β-D-GLUCOSIDASE CHEMICALLY BOUND TO MICROCRYSTALLINE CELLULOSE*†

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ABSTRACT

Almond β-D-glucosidase reacts with cellulose carbonate to give an enzymically active, insoluble, enzyme derivative. The coupling reaction is pH-dependent, the optimum being at pH ~7.8. The bound enzyme is more stable than the free form to incubation at 37°. The pH–activity profile for the bound is similar to that for the free enzyme.

INTRODUCTION

Enzymes bound to insoluble, solid matrices promise to be of considerable value both in industry and in research, because they are readily recoverable for re-use, and because they often possess greater stability than the native forms. In general, enzymes have been attached to a solid phase by the tyrosine residues (by coupling with a diazotizable, solid derivative) or the amino groups of the protein. Barker, Somers, and Epton[1,2] found that microcrystalline cellulose is a convenient support for solid-phase enzymes. Enzymes were coupled by their amino groups to cellulose in the form of its 3-(*p*-aminophenoxy)-2-hydroxypropyl ether by intermediate activation with thiophosgene. This procedure requires several steps starting from cellulose, and therefore an alternative method, requiring fewer intermediate stages, was sought for coupling enzymes to microcrystalline cellulose.

Doane *et al.*[3,4] observed that the *trans*-cyclic carbonates of D-glucose derivatives can be opened by nucleophilic attack by amines, giving the stable urethan linkage; this suggested that such carbonates prepared from cellulose[5] might react with the amino groups of enzymes, and thus afford stable, cellulose-bound enzyme preparations. This paper describes the reaction of almond β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) with cellulose carbonate to give a stable, bound enzyme.

EXPERIMENTAL

Cellulose carbonate.[5] — A suspension of microcrystalline cellulose (20 g, Sigmacell 38) in a mixture of dimethyl sulphoxide (600 ml), *p*-dioxane (peroxide-free,

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30 ml), and triethylamine (160 ml) was cooled in ice-water, ethyl chloroformate (320 ml) was added with stirring during 20 min, and the mixture was stirred for 15 min. The mixture was then made neutral with concentrated hydrochloric acid, and poured into 90% aqueous ethanol (1 litre). After thorough mixing with a top-drive macerator, the solid product was filtered off and washed with 90% aqueous ethanol (2 litres), ethanol (2 litres), and ether (1.5 litres). After air-drying, the cellulose carbonate was stored *in vacuo* over phosphorus pentaoxide. Quantitative infra-red spectroscopy, as already described[5], indicated that ~ 12.2% of the D-glucose residues in the cellulose derivative carried *trans*-cyclic 2,3-carbonate groups.

Coupling of β-D-glucosidase with cellulose carbonate. — Cellulose carbonate (200 mg) was added to a solution of β-D-glucosidase (ex. sweet almonds, Koch–Light Laboratories, Ltd., 1.0 mg) in phosphate buffer (0.05M, pH 6.10, 10 ml), and the mixture was stirred for 3 h at 0°. The solid was recovered by centrifugation and subjected to five cycles of alternate washing with acetate buffer (0.2M, pH 5.0, 10 ml) and a solution of M sodium chloride and M sucrose in the same buffer (10 ml). Finally, the solid was washed twice with the acetate buffer (10 ml), and stored at 5° as a suspension in this buffer.

A control experiment with cellulose instead of cellulose carbonate was performed in the same way.

Couplings by the same method were conducted at various pH values by use of the following buffers: phosphate (0.05M, pH 7.05), phosphate (0.05M, pH 8.10), borate (0.05M, pH 9.00), and borax (0.05M, pH 9.90). In each instance, a control experiment with cellulose instead of cellulose carbonate was carried out.

Determination of β-D-glucosidase activity. — A suspension of the enzyme–cellulose carbonate product (20 mg) in acetate buffer (0.2M, pH 5.0, 1.0 ml) was added to a solution of *o*-nitrophenyl β-D-glucopyranoside (20 mg/ml, 0.50 ml) in the same acetate buffer, and the mixture was magnetically stirred for 1 h at 37°. (The same rate of stirring was employed in all comparable experiments.) An aliquot (0.50 ml) of the reaction mixtures was then added to sodium carbonate solution (0.2M, 0.50 ml), the solid was removed by centrifugation, and the absorbance (A) of the solution at 420 nm was determined. The liberation of *o*-nitrophenol was calculated by reference to a calibration graph prepared by use of solutions of *o*-nitrophenol in 0.1M aqueous sodium carbonate. Products from the control experiments were assayed in the same way. It was established that, if aliquots of the enzyme–cellulose carbonate suspension were taken, they gave reproducible results.

The activities of the cellulose carbonate–β-D-glucosidase complexes, prepared at different pH values under the standard conditions described, are shown in Fig. 1, together with the activities of samples of cellulose treated with β-D-glucosidase at various pH values under the same conditions.

Stability of cellulose carbonate with respect to pH. — Cellulose carbonate (200 mg) was added to citrate buffer (0.05M, pH 4.0, 10 ml), and the mixture was stirred for 30 min at 0°. The solid was then recovered by centrifugation, and was washed successively with water (2 × 10 ml), ethanol (2 × 10 ml), and ether (10 ml),

air-dried, and kept *in vacuo* over phosphorus pentaoxide. Similar portions of cellulose carbonate were incubated at various pH values in the same way.

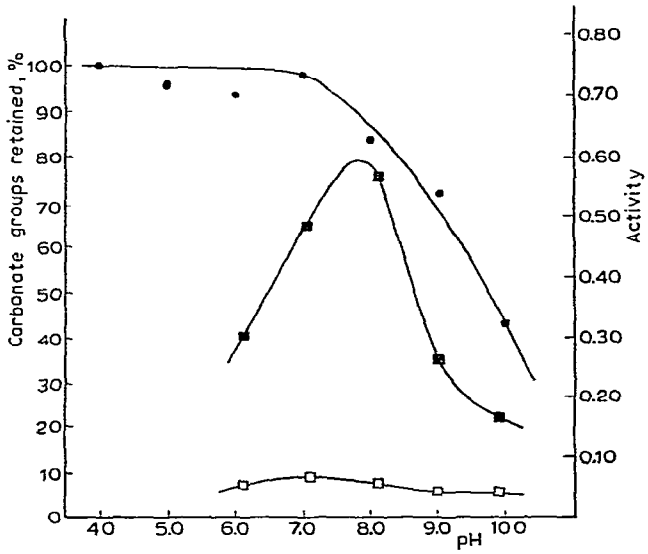

Fig. 1. Activity of solid-phase enzyme prepared from cellulose carbonate and β-D-glucosidase at various pH values. (The activity of the preparation is expressed in μmole of *o*-nitrophenol liberated/ml per h by 10 mg of complex at 37°. The fraction of carbonate groups retained is in %, relative to the sample from pH 4.0. The pH is that at which the complex was prepared and the cellulose carbonate was incubated. ■, β-D-Glucosidase activity of product from reaction of β-D-glucosidase with cellulose carbonate; □, β-D-glucosidase activity of controls obtained by incubation of cellulose with β-glucosidase; ●, content of *trans*-cyclic carbonate groups in cellulose carbonate after incubation for 30 min at 0° at the pH values indicated, as determined by absorption at 1810 cm^{-1}.)

About 5 mg of each well-dried sample was accurately weighed, mixed with potassium bromide (300 mg), and pressed into a pellet for i.r. examination. The content of cyclic carbonate in each preparation after incubation was determined by reference to the absorption at 1810 cm^{-1}. Fig. 1 shows the *trans*-cyclic carbonate content of preparations of cellulose carbonate after incubation at various pH values, and demonstrates the instability of the *trans*-cyclic carbonate group at the higher pH values employed.

pH–Activity profiles for bound and free β-D-glucosidase. — β-D-Glucosidase coupled to cellulose carbonate at pH 8.0 was used for this experiment. The solid-phase enzyme (10 mg) was washed with the chosen buffer (2 × 10 ml), and was then suspended in the buffer (0.50 ml). A solution of *o*-nitrophenyl β-D-glucopyranoside (2 mg/ml, 0.50 ml) in water was then added, and the mixture was stirred for 30 min at 37°. An aliquot (0.50 ml) was added to sodium carbonate solution (0.2M, 0.50 ml), and A_{420} was determined. The buffer solutions used were: citrate–phosphate, pH 2.5; citrate, pH 3.0, 3.5, 4.0, 4.5, 5.5, 6.0; phosphate, pH 6.5, 7.0, 7.5, 8.0, and 8.5. Each had an initial concentration of 0.1M (*i.e.*, the final concentration was 0.05M).

A solution of free β-D-glucosidase (2.5 μg/ml, in the appropriate buffer, 0.50 ml)

was added to a solution of *o*-nitrophenyl β-D-glucopyranoside (2.0 mg/ml, 0.50 ml) in water, and the mixture was incubated, and assayed for release of *o*-nitrophenol as just described. The same buffer solutions were used for the pH–activity profiles of both the free and the bound enzymes. It was established that in no case did the buffer solution influence the colorimetric determination of the release of *o*-nitrophenol. Fig. 2 shows the pH–activity profiles of the free and coupled enzymes.

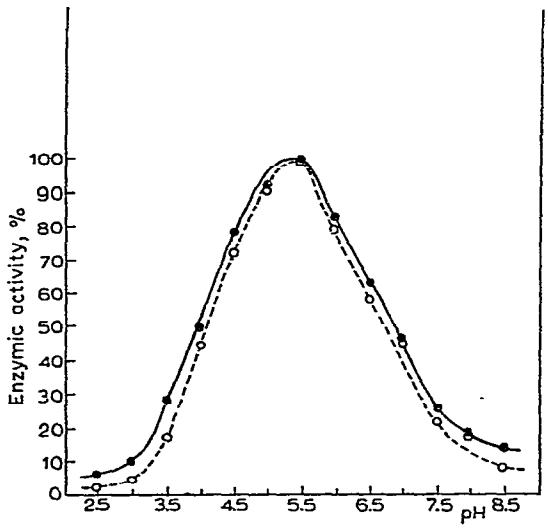

Fig. 2. pH-Activity profiles for β-D-glucosidase. (The enzymic activity is given in % of the maximum at pH 5.5. ●, free enzyme; ○, enzyme attached to cellulose carbonate.)

Stability of bound β-D-glucosidase at 37°. — A sample of the bound β-D-glucosidase preparation (200 mg, prepared at pH 8.0) in acetate buffer (0.2M, pH 5.0, 10 ml) containing one drop of toluene was incubated at 37°, with shaking. Aliquots (1.0 ml, 20 mg of solid) were removed at suitable time-intervals, and the activity of the solid was assayed as already described.

Free β-D-glucosidase (4 μg/ml) in acetate buffer (0.2M, pH 5.0) was incubated in the same way, and aliquots (1.0 ml) were assayed at intervals.

Fig. 3 shows the effect of prolonged incubation at 37° on free β-D-glucosidase and on β-D-glucosidase coupled to cellulose carbonate. After 4 days (not shown in Fig. 3), the coupled enzyme had retained 38% of its original activity, whereas the activity of the free enzyme was 0.4% of the original value.

Attempted determination of protein bound to cellulose. — Attempts were made to determine the protein bound to cellulose by acid hydrolysis and ninhydrin assay according to the method described by Barker *et al.*[2]. The analytical system of a Technicon Autoanalyser[6] was used for the measurement of ninhydrin-produced colour. However, blank values obtained from cellulose alone were so high that satisfactory protein analyses could not be made. The results obtained indicated that

in no case did any product contain more than 200 μg of protein per 100 mg of cellulose.

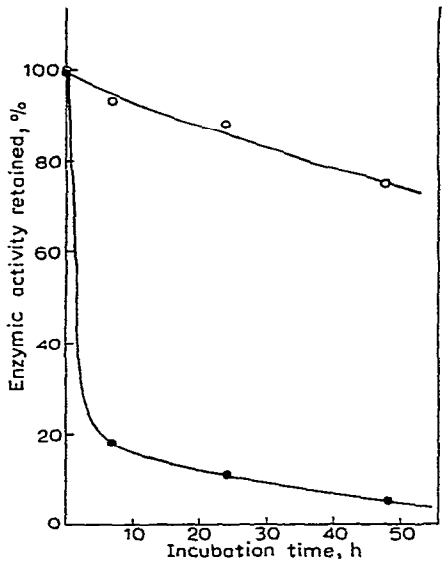

Fig. 3. Loss of activity of β-D-glucosidase on incubation at 37° at pH 5.0. (The enzymic activity retained is given as % of the original activity. ●, free enzyme; ○, enzyme attached to cellulose carbonate.)

DISCUSSION

It is clear that β-D-glucosidase becomes coupled in an active form to the cellulose derivative. That the coupling between the enzyme and the cellulose derivative involves a covalent link rather than hydrogen bonds or physical adsorption is indicated by (*a*) the retention of activity by the products even after application of thorough washing procedures, and (*b*) the results obtained when cellulose itself was incubated with the enzyme (see Fig. 1). These very low activities obtained with cellulose alone probably represent residual, physically adsorbed enzyme.

The chemical reaction between the enzyme and the cellulose carbonate is obviously pH-dependent, maximum combination occurring at pH ~7.8. The relation between the specific activity of the product and the pH at which it was prepared can reasonably be explained in the following way. Below pH 7.8, the activity of the product decreases with decreasing pH. The lower half of the pH–activity profile (Fig. 1, solid squares) probably reflects the degree of protonation of the amino groups, on the surface of the protein, that attack the carbonate groups to form urethan links. At lower pH values, these groups will be protonated to a considerable extent and, therefore, not effective as nucleophiles. As the pH is increased, the proportion of these groups present in the unprotonated form also increases.

An explanation for the upper portion of the curve (pH >7.8) became apparent when the stability of cellulose carbonate at various pH values was examined. As shown in Fig. 1, the carbonate appears to be relatively stable at pH values between 7 and 4, in that the carbonate content is virtually unchanged. At higher pH values, however, the carbonate content decreases as the pH of the incubation medium is increased. Clearly, the higher the pH, the greater the rate of hydrolysis of the cyclic carbonate groups. At higher pH values, therefore, hydrolysis of the carbonate groups (by attack either by water or by hydroxide ions) competes increasingly with attack by the nucleophilic groups on the protein molecule.

On this basis, it seems probable that the activity of the cellulose carbonate–enzyme complexes prepared at different pH values reflects the proportion of enzyme bound. An alternative explanation for the upper (pH >7.8) portion of the curve might be that, at higher pH values, the enzyme becomes progressively inactivated. However, we have shown that, when incubated at 4° and pH 10.0 for 3 h, this β-D-glucosidase does not lose activity relative to a sample incubated at pH 5.35.

Support for these explanations could be obtained from accurate determinations of the protein bound to the cellulose. The ninhydrin assay as described did not give results sufficiently accurate for this purpose.

The lack of a suitable method for determination of cellulose-bound protein in this complex has meant that no estimate has been made of the activity per mg of protein of β-D-glucosidase bound to the solid phase, as compared with that of the native enzyme. However, this lack did not preclude a study of the relative stabilities of the free and bound enzymes on incubation at 37°. The results (see Fig. 3) showed clearly that the β-D-glucosidase attached to cellulose carbonate is considerably more stable than it is in the free state. Indeed, even after 4 days, the bound enzyme still retained 38%, whereas the free enzyme possessed only 0.4%, of its original activity. This degree of stabilization may be due to the lower likelihood of spontaneous denaturation when the enzyme is attached to a rigid solid phase, or it may result from decreased accessibility of the coupled enzyme to inactivating enzymes present as contaminants in the enzyme preparation.

From the foregoing discussion, it is apparent that the groups on the protein that are involved in the enzyme–solid phase linkage are capable of existing in protonated or unprotonated forms according to the pH. Reaction of these groups with the cyclic carbonate (especially if they are near the active site) might be expected to influence the catalytic properties of the enzyme, including variation of catalytic activity with pH. As may be seen in Fig. 2, the pH–activity profiles of bound and free β-D-glucosidase are virtually identical, showing that the coupling process has very little influence on this aspect of the enzyme's activity. This conclusion is in contrast with the results obtained by Goldstein *et al.*[7], who found that the pH–activity profile of trypsin is displaced considerably on attachment to a polyanionic solid phase[8]. It is interesting that the optimum in the curves at pH 5.5 is significantly higher than that reported by Hofstee[9] for almond β-D-glucosidase with a different substrate (salicylyl β-D-glucopyranoside).

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