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Characteristics of invertase immobilized on three different types of supports

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Invertase was immobilized on three different supports, namely, diethylaminoethyl-cellulose (DEAE-cellulose), polyacrylamide and hen-egg white. The amount and yield activity of immobilized enzyme were estimated. The effect of enzyme concentration on the activity of immobilized invertase was studied.

The immobilized enzyme preparations followed Michaelis–Menten behaviour. The Michaelis constant was estimated. The free enzyme and that immobilized on DEAE-cellulose had an optimum pH at 4·5, while it was 4 for enzyme fixed on polyacrylamide and hen-egg white. Optimum temperature was 40°C for both free and immobilized invertase on DEAE-cellulose, while it was 50°C for enzyme fixed on polyacrylamide and hen-egg white. Thermal stability was estimated; it was found that the immobilized enzyme preparations were more stable at 60°C than the free one.

INTRODUCTION

Enzymes or cells are often used in an immobilized form in industry because it is very difficult to reuse the free enzymes or to use them continuously. A wide variety of methods has been described for the attachment of enzymes to supporting structures (Filippusson & Hornby, 1970). A good carrier for immobilized enzymes must be a stable material which possesses versatile chemical properties. Also, it must be inexpensive, especially when it is used in commercial processes. Invertase is of great importance to the food industry. It has been immobilized on some organic and inorganic supports (Mason & Weetall, 1972; Boudrant & Cheftel, 1975). The present work presents some characteristics of some inexpensive and novel carriers for immobilization of invertase, using simple techniques. Three carriers have been chosen to be used as support materials. Diethylaminoethyl-cellulose (DEAE-cellulose) is inexpensive, chemically stable and is resistant to microbiological contamination (Chen & Tsao, 1970). Invertase was immobilized on DEAE-cellulose by adsorption. This technique is simple, mild and reversible, permitting reuse of both enzyme and the support (Huitran & Limon-Lason, 1978). The second carrier used was polyacrylamide gel. It is the polymer most often used for immobilization of enzymes by entrapping. The third carrier was hen-egg white, a novel support for immobilization of enzymes. It is nontoxic and inexpensive, which makes it an economical support for enzyme immobilization.

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MATERIALS AND METHODS

Materials

Invertase (β-D-fructofuranosidase, EC 3.2.1.26) from baker's yeast grade V, was purchased from Sigma Chemical Co., USA. 3,5-Dinitrosalicylic acid was obtained from BDH Ltd, UK. DEAE-cellulose was obtained from Whatman Chemicals, UK. Acrylamide, crystallized pure, *N,N*-methylenebis(acrylamide) and ammonium persulphate were purchased from Serva Feinbiochemica Co. Dimethylaminopropionitrile (DMAPN) and glutaraldehyde were obtained from BDH Ltd, UK. Sucrose was obtained from Koch Light Co., UK. Hen eggs were obtained from the local market. All other chemicals were analytical grade.

Methods

Immobilization of invertase was carried out by using the following carriers.

DEAE-cellulose

Invertase was immobilized on DEAE-cellulose according to the method described by Huitran & Limon-Lason (1978). A suspension of DEAE-cellulose was prepared by suspending 1 g DEAE-cellulose in 100 ml distilled water and stirring gently for 5 min at 4°C, using a magnetic stirrer. A solution of invertase (80 mg in 8 ml distilled water) was added to the suspension of DEAE-cellulose and stirred gently for 30 min at 4°C. The enzyme support complex obtained was filtered and washed three times with 100 ml distilled water through filter paper (Whatman No. 1) to remove the uncoupled enzyme, then suspended in 50 ml distilled water and kept at 4°C.

Polyacrylamide

The method described by Kobayashi *et al.* (1975), was followed, with some modifications. Polyacrylamide gel was prepared by dissolving 23·67 g acrylamide and 1·24 g *N,N*-methylenebis(acrylamide) in 100 ml phosphate buffer pH 7. The enzyme solution (35 mg of invertase dissolved in 3·5 ml of the same buffer) was added to an equal volume of polyacrylamide gel, and the suspension was shaken mechanically for 10 min at room temperature. The suspension (enzyme + gel constituent) was cooled in an ice bath for 5 min, then 1 drop of DMAPN was added. After 2 min, 0·5 ml of 5% ammonium persulphate was added to the mixture. The cooling was continued for 20 min until a gel was formed. The gel obtained was then shattered by passage through a syringe needle and washed with 100 ml phosphate buffer pH 7 to remove free enzyme, then it was filtered (Whatman No. 7). The immobilized enzyme preparation was kept at 4°C.

Hen-egg white

Invertase was attached to hen-egg white according to the method of D'souza and Nadkarui (1981). A hen egg white was broken and the egg white was carefully separated from yolk. A 35 ml sample of egg white was mixed thoroughly with a solution of invertase (35 mg in 3·5 ml distilled water) and then 3·5 ml of glutaraldehyde (25%) were added. The mixture was stirred well for 3 min with a magnetic stirrer and allowed to stand for 2·5 h at room temperature. The hard gel obtained was then shattered by passing through a syringe needle, and washed with 100 ml distilled water and filtered. The free aldehyde groups present in the gel were blocked by suspending the gel overnight at 4°C in 100 ml phosphate buffer (pH 7) containing 0·1M lysine. The immobilized preparation was washed with distilled water, filtered and stored at 4°C.

Progress of sucrose hydrolysis by immobilized invertase

Twenty g (wet wt) sample of the enzyme support complex was poured into a glass column of length 32 cm and i.d. 1·5 cm, purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The substrate (0·25M) in acetate buffer pH 4·5 was then passed through the column. The reducing sugars produced were then determined by the 3,5-dinitrosalicylic acid method described by Brunner (1964) (Fig. 1).

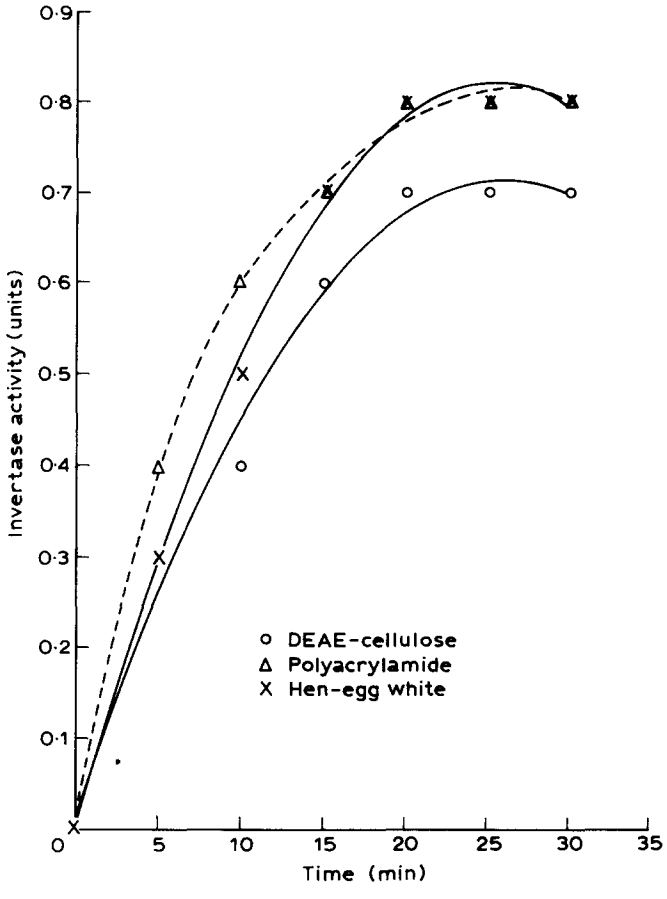

Fig. 1. Progress of sucrose hydrolysis by immobilized invertase.

Amount and yield of bound enzyme

The amount and yield of immobilized invertase were calculated according to Imai *et al.* (1986*a*), using the following parameters:

$$\text{Immobilization amount} = A - B$$

$$\text{Immobilization yield of enzyme} = (A - b/A) \times 100$$

where A is the total amount of enzyme (mg) added to the immobilization solution, and B is the amount of residual enzyme in the immobilization and washing solutions, after the immobilization procedure. Both A and B were evaluated from the amount of reducing sugars produced enzymatically in the corresponding solutions. The activity yield was calculated from the equations reported by Boudrant & Cheftel (1975) as follows:

$$\frac{\text{quantity of adsorbed invertase}}{\text{quantity of added invertase}} \times \frac{\text{enzymic activity of adsorbed invertase}}{\text{enzymic activity of the same quantity of free invertase}}$$

Assay of invertase activity

Invertase activity was estimated by the 3,5-dinitrosalicylic acid method described by Bruner (1964). The experiment was carried out by stirring the enzyme (100 mg immobilized enzyme preparations or an aliquot of free enzyme solution (100 mg enzyme/10 ml acetate buffer (pH 4·5) containing invertase activity equal to that present in the immobilized preparations) in 5 ml sucrose in 0·2M acetate buffer pH 4·5 and incubated for 15 min at 37°C. An aliquot of 0·5 ml was pipetted into a test tube, and the enzymic process was stopped by adding 2 ml dinitrosalicylic reagent (1 g 3,5-dinitrosalicylic acid in 20 ml sodium hydroxide and diluting with 50 ml distilled water) and maintaining for 5 min on a boiling water bath, cooling and diluting to 30 ml with distilled water. The optical density was measured at 540 nm, using a Specol spectrocolorimeter. A blank was prepared under the same conditions. A standard curve was prepared, using pure sucrose. The enzyme activity was expressed in terms of units. One unit of enzyme was defined, according to Hardil & Svec (1981), as 1 μmol sucrose inverted/min/mg of β-D-fructofuranosidase (invertase).

RESULTS AND DISCUSSION

Amount and yield of invertase immobilized on different supports

Table 1 indicates that the yield of immobilized invertase reached about 100% for the three supports used. This indicates that all the enzyme used in the immobilization process was completely bound to the support. The important factor is the activity yield and efficiency of activation, because the total amount of immobilized invertase may not be totally active. It is clear that DEAE-cellulose had the greatest activity yield, followed by hen-egg white, then polyacrylamide. Invertase immobilized on DEAE-cellulose retained about 75% of its activity compared with the free enzyme. The yield of the activity of immobilized enzyme varies according to the type of enzyme, the physical and chemical properties of the support, and the immobilization technique used. Suzuki *et al.* (1966) found that the bound invertase on DEAE-cellulose showed an activity corresponding to 30 to 50% of that of the free enzyme. Maeda & Suzuki (1973) found that invertase immobilized to

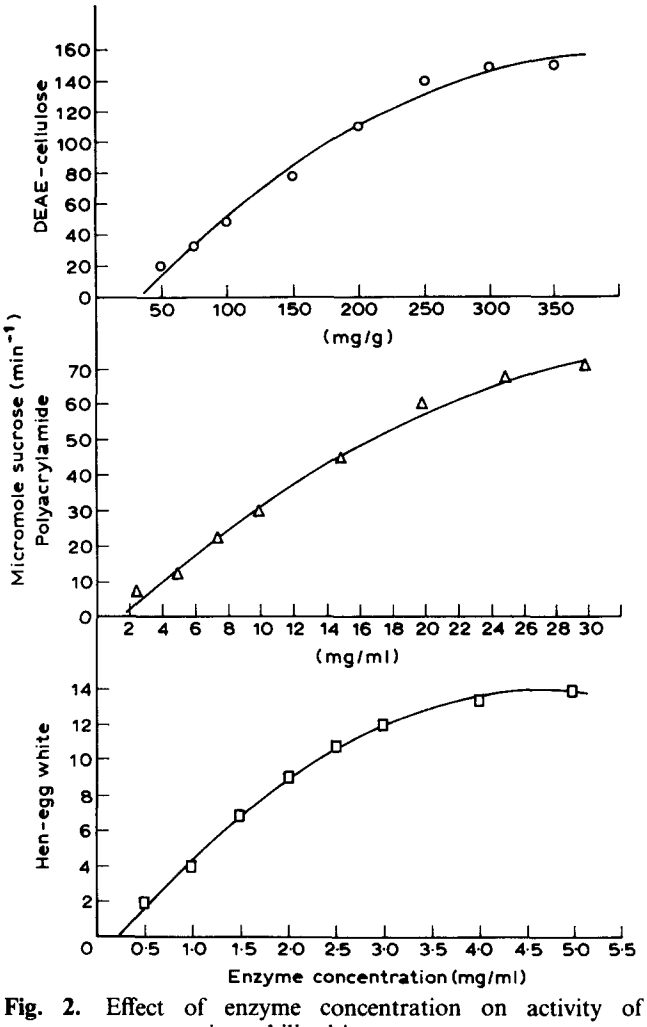

Fig. 2. Effect of enzyme concentration on activity of immobilized invertase.

DEAE-cellulose was less active than native enzyme and *c.* 55 to 70% of the enzyme activity was lost on binding. The amount of invertase adsorbed on bead cellulose, containing weak basic *N,N*-diethylamino-2-hydroxypropyl groups, was proportional to porosity and to the exchange capacity of the ion exchanger. Hardil & Svec (1981) showed that invertase fixed on hen-egg white retained about 25% of its original activity.

Effect of enzyme concentration

The effect of the enzyme concentration was studied by using invertase solutions of increasing concentrations.

Table 1. Amount and yield of invertase immobilized on different supports

Support used	Amount of added invertase (mg)	Amount of bound invertase (mg)	Yield of immobilization (%)	Activity[a]		Activity yield (%)
				Added	Bound	
DEAE-cellulose	80·0	80·0	100·0	111·1	83·3	75·0
Polyacrylamide	175·0	174·9	100·0	111·1	34·5	31·1
Hen egg-white	30·0	30·0	100·0	111·1	46·8	42·0

[a] Units: μmol sucrose hydrolysed/min/mg enzyme/g support.

The ratio, mg invertase/g DEAE-cellulose, varied between 50 and 350, while the ratio, mg enzyme/ml polyacrylamide gel, varied between 2·5 to 30 and that per ml of hen-egg white ranged between 0·5 and 5. Figure 2 shows that the activity of immobilized invertase increased by increasing enzyme concentration, then the reaction velocity approached a constant. DEAE-cellulose was the most active support for fixing invertase since 1 g of DEAE-cellulose could adsorb up to 350 mg of invertase. No activity of the added enzyme was found in filtrates made in this range. Immobilization of invertase in polyacrylamide gel showed that about 30 mg of enzyme could be entrapped per ml of gel. Kreen *et al.* (1973) reported that the amount of enzyme fixed on polyacrylamide depends on the size of the gel particles. It was noticed that hen-egg white could bind about 5 mg/ml of gel.

Effect of substrate concentration

Figure 3 shows that the activity of both free and immobilized invertase increased by increasing the concentration of substrate until a plateau was reached, and thus followed the normal effect of a substrate in an enzymic reaction. Hardil & Svec (1981) showed that the activities for both the native and immobilized invertase on *N,N*-diethylaminoethyl-cellulose-2-hydroxypropyl-cellulose showed sharp decreases. The correlation between rate of reaction and corresponding sucrose concentration

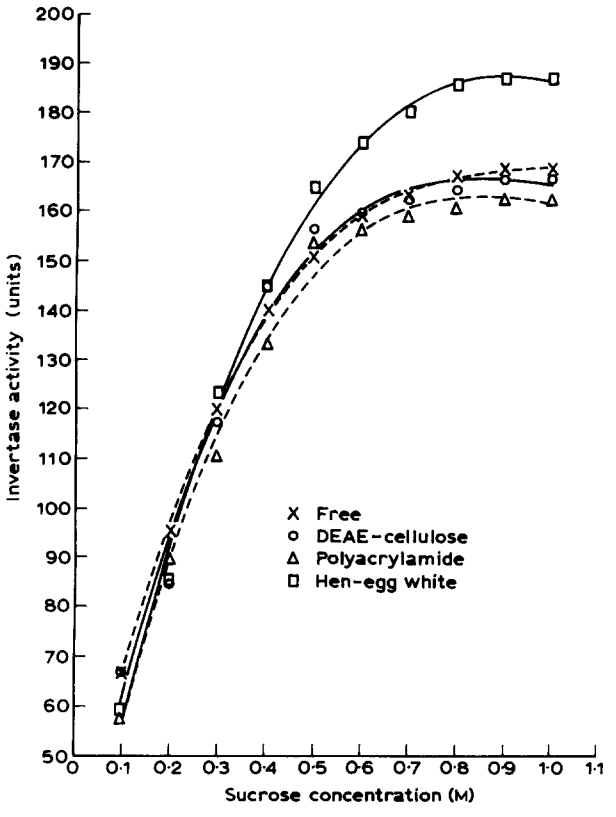

Fig. 3. Effect of substrate concentration on activity of free and immobilized invertase.

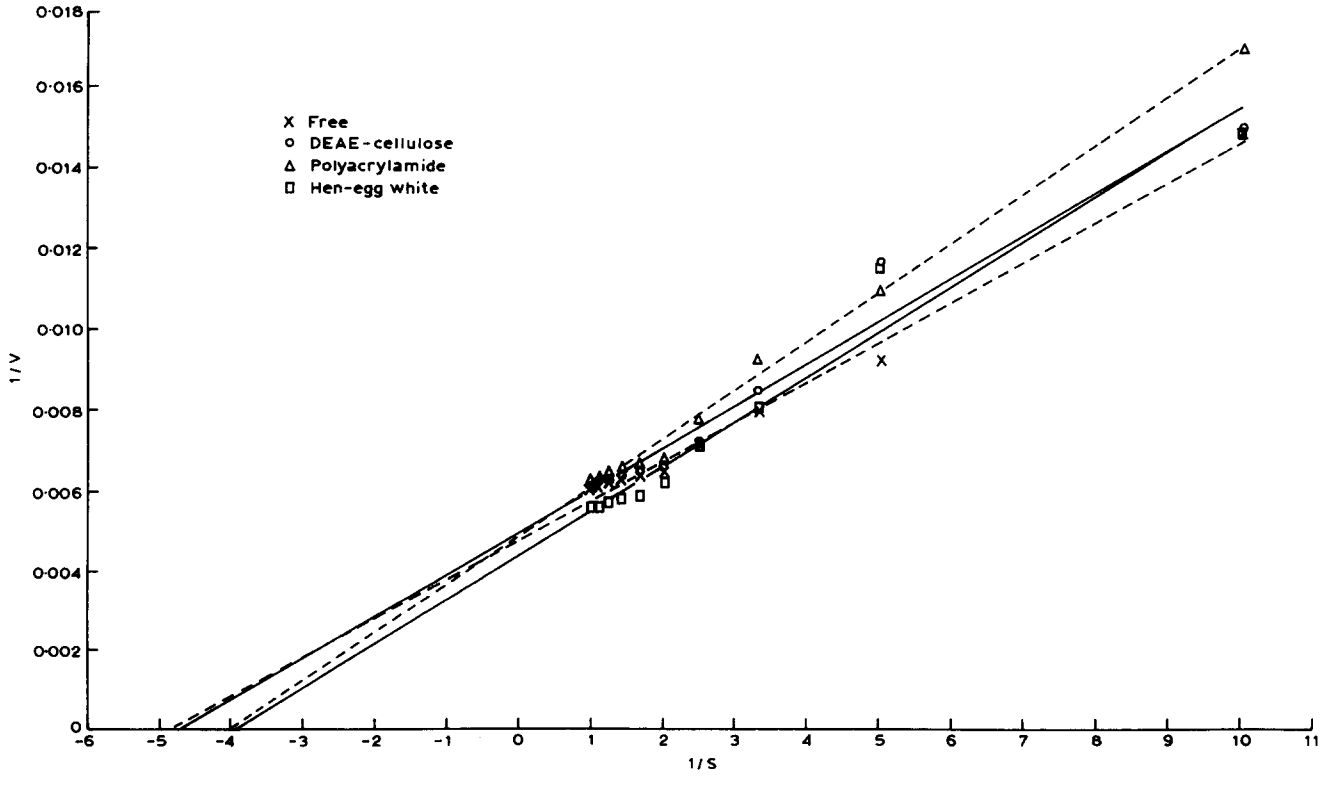

Fig. 4. Calculation of Michaelis constant and maximum velocity of free and immobilized invertase.

is shown in Fig. 4 by reciprocal plots. The K_m was calculated from Lineweaver–Burk plots. The K_m of the free enzyme was 0·21M, while the apparent K_m was 0·21, 0·24 and 0·24 for enzyme immobilized on DEAE-cellulose, polyacrylamide and hen-egg white, respectively. These results show that the immobilization of invertase on DEAE-cellulose had no effect on the apparent K_m value, while that value for enzyme immobilized on both polyacrylamide and hen-egg white was higher than that of the free enzyme. It increased by a factor of 1·14. These results are in agreement with those reported by other workers (D'souza & Nadkarni, 1981; Imai *et al.* 1986*b*; Simionescu *et al.* 1987), who stated that the apparent K_m was increased when the enzyme was attached to many types of carriers. In all cases, the increase in K_m value was attributed to electrostatic interaction between the carrier and the substrate.

pH activity profile

Figure 5 shows that the maximum activity of invertase immobilized on DEAE-cellulose was at pH 4·5. It was similar to that obtained for the free enzyme. There is a shift in the pH activity curves of invertase immobilized on both polyacrylamide and hen-egg white towards the acidic side, being about pH 4. This shift may be due to a pH difference between the solution and the close proximity of the gel matrix. Thornton *et al.* (1975) reported that immobilization of invertase on hornblende caused a shift of the optimum pH of the adsorbed enzyme towards the acidic direction. D'souza & Nadkani (1981) reported that the broadening of the pH optimum towards the acidic side could be attributed to the fact that hen-egg white is basic in nature.

Effect of temperature

The dependence of invertase activity on temperature is shown in Fig. 6. The optimum temperature for both the free enzyme and that attached to DEAE-cellulose was obtained at 40°C. Hardil and Svec (1981) reported that the maximum activity for both free and immobilized invertase on bead *N,N*-diethylamino-2-hydroxypropyl-cellulose was obtained in the range 30°–50°C. The optimum activity temperature for both invertase entrapment in polyacrylamide and hen-egg white was shifted to about 10°C higher, and change of the temperature dependence curves was observed. Tomar & Prabhu (1985) found that invertase fixed on bentonite had a higher optimum temperature (60°–65°C) and a higher thermal stability compared to that of the free enzyme. The heat stability of immobilized invertase was measured in two ways: firstly, thermal treatment was carried out by keeping the free and immobilized enzyme preparations, for 30 min, in 0·2M acetate buffer solution (pH 4·5) at several temperatures ranging

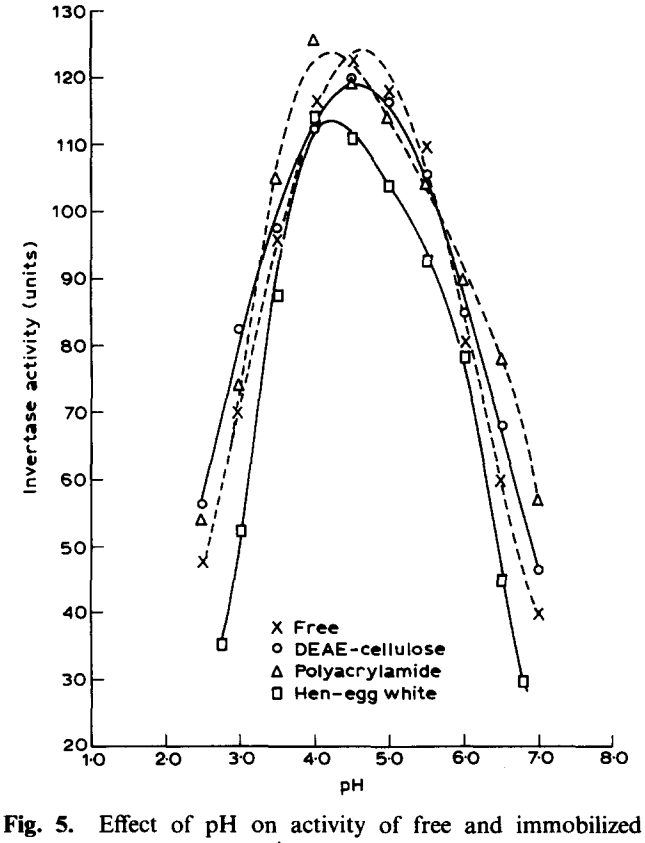

Fig. 5. Effect of pH on activity of free and immobilized invertase.

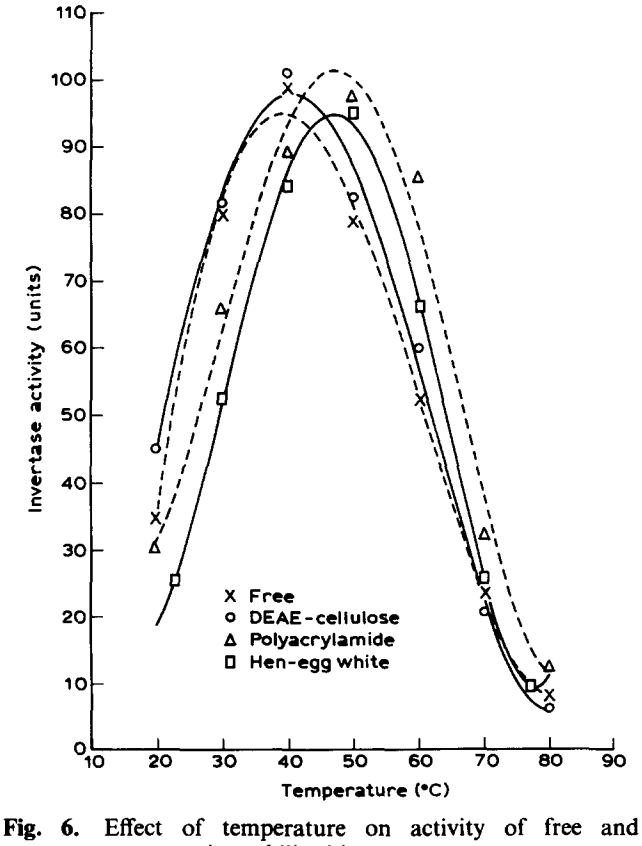

Fig. 6. Effect of temperature on activity of free and immobilized invertase.

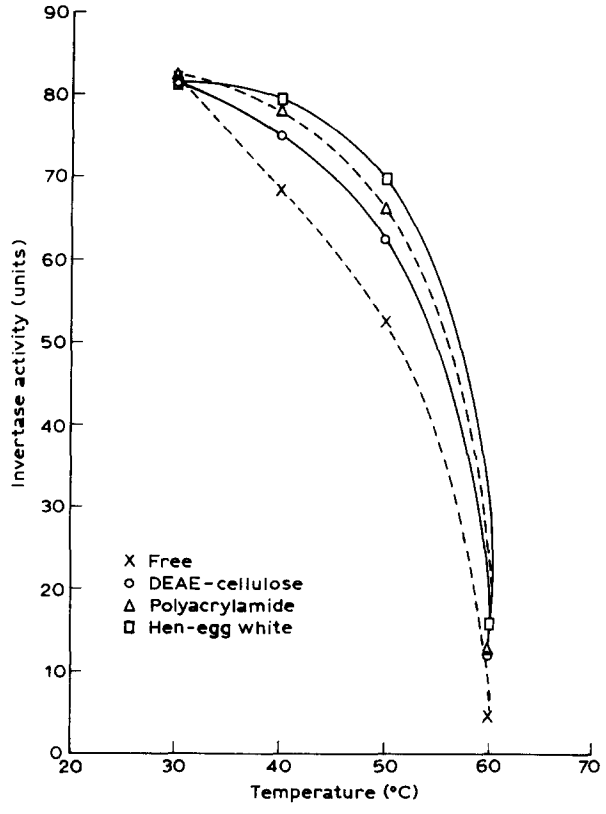

Fig. 7. Thermal stability of free and immobilized substrate.

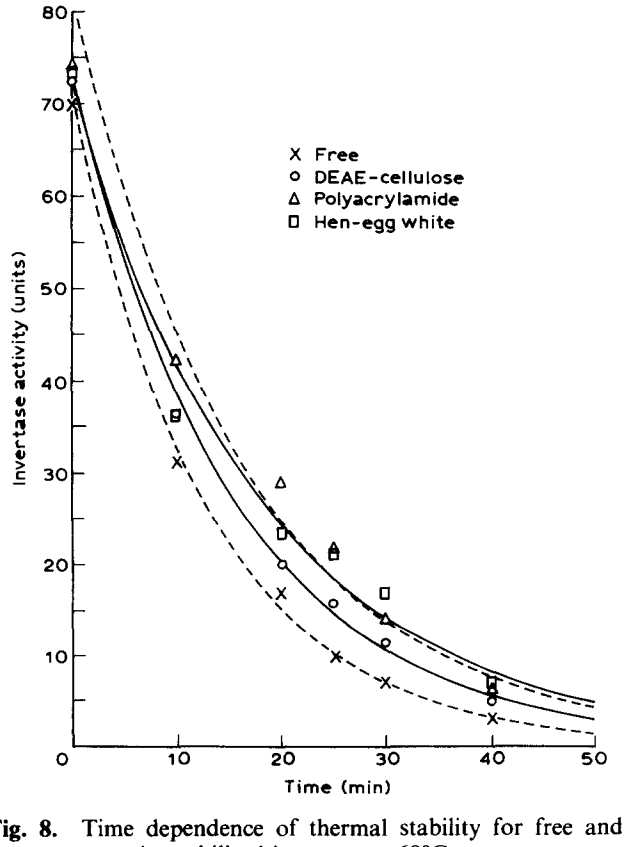

Fig. 8. Time dependence of thermal stability for free and immobilized invertase at 60°C.

between 30° and 70°C. As shown in Fig. 7, the free enzyme became unstable above 40°C, while immobilized enzyme preparations were stable over the temperature range 30°–50°C. Above 50°C the activity diminished rapidly. At 60°C, the activity of immobilized invertase decreased to 20·0, 16·0 and 15·0% for enzyme immobilized on hen-egg white, polyacrylamide and DEAE-cellulose, respectively, while that of the free enzyme fell to 6%. Thornton *et al.* (1975) found that the invertase (free and immobilized on hornblende) was increasingly unstable above 55°C.

Secondly, the free enzyme and the immobilized enzyme preparations were kept in 0·2M acetate buffer solution (pH 4·5) at 60°C for various times. Figure 8 shows that immobilized invertase preparations were more stable than the free enzyme. It is clear that invertase immobilized on hen-egg white retained about 23% of its original activity when kept at 60°C for 30 min, while the free enzyme retained about 10%. It was evident that the thermal stability was improved by immobilization.

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