The Preparation and Properties of Yeast β -Fructofuranosidase Chemically Attached to Polystyrene

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1. A process is described for the chemical attachment of an enzyme to the surface of a polystyrene matrix. 2. By this process yeast β -fructofuranosidase was chemically attached to the surface of both polystyrene beads and polystyrene tubes. 3. The kinetics of sucrose hydrolysis by β -fructofuranosidase and polystyrene-supported β -fructofuranosidase were compared. 4. The pH-activity curve of the polystyrene-supported enzyme shows a marked difference from that of the free enzyme in solution. 5. The inhibitor dissociation constant with respect to tris is increased, whereas the inhibitor dissociation constant with respect to aniline is decreased when the enzyme is attached to polystyrene. 6. Differences between the properties of the bound and free enzyme are discussed in terms of a micro-environmental effect arising from the hydrophobic nature of the polystyrene support.

A wide variety of methods have been described for the attachment of enzymes to supporting structures (Silman & Katchalski, 1966). For many of the resulting products it has been shown that the supporting material can have a modifying effect on the kinetics of the attached enzyme (Goldstein, Levin & Katchalski, 1964; Hornby, Lilly & Crook, 1968). Effects such as this have been explained in terms of the supporting matrix generating a micro-environment about the bound enzyme that is dictated by the physical and chemical nature of the support. The present work describes the effect of attachment to polystyrene on the activity of an enzyme. In this case differences in the activity of the free enzyme and the supported enzyme may be correlated with the generation of a micro-environment by the hydrophobic polystyrene support.

So far enzymes have been insolubilized by their attachment to either beads or fibres, and in this form used in either packed beds or stirred tank reactors (Lilly & Sharp, 1968), or else they have been insolubilized by their attachment to porous sheets (Sharp, Kay & Lilly, 1969). The present work describes the attachment of an enzyme to a polystyrene structure. Although the attachment of enzymes to polystyrene has been previously reported (Grubhofer & Schleith, 1954), the conditions used for the preparation of the support have always incurred a deterioration of the shape of the initial polymeric structure. The process described here is a modification of existing methods such that the integrity of the gross polystyrene structure is at all times preserved. Thus it has been possible to

attach an enzyme covalently to the inner surface of a tube and to use the enzyme in this form in a continuous flow process.

MATERIALS AND METHODS

Enzymes. β -Fructofuranosidase from baker's yeast (grade VI, Sigma Chemical Co., St Louis, Mo., U.S.A.) was used without further purification in all experiments.

Preparation of polynitrostyrene beads. Polystyrene beads (a gift from the Shell Chemical Co. Ltd., London S.E.1, U.K.) were suspended in 47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84) at 0°C and vigorously stirred for 20 min. The nitration was then stopped by pouring the reaction mixture into a large volume of water, and the product was thoroughly washed with water.

Preparation of polyaminostyrene beads. Polynitrostyrene beads were suspended with stirring in 6% (w/v) $\text{Na}_2\text{S}_2\text{O}_4$ –2M-KOH for at least 2 h at 70°C. The product was then thoroughly washed in dil. HCl and finally in water.

Preparation of polyaminostyrene tubes. Polystyrene tubes (2 mm internal diameter) were a gift from B.P. Chemicals (U.K.) Ltd., London W.1, U.K. The conditions used for the preparation of the surface-aminated tubes were essentially the same as those used for the preparation of the polyaminostyrene beads. Nitration was affected by perfusion of the tubes at 0°C for 20 min in 47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84). Reduction was then brought about by perfusion with 6% (w/v) $\text{Na}_2\text{S}_2\text{O}_4$ –2M-KOH for 4 h at 70°C. The tubes were finally washed in dil. HCl and then in water.

Attachment of β -fructofuranosidase to polyaminostyrene beads. Polyaminostyrene beads were first diazotized by suspending 3 g of them, with rapid stirring, in 300 ml of 0.6M-HCl at 0°C. Samples (10 × 2 ml) of ice-cold 20%

(w/v) NaNO_2 in water were added over a period of 14 min. The resulting polystyrene-diazonium chloride was filtered on a sintered glass funnel and washed quickly with mm-HCl , and immediately thereafter transferred to a solution containing 560 mg of β -fructofuranosidase in 58 ml of 50 $\text{mm-Na}_4\text{P}_2\text{O}_7$ adjusted to pH 8.0 at 0°C with 2 M-HCl . The suspension was stirred at 0°C for 2 h and then at 4°C for a further 2 h, after which time the product was recovered by filtration and washed with water, 0.5 M-NaCl , mm-HCl and water (1 litre of each). The preparation was stored as a suspension in water at 4°C .

Attachment of β -fructofuranosidase to polyaminostyrene tubes. The interior of a 2 m length of polyaminostyrene tubing, in the form of a coil, was washed through with 0.6 M-HCl and then diazotized by perfusion with 1.5% (w/v) NaNO_2 –0.6 M-HCl at 0°C for 20 min at a flow rate of 3.3 ml/min, after which time the tube was washed through with 0.6 M-HCl followed by mm-HCl . Then the tube was immediately filled with a solution containing 200 mg of β -fructofuranosidase in 20 ml of 50 $\text{mm-Na}_4\text{P}_2\text{O}_7$ adjusted to pH 8.0 with 2.0 M-HCl , and the solution was re-circulated through the tube at a flow rate of 3.3 ml/min for 2 h at 0°C . The tube, still containing the enzyme, was then sealed at both ends and stored at 4°C for a further 3 h. Finally, the tube was washed thoroughly with water, 0.5 M-NaCl , mm-HCl and water (1 litre of each). The tube was stored in water at 4°C .

Determination of enzymic activity. The activity of both free β -fructofuranosidase and polystyrene-supported β -fructofuranosidase was measured by following the rate of hydrolysis of sucrose by the appearance of reducing sugar. Reducing sugar was determined either by the dinitrosalicylic acid method (Bruner, 1964) or by the alkaline ferri-cyanide method (Linker, 1966). Unless otherwise stated the activity of free β -fructofuranosidase was determined at 25°C and pH 5.0 in acetate buffer (10.1) (Datta & Grzybowski, 1961). All assays were started by the addition of the enzyme (final assay volume 2.0 ml) and stopped by the addition of 2.0 ml of the alkaline reagent used for the determination of the reducing sugar.

The polystyrene-bead-supported β -fructofuranosidase was assayed at 25°C and pH 5.0 in acetate buffer (10.1) (Datta & Grzybowski, 1961) in a total assay volume of 30 ml unless otherwise stated. All assays were performed in a continuously stirred reaction vessel, the rate of stirring being kept constant throughout all experiments.

The polystyrene-tube-supported β -fructofuranosidase was used throughout in the form of a coil. Assays were carried out by perfusion of the coil with the substrate solution at a predetermined flow rate followed by the determination of the reducing sugar in the effluent. Unless otherwise stated all assays were carried out at 25°C and pH 5.0 in acetate buffer (10.1) (Datta & Grzybowski, 1961). Both the substrate solution and the coil were held throughout in a constant-temperature bath and in all experiments care was taken to ensure complete equilibrium of the system before assays for reducing sugar in the effluent were undertaken.

The pH-activity curves of both free and polystyrene-bound β -fructofuranosidase were compiled from results obtained at 25°C as described above. In each case, however, a citrate-phosphate-borate buffer was used in place of the acetate buffer. For this, an equimolar mixture of NaH_2PO_4 , citric acid and H_3BO_3 was titrated to the approximate pH desired with either 2 M-HCl or 2 M-NaOH , the buffer solutions were then diluted so that the final concentration of all buffer ions was in each case 20 mM and finally the pH of the diluted mixtures was noted.

Determination of chemically attached protein. The protein content of the preparations was determined by hydrolysis in 6 M-HCl for 42 h at 120°C , followed by determination of the amino acids in the neutralized hydrolysates by the method of Cocking & Yemm (1954). A standard curve was obtained by treating known amounts of β -fructofuranosidase in a similar manner.

RESULTS

Table 1 shows the effect of varying the acid composition, temperature and reaction time of the initial nitration process on the degree of nitration of

Table 1. *Effect of acid composition, reaction time and temperature on the nitration of polystyrene*

Expt. no.	Composition of nitration mixture	Temperature ($^\circ\text{C}$)	Time (min)	% nitration*	Comments
1	47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	–10	20	0.25	Insufficient substitution
2	47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	0	20	2.8	
3	47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	10	20	5.8	Partial solubilization
4	47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	0	10	1.0	Insufficient substitution
5	47% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	0	60	6.8	Partial solubilization
6	30% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	0	20	1.5	Insufficient substitution
7	60% (v/v) HNO_3 (sp.gr. 1.42)– H_2SO_4 (sp.gr. 1.84)	0	20	1.25	Insufficient substitution
8	Fuming HNO_3 (sp.gr. 1.50)	0	20	14.5	Solubilization

* Calculated as $(100 \times \text{no. of nitro groups})/(\text{no. of styrene monomers})$.

the polystyrene. The results show that both prolonged nitration at 0°C and nitration at temperatures higher than 0°C increase the degree of nitration but simultaneously result in a deterioration in the polystyrene in that the structure undergoes at least partial solubilization. At temperatures lower than 0°C and nitration times of less than 20 min there was no solubilization of the polystyrene but the degree of nitration was correspondingly lower. The conditions described for Expt. 2 produced an adequate degree of substitution without solubilization of the polystyrene and were therefore used in all subsequent experiments.

The protein in all polystyrene-supported preparations of β -fructofuranosidase is believed to be covalently attached to the support, since prolonged subsection of the preparations to the washing procedures prescribed above does not remove the enzymic activity. The protein content of all preparations of β -fructofuranosidase attached to polystyrene beads was within the range 0.5–1.0% (w/w). Polystyrene-bead-supported β -fructofuranosidase was always assayed in a continuously stirred reaction vessel. Small deviations from the stirring rate that was used in all experiments did not have any significant effect on the rate of sucrose hydrolysis by the insoluble enzyme. In all experiments with polystyrene-tube-supported β -fructofuranosidase the extent of sucrose hydrolysis never corresponded to more than 10% of the original substrate solution. For rates of substrate perfusion through the enzyme coil, within the range 0.5–5.0 ml/min, the percentage hydrolysis of the sucrose was always inversely proportional to the flow rate.

The effect of substrate concentration on the hydrolysis of sucrose by free β -fructofuranosidase and polystyrene-bound β -fructofuranosidase was investigated. The results of these experiments, presented in Fig. 1 by the method of Lineweaver & Burk (1934), show that the polystyrene-supported β -fructofuranosidase preparations obey Michaelis-Menten kinetics. The values for the Michaelis parameters of the free and bound enzyme, as calculated from the results shown in Fig. 1, are shown in Table 2. These results show that the K_m with respect to sucrose is increased over twofold when the enzyme is attached to either polystyrene beads and assayed in a continuous stirred reactor, or when the enzyme is attached to a polystyrene tube and assayed at a flow rate of 1.18 ml/min. Table 2 also shows that the value for V_{max} of the enzyme is decreased ninefold when the enzyme is attached to polystyrene beads.

The effect of pH on the rate of hydrolysis of sucrose by β -fructofuranosidase attached to polystyrene beads and by free β -fructofuranosidase, both in the presence and absence of 40% (v/v)

Fig. 1. Effect of substrate concentration on the hydrolysis of sucrose by β -fructofuranosidase in free solution and chemically attached to polystyrene. Δ , β -Fructofuranosidase in free solution; v is expressed as μmol of sucrose hydrolysed/min per μg of enzyme. $\bullet$, Polystyrene-tube-supported β -fructofuranosidase; v is expressed as $5 \times 10^{-2} \times \mu\text{mol}$ of sucrose hydrolysed/ml of effluent per m of tube at a flow rate of 1.18 ml/min. $\blacksquare$, Polystyrene-bead-supported β -fructofuranosidase; v is expressed as μmol of sucrose hydrolysed/min per μg of bound enzyme.

Table 2. Values of K_m and V_{max} for the hydrolysis of sucrose by β -fructofuranosidase in free solution or chemically attached to polystyrene

Preparation	K_m (mM)	V_{max}
β -Fructofuranosidase	17–20	0.016*
Polystyrene-bead-supported β -fructofuranosidase	40	0.0018*
Polystyrene-tube-supported β -fructofuranosidase	45	0.082†

* Expressed as μmol of sucrose hydrolysed/min per μg of enzyme, free or bound.

† Expressed as μmol of sucrose hydrolysed/ml of effluent per m of tube at a flow rate of 1.18 ml/min.

dioxan, was investigated. The results of this experiment (Fig. 2) show that the pH-activity curve of β -fructofuranosidase is constricted, without any significant alteration of the pH optimum, either when it is chemically attached to polystyrene beads or when it is assayed in the presence of dioxan.

The effect of the cationic inhibitors aniline and tris on the activity of β -fructofuranosidase and the polystyrene-tube-supported enzyme was studied.

Fig. 2. Effect of pH on the hydrolysis of sucrose by β -fructofuranosidase in free solution (●), β -fructofuranosidase chemically attached to polystyrene beads (▲) and β -fructofuranosidase in free solution in the presence of 40% (v/v) dioxan (■). All assays were performed at 25°C in the presence of citrate-phosphate-borate buffer prepared as described in the text.

The results of this experiment (Fig. 3) show that the non-competitive nature of the inhibition is unchanged when the enzyme is chemically attached to polystyrene. However, the results do show that K_i for aniline is decreased from 0.94 to 0.39 mM when the enzyme is attached to polystyrene, whereas the corresponding constant for tris is increased from 0.45 to 1.10 M.

Preparations of β -fructofuranosidase chemically attached to both polystyrene beads and tubes have been used over a period of several weeks without any significant loss in activity, the preparations being stored in water at 4°C when not in use. A preparation of polystyrene-bead-supported enzyme did, however, lose all its activity after 3 months when stored in the dry state.

DISCUSSION

When β -fructofuranosidase is chemically attached to polystyrene beads there is an approximate ninefold decrease in V_{max} and an approximate twofold increase in the K_m with respect to sucrose. Decreases in enzymic activity, resulting from the attachment of an enzyme to an insoluble support, have been previously reported and have been ascribed to the supports preventing access to the active site of the bound enzyme or else to the enzymes being bound to the support in an inactive conformation (Hornby, Lilly & Crook, 1966). The twofold increase in K_m that is observed when the enzyme is attached to polystyrene may be due to the particulate nature of the insoluble enzyme

Fig. 3. Non-competitive inhibition by tris and aniline of the hydrolysis of sucrose by β -fructofuranosidase in free solution (a) and chemically attached to a polystyrene tube (b). For the enzyme in free solution v is expressed as μ mol of sucrose hydrolysed/min per μ g of enzyme and for the enzyme attached to the polystyrene tube v is expressed as μ mol of sucrose hydrolysed/ml of effluent per m of tube at a flow rate of 1.18 ml/min. (a) ▲, β -Fructofuranosidase in free solution; ●, β -fructofuranosidase in free solution assayed in the presence of 0.25 M tris; ■, β -fructofuranosidase in free solution assayed in the presence of 0.15 M aniline. (b) □, Polystyrene-tube-supported β -fructofuranosidase; ○, polystyrene-tube-supported β -fructofuranosidase assayed in the presence of 0.15 M tris; Δ, polystyrene-tube-supported β -fructofuranosidase assayed in the presence of 0.15 M aniline.

preparation; the enzyme attached to the surface of the polymer may be considered to be isolated from the bulk of the substrate solution by a diffusion layer. It has been shown that effects of this type can bring about an elevation of the experimentally observed K_m (Lilly, Hornby & Crook, 1966). Alternatively, the attachment of the enzyme to the surface of a hydrophobic structure such as polystyrene may be creating an environment about the bound enzyme that does not favour equal partition of the substrate between the bulk of the substrate solution and the surface of the polymer matrix. If the partition were such that at equilibrium the concentration of substrate is less in the environment of the bound enzyme than that measured in the bulk of the substrate solution, then an increase in the observed K_m would ensue.

Fig. 2 shows that the pH-activity curve of β -fructofuranosidase is constricted, without any significant displacement of the pH optimum, when the enzyme is attached to polystyrene. A qualitatively similar effect is also observed when the enzyme is assayed in the presence of an organic solvent. Because of the similarity of these two effects, it is possible that the bound β -fructofuranosidase may be considered to be in an environment of depressed polarity, due to the close presence of the hydrophobic polystyrene matrix.

Arguments similar to those used above would also explain the observed differences between the inhibitor dissociation constants of the free and bound enzyme for aniline and tris. The almost threefold increase of K_i for tris that results from the binding of the enzyme to polystyrene could be explained in terms similar to those used above to account for the elevation of the observed K_m for sucrose when the enzyme is insolubilized. The decrease in the K_i for aniline can be explained in terms of the polystyrene matrix creating a micro-environment about the bound β -fructofuranosidase that favours partition of the aromatic inhibitor aniline at the surface of the support, due to the hydrophobic nature of the polystyrene. If this were so, then the concentration of aniline around the bound enzyme would be greater than that measured in the bulk of the assay solution and consequently there would be a decrease in the observed K_i . Previously reported micro-environmental effects on the activity of water-insoluble enzymes have all arisen from the existence of ionizable groups on the supporting polymer matrix and have been used to explain differences between the observed K_m of free and bound enzyme for charged substrate molecules (Goldstein *et al.* 1964). Micro-environmental effects such as that suggested above, which arise solely from the hydrophobic nature of the support, have not yet been observed elsewhere.

The present work also describes a method for attaching enzymes to the surface of polystyrene by a process that in no way impairs the integrity of the initial polymeric structure. With previously reported methods for the attachment of proteins to

polystyrene (Gyenes & Sehon, 1960) this was not possible, since the severe conditions described for the initial nitration of the polystyrene always involved a deterioration in the shape of the polystyrene due to its solubilization in the course of the nitration. By using the present process β -fructofuranosidase was attached to the inside surface of a coiled polystyrene tube and the use of the enzyme in this form was demonstrated. A possible extension of this work could be the involvement of enzymes attached to tubes in continuous automated analysis.

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REFERENCES

- Bruner, R. L. (1964). In *Methods in Carbohydrate Chemistry*, vol. 4, p. 67. Ed. by Whistler, R. L. London: Academic Press (Inc.) Ltd.
- Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, 12.
- Datta, S. P. & Grzybowski, A. K. (1961). In *Biochemists' Handbook*, p. 19. Ed. by Long, C. London: E. and F. N. Spon Ltd.
- Goldstein, L., Levin, Y. & Katchalski, E. (1964). *Biochemistry, Easton*, **3**, 1913.
- Grubhofer, N. & Schleith, L. (1954). *Hoppe-Seyler's Z. physiol. Chem.* **297**, 108.
- Gyenes, L. & Sehon, A. H. (1960). *Can. J. Biochem.* **38**, 1235.
- Hornby, W. E., Lilly, M. D. & Crook, E. M. (1966). *Biochem. J.* **98**, 420.
- Hornby, W. E., Lilly, M. D. & Crook, E. M. (1968). *Biochem. J.* **107**, 669.
- Lilly, M. D., Hornby, W. E. & Crook, E. M. (1966). *Biochem. J.* **100**, 718.
- Lilly, M. D. & Sharp, A. (1968). *Chem. Engr, Lond.*, **215**, CE 12.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Linker, A. (1966). In *Methods in Enzymology*, vol. 8, p. 650. Ed. by Neufeld, E. F. & Ginsburg, V. London: Academic Press (Inc.) Ltd.
- Sharp, A., Kay, G. & Lilly, M. D. (1969). *Biotechnol. & Bioengng*, **11**, 363.
- Silman, I. H. & Katchalski, E. (1966). *A. Rev. Biochem.* **35**, 633.