[6]. The differences in the pH optima for maximal coupling of enzyme activity may reflect differences in the nature of the glasses used.

Overall, these results show that titanium chelation of glass for insolubilisation of β-D-glucosidase does not significantly increase the activity of the product, and that an enzymically active surface may be produced without deliberate pre-treatment of the glass. The phenomenon may have application in industrial processes and in the simple and rapid production of miniature reactors, for example, for routine clinical assays. The initial activities of the products obtained (up to 50 units/g of matrix) compare very favourably with those obtained for β-D-glucosidase attached to cellulose *trans*-2,3-carbonate[7,8] (5.4 units/g) and poly(allyl cyclic carbonate)[9,10] (14 units/g).

Apart from the specific consideration of insolubilisation of β-D-glucosidase, the results have some general implications in terms of the binding of proteinaceous materials to glass. Presumably, any such material may become bound to glass in a like manner, and problems of partial losses and inadvertent generation of biologically active surfaces may arise, particularly when small amounts are being handled. In this respect, whilst "chromic acid", on account of its ionic and oxidative properties, is an effective cleansing agent for laboratory glassware, it should be noted that the cleansed surface is still susceptible to adsorption of proteinaceous materials from aqueous solution.

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