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## Note

# The preparation and properties of amyloglucosidase chemically attached to polystyrene beads

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Amyloglucosidase [EC 3.2.1.2, α-(1→4)-glucan glucohydrolase], an enzyme of considerable industrial interest, has previously been immobilised by chemical coupling to DEAE-cellulose[1] and to a diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose[2], and physically coupled to DEAE-cellulose[3]. Previous work[4] with alpha-amylase (an endo-amylase) chemically coupled to polystyrene revealed interesting effects of the immobilisation on the action pattern of the enzyme. We now report on the chemical coupling of amyloglucosidase (an exo-amylolytic enzyme) to polystyrene beads.

## EXPERIMENTAL AND RESULTS

The mold *Rhizopus delemar* (CMC 44,245) was grown in submerged culture in a 14L New Brunswick Fermentor for 3 days in 12-l batches at 30°, with aeration at 10 l/min and agitation at 400 revs/min. The culture medium was 2% malt extract and 1% yeast extract (Oxoid Ltd) at pH 5.4. After 3 days, the culture was filtered through a cheese cloth and decreased in volume to (1.5 l) by rotary evaporation at 40°/ ~15 mmHg. The cooled (4°) concentrate was added, with stirring, to 1 vol. of acetone at −15°. The protein precipitate was collected by centrifugation, and a solution in 50mM sodium acetate buffer (pH 5) was exhaustively dialysed against the same buffer. To remove alpha-amylase activity, the protein was chromatographed[5] on Sephadex G-50 and then on Amberlite IRC-50 resin. The amyloglucosidase fraction, so isolated, was shown to be free of alpha-amylase by the oxidised amylose method[6]. The enzyme solution was then lyophilised. For coupling experiments, except where otherwise stated, the enzyme was dissolved in 100mM borate buffer (pH 8.4).

Polystyrene beads (~1.5 mm diameter; a gift from the Shell Chemical Co., London) were nitrated by rapid stirring in a mixture of 45% of conc. nitric acid (sp. gr. 1.42) and 55% of sulphuric acid (sp. gr. 1.84) for 20 min at 0°, followed by washing with distilled water to remove traces of acid. The resultant polynitrostyrene

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beads were treated with 6% sodium dithionite in 2M sodium hydroxide for 5 h at 90° to yield polyaminostyrene beads, which were washed with water, M hydrochloric acid, and water, and then dried *in vacuo* over silica gel.

Polyaminostyrene beads (500 mg) were stirred with 0.6M hydrochloric acid containing 0.2% of sodium nitrite for 20 min at 0°. The diazotised beads were collected, washed with distilled water, and immediately added to a stirred solution of the enzyme (protein concentration, 0.3–7.5 mg/ml) at 4°. After slow stirring for 12 h, the enzyme-coupled beads were collected, washed with distilled water (2 l), and then washed exhaustively with M sodium chloride to remove physically attached protein. The enzyme beads were stored as a suspension in water at 4°.

Chemically attached protein was assayed by acid hydrolysis, followed by determination[7] of the amino acids in the neutralised hydrolysates. Results were calculated by using a standard plot obtained with amyloglucosidase.

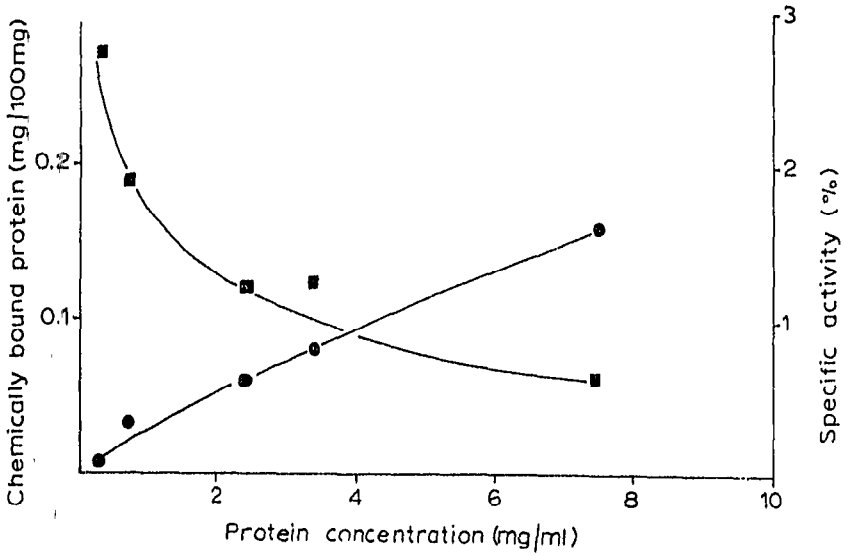
Amyloglucosidase activity was determined by measuring the rate of release of D-glucose from a 1% solution of soluble starch in 100mM acetate buffer (pH 5). D-Glucose concentrations were measured by the dinitrosalicylate method[8]. For measurements of polystyrene–amyloglucosidase activity, a constantly stirred reactor was used.

The effect of varying the protein concentration on the amount of protein chemically bound and the resultant specific activity is shown in Fig. 1. Likewise, the effect of the pH of the coupling mixture is shown in Fig. 2.

The Michaelis constant ($K_m$) of the polystyrene–amyloglucosidase was determined at 37° and pH 5, with soluble starch as the substrate, using a Lineweaver–Burk plot of inverse initial velocities against inverse substrate concentrations. An average value of 0.6% was obtained. Values of 0.05% were obtained for the soluble amyloglucosidase.

The action pattern of amylolytic enzymes[4] can be investigated by plotting the blue value (iodine-staining power) of a reaction mixture against the reducing power liberated at intervals during the course of amylolysis. Such measurements were carried out for both polystyrene–amyloglucosidase and soluble amyloglucosidase. Blue values were measured at 610 nm on aliquots (0.2 ml), after adding 0.1M iodine–potassium iodide in 0.1M hydrochloric acid (2 ml) and diluting to 50 ml with distilled water. Values were expressed as percentages of the initial value. Reducing sugar measurements were carried out on aliquots, as described previously for D-glucose, and expressed as percentages of the maximal values obtainable after exhaustive hydrolysis. Fig. 3 shows the patterns obtained for the immobilised enzyme and the free enzyme.

Polystyrene–amyloglucosidase beads packed in a column (5 × 1 cm) were used to investigate the relationship between the rate of flow of substrate through the column and the percentage conversion into products. Experiments were carried out at 3 concentrations (0.05, 0.1, and 0.5%) of soluble starch. The results are shown in Fig. 4. The action pattern effect was also investigated using the packed bed of insoluble enzyme. No difference was observed between the packed bed experiments and the stirred reactor experiments.



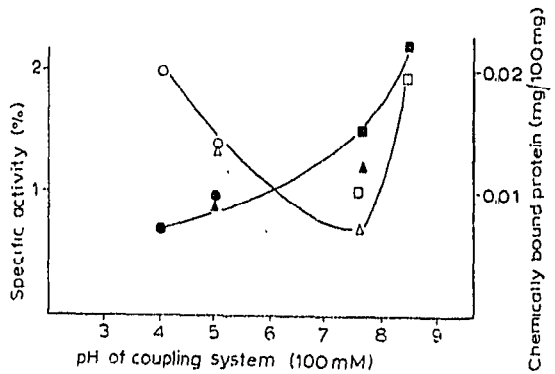




Fig. 1. The effect of protein concentration on the amount of chemically bound protein (●) and the resulting specific activity (■). Coupling was carried out in 100mM borate buffer (pH 8.4).

Fig. 2. Effect of the pH of the coupling system on the amount of protein bound (full symbols), and on the resulting specific activity (open symbols). Concentration of protein, 0.5 mg/ml. Buffer systems: acetate (●, ○), borate (■, □), and phosphate (▲, △).

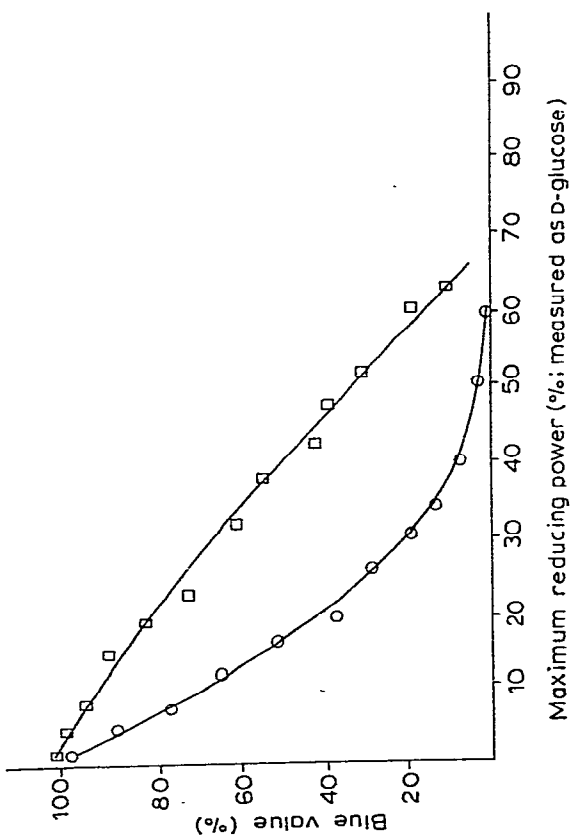



Fig. 3. Action-pattern curves for polystyrene–amyloglucosidase (□) and for soluble amyloglucosidase (○).

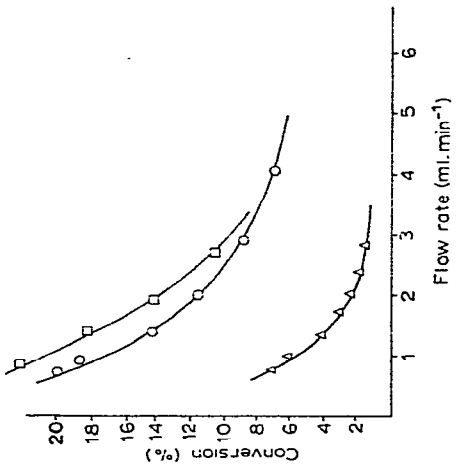



Fig. 4. Relationship between flow rate and percent conversion into products, using a packed bed of polystyrene–amyloglucosidase. Soluble starch was used as substrate: □, 0.05%; ○, 0.1%; △, 0.5%.

## DISCUSSION

Enzymically active preparations of polystyrene–amyloglucosidase have been prepared with a maximal specific activity of 2.37% of that of the free enzyme. This specific activity is very low in comparison with the values (16–55%) reported for amyloglucosidase physically bound to DEAE-cellulose[1], and those (16–25%) for the enzyme coupled to cellulose[2]. It is of interest that Barker *et al.*[2] noted a greater specific activity for exo-amylolytic enzymes [beta-amylase and gamma-amylase (amyloglucosidase)] coupled to cellulose than for a coupled endo-amylolytic enzyme (alpha-amylase). These authors reported values of 16–25% for the activity of the exo-amylolytic enzymes and only 4–5.3% for the endo-amylolytic enzyme (alpha-amylase). One of us[4] has previously reported a specific activity of ~8% for polystyrene–alpha-amylase as compared with the free enzyme. For amylolytic enzymes coupled to polystyrene, it appears, therefore, that exo-amylolytic activity retained on coupling is not greater than endo-amylolytic activity.

The decrease in specific activity, which was apparent with increase in content of bound protein in preparations of the polystyrene–amyloglucosidase beads, is probably explained by overcrowding of the enzyme molecules on the surface of the beads. One of the particular disadvantages of the solid bead is the relatively low ratio for surface area to weight and the consequent low loading of protein that can be achieved with retention of reasonable activity.

The pH of the coupling mixture appears to exert effects on both the amount of protein chemically bound and the resultant specific activity. At pH 4, there is <50% of the binding that occurs at pH 8.4, presumably because of unfavourable ionization of the enzyme protein, whereas the specific activities are similar and maximal at each pH. It appears, therefore, that coupling is most efficient at pH 8.4.

Polystyrene–amyloglucosidase showed a $K_m$ with respect to starch of 0.6%, this value being an ~10-fold increase in $K_m$ compared with the soluble enzyme. Filippusson and Hornby[10] demonstrated a two-fold increase in the $K_m$ of yeast $\beta$-D-fructofuranosidase with respect to sucrose following coupling to polystyrene. The much greater increase reported here may be related to the macromolecular nature of the substrate.

The greater degree of multiple action (greater production of reducing power for a given fall in blue value) exhibited by polystyrene–amyloglucosidase, compared with the free enzyme, can be attributed to the same steric effects caused by the macromolecular support material as were considered responsible for the change in action pattern observed with polystyrene alpha-amylase[4]. For alpha-amylase, coupling to polysyrene resulted in an increase in D-glucose liberation, relative to other products, during $\alpha$-amylolysis of starch. As D-glucose is considered to be the sole hydrolytic product of amyloglucosidase action on starch, such an effect would not be possible. The most probable explanation is an increased number of catalytic events (each of which liberated a D-glucose molecule) during the lifetime of an enzyme–substrate complex.

The packed column experiments demonstrate that a polystyrene–amyloglucosidase column can be used for the hydrolysis of starch on a continuous basis. The nature of the polystyrene beads allows rapid, uniform flow of substrate; however, the low specific activity of the preparation would probably deprive the method of commercial value.

ACKNOWLEDGMENTS

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