Thermal stabilization of glucose oxidase and glucoamylase by physical entrapment

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Physical entrapment was used as an approach to achieve thermal stabilization of enzymes. The $t_{1/2}$ values for the thermoinactivation of glucose oxidase and glucoamylase were increased several-fold by their entrapment in polyacrylamide gels. In polyacrylate gels the individual enzymes behaved differently, probably owing to microenvironmental effects arising by the polyelectrolyte nature of the carrier.

Enzymes bound to artificial membranes or to some polymer particles have been used as model systems for studying the microenvironmental effects on the mode of action of enzymes in their native milieu *in vivo* (Levin *et al.*, 1964; Goldstein *et al.*, 1964; Silman & Karlin, 1967; Goldman *et al.*, 1968). In addition, these immobilized enzyme derivatives can serve as specific and easily removable biocatalysts in continuous enzymic processes. The major drawback in the commercial exploitation of immobilized enzymes is the loss of their catalytic activity over a period of time, which has often been attributed to thermal denaturation of the enzyme. This problem, when solved, allows the preparation of highly thermostable immobilized enzyme derivatives for industrial purposes.

Several attempts were made by earlier workers to stabilize enzymes by covalently attaching them to carriers (Gabel *et al.*, 1970; Gabel, 1973) or by physical or chemical entrapment (Martinek *et al.*, 1977*a,b*). However, the results obtained were of limited application, and no generalizations on the principles of thermal stabilization could be elaborated. The enzymes used in their studies were mainly the proteolytic enzymes trypsin and chymotrypsin, which on immobilization would result in the inhibition or prevention of their autolytic activity, thereby affording apparent stabilization (Klibanov, 1979).

To circumvent this problem of thermal inactivation of enzymes, we have used physical entrapment as an approach to enhance the conformational stability of the enzymes. This was achieved by the formation of weak non-covalent interactions between the enzyme and the polymer gel matrix, thus retarding its thermal denaturation. In the present paper we discuss the results of the thermal stabilization of glucose oxidase (β -D-glucose-O₂ oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* and

glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) from *Thermomyces lanuginosus* and *Rhizopus* sp. by their physical entrapment in polyacrylamide gels.

Experimental

Materials

Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, ammonium persulphate, Tris, purified glucose oxidase from *A. niger* and crude glucoamylase from *Rhizopus* sp. were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylic acid and soluble starch were the products of E. Merck, Darmstadt, West Germany.

Purification of glucoamylases

Glucoamylase was purified from the culture filtrates of the thermophilic fungus *T. lanuginosus* ML-M as described previously (Basaveswara Rao *et al.*, 1979).

Glucoamylase from *Rhizopus* sp. was purified to homogeneity by the following procedure. The crude enzyme powder from *Rhizopus* sp. (200 mg) was extracted with 5 ml of 50 mM-sodium acetate buffer, pH 5.0, and the insoluble material was sedimented and discarded. The supernatant was loaded on to a Sephadex G-25 column (3 cm $\times$ 27 cm) that was pre-equilibrated with 50 mM-sodium acetate buffer, pH 5.0, and eluted with the same buffer. The protein fractions were pooled and loaded on to a DEAE-cellulose column (1.5 cm $\times$ 16 cm) that had been equilibrated with 50 mM-sodium acetate buffer, pH 5.0, and washed with the same buffer. Unlike the glucoamylase from *T. lanuginosus*, the *Rhizopus* glucoamylase did not bind to DEAE-cellulose and was eluted in the unadsorbed fraction. The volume of the enzyme fraction was decreased by

dialysis against solid sucrose, and the concentrated enzyme was passed through a Bio-Gel P-100 column (1.8 cm $\times$ 76 cm) that had been equilibrated with 50 mM-sodium acetate buffer, pH 5.0, and eluted with the same buffer. The active fractions were pooled and concentrated by dialysis against solid sucrose. The enzyme solution was then dialysed against 1 M-Tris/glycine buffer, pH 8.6, for 15 min, and analytical polyacrylamide-disc-gel electrophoresis was performed. A 100 μ l sample, containing about 100 μ g of protein, was applied to each gel (7%, w/v) tube (0.5 cm $\times$ 10 cm), and electrophoresis was performed in 1 M-Tris/glycine buffer, pH 8.6, at 100 V for 3–4 h. The protein bands were located by staining one of the gels with Coomassie Brilliant Blue G at the end of the electrophoresis run. The remaining gels were cut corresponding to the major protein band (closest to the origin), which was found to exhibit the enzyme activity. The gel discs were finely macerated with a glass rod, and the enzyme was eluted overnight with 200 mM-sodium acetate buffer, pH 5.0, extensively dialysed against distilled water and stored frozen at -20°C .

Entrapment of glucose oxidase and glucoamylase into polyacrylamide and polyacrylate gels

To 5 g of either acrylamide or sodium acrylate and 143 mg of *NN'*-methylenebisacrylamide, 3 ml of the enzyme (110 μ g of glucose oxidase, 62 μ g of *T. lanuginosus* glucoamylase or 55 μ g of *Rhizopus* glucoamylase) in 1 M-Tris/HCl buffer, pH 8.6, was added and the volume was made up to 10 ml with the same buffer. Polymerization mixtures in the range 5–50% (w/v) total monomer (acrylamide or sodium acrylate) concentrations (50% is the solubility limit of the monomers) were prepared by taking appropriate portions from the above stock solution and diluting each to 1.0 ml with the enzyme solution, keeping its concentration constant. Polymerization was initiated by the addition of 10 μ l of *NNN'*-tetramethylethylenediamine (30%, v/v) and 10 μ l of ammonium persulphate (30%, w/v) and was conducted at 4°C . Although polymerization was almost complete in 5–10 min, the gels were kept at 4°C for a further period of 6–12 h to ensure complete gelling and entrapment.

Processing of the gels

The gels were first minced with a glass rod, suspended in 30 ml of 0.2 M-sodium acetate buffer, pH 5.0, containing 1 M-NaCl and homogenized in a Potter-Elvehjem homogenizer. The gel suspension was centrifuged at 10000 g for 20 min at 4°C , and the supernatant, containing enzyme that had not been trapped in the gels, was discarded. This washing procedure was repeated three times with the same buffer, the final supernatant being free of the enzyme activity. The gels were then washed thrice

with 30 ml of 10 mM-sodium acetate buffer, pH 5.0, to remove NaCl and were finally suspended in the same buffer.

Enzyme assays

Entrapped glucose oxidase was assayed by incubating a portion of the gel suspension, in a total reaction mixture, of final volume 1.0 ml, containing 10 mM-sodium acetate buffer, pH 5.0, 55 mM-glucose and 10 mM-EDTA at 37°C for 20 min with occasional shaking. The reaction was terminated by transferring the tubes to a boiling-water bath for 10 min, and the amount of H_2O_2 formed was determined by using the peroxidase method (McComb & Yushok, 1958). One unit of the enzyme is defined as the amount that oxidizes 1 μ mol of β -D-glucose to D-gluconic acid and H_2O_2 per min under the conditions of assay.

Entrapped glucoamylase was assayed by incubating a portion of the gel suspension, in a total reaction mixture, of final volume 1.0 ml, containing 10 mM-sodium acetate buffer, pH 5.0, and 1% soluble starch for 60 min with occasional shaking. For *Rhizopus* glucoamylase (mesophilic), the reaction was performed at 37°C and for the *T. lanuginosus* glucoamylase (thermophilic) it was performed at 50°C . The reaction was terminated by transferring the tubes to a boiling-water bath for 20 min, and the amount of glucose formed was determined by a coupled assay involving the use of the glucose oxidase/peroxidase method (McComb & Yushok, 1958) with minor modifications (Basaveswara Rao *et al.*, 1981). One unit of the enzyme is defined as the amount that liberates 1 μ mol of glucose from soluble starch per min under the conditions of assay.

Soluble enzymes were assayed under the same conditions as those described above. The reaction was linear up to 2 h under these conditions. The increase in the reaction rate with increasing substrate concentrations, when fixed amounts of the entrapped enzymes were used, indicated that the concentrations employed in the enzyme assays (55 mM-glucose for glucose oxidase, 1% soluble starch for the glucoamylases) were well above the saturating values. These concentrations were used in all the studies to overcome any diffusional limitations of the substrates into the gels.

Thermal-stability experiments

Thermal stability of the enzymes was determined by incubating the trapped enzyme/gel suspensions (gel concentration ranging from 5 to 50%) or the soluble enzymes at defined temperatures in the range 40 – 75°C in 10 mM-sodium acetate buffer, pH 5.0, in a constant-temperature water bath. Samples were removed at appropriate intervals, that at zero time serving as control, and were cooled immediately in ice. The residual activities were determined and are

expressed as the fractional percentages of the control values.

Results

The yield of trapped enzyme activities increased with increasing total monomer concentration up to a maximum value and then decreased (Table 1). A sharp decrease in the trapped activities was found at high acrylamide concentrations. Thus the trapped glucose oxidase and *Rhizopus* glucoamylase activities recovered in the gels were decreased to zero

when 50% acrylamide was employed. Also, the yields of trapped activities of glucose oxidase and *T. lanuginosus* glucoamylase decreased at high acrylate concentrations, but the decrease was not as sharp as when acrylamide was used. However, with *Rhizopus* glucoamylase the yield of trapped activity remained constant between 20% and 40% acrylate concentrations and increased at 50% acrylate concentration.

The kinetic curves of the thermal stability of the enzymes in their native and entrapped forms are shown in Fig. 1. The process of thermoinactivation

Table 1. Effect of total monomer concentrations on the entrapment of glucose oxidase and glucoamylase
Experimental details are given in the text.

Concn. of total monomer (% w/v)	Yield of trapped enzyme activities in polyacrylamide gels (%)			Yield of trapped enzyme activities in polyacrylate gels (%)		
	Glucose oxidase	Glucoamylase (<i>T. lanuginosus</i>)	Glucoamylase (<i>Rhizopus</i> sp.)	Glucose oxidase	Glucoamylase (<i>T. lanuginosus</i>)	Glucoamylase (<i>Rhizopus</i> sp.)
5	9.3	5.6	37.1	—*	—*	—*
10	18.5	14.4	60.2	22	1.02	11.8
20	27.4	18.6	38	41.6	3.1	27.7
30	12.8	31.4	25	41.4	3.2	25.1
40	3.7	18.2	4.6	30.4	2.7	27.3
50	0.2	2.8	0	19.5	2.2	41.0

* Gels formed at 5% acrylate concentration were very soft, of soluble nature and hence could not be recovered by centrifugation.

Fig. 1. Kinetics of thermoinactivation of enzymes in semi-logarithmic plots

The experiment was conducted as described in the Experimental section. Approx. 15 munits of glucose oxidase or 5 munits of glucoamylases were employed for each assay in all the thermostability experiments. (a) Thermal inactivation of glucose oxidase at 70°C. ○, Enzyme entrapped in 20% polyacrylamide gels; ●, enzyme entrapped in 20% polyacrylate gels; Δ, native enzyme. (b) Thermoinactivation of *T. lanuginosus* glucoamylase at 70°C. ○, Enzyme entrapped in 40% polyacrylamide gels; ●, enzyme entrapped in 40% polyacrylate gels; Δ, native glucoamylase. (c) Thermal inactivation of *Rhizopus* glucoamylase at 54°C. ○, Enzyme entrapped in 40% polyacrylamide gels; ●, enzyme entrapped in 40% polyacrylate gels; Δ, native glucoamylase.

Fig. 2. Effect of gel concentration on the half-lives ($t_{1/2}$) of thermoinactivation of enzymes

The experiment was conducted at 60°C with glucose oxidase (a), at 70°C with *T. lanuginosus* glucoamylase (b) and at 48°C with *Rhizopus* glucoamylase (c). Experimental details are given in the text. ○, Enzymes entrapped in polyacrylamide gels; ●, enzymes entrapped in polyacrylate gels; △, native enzymes.

of glucose oxidase and the glucoamylases was found to follow first-order kinetics. The enzymes entrapped in polyacrylamide gels were more stable to temperature inactivation when compared with the soluble enzymes, whereas the thermal stability of those entrapped in polyacrylate gels varied with individual enzymes. The increase in the thermal stability was about 7-fold with *T. lanuginosus* glucoamylase (Fig. 1b) but varied very slightly with glucose oxidase (Fig. 1a), whereas with *Rhizopus* glucoamylase the polyacrylate-gel-entrapped enzyme was inactivated much faster than the native soluble enzyme (Fig. 1c).

Fig. 2 depicts the effect of gel concentration on the half-lives ($t_{1/2}$) of the thermoinactivation of the enzymes. The $t_{1/2}$ is defined as the time required for 50% inactivation, and is calculated from the plots as obtained in Fig. 1 for different gel concentrations, with incubation at different temperatures in the range 40–75°C. The $t_{1/2}$ values of the enzymes entrapped in polyacrylamide gels increased several-fold when compared with their soluble counterparts. The $t_{1/2}$ value increased about 12-fold for glucose oxidase entrapped in 20% gels (Fig. 2a), by about 6-fold for *T. lanuginosus* glucoamylase entrapped in 40% gels (Fig. 2b) and about 5-fold for *Rhizopus* glucoamylase entrapped in 40% gels (Fig. 2c). On the other hand, for the enzymes entrapped in polyacrylate gels the $t_{1/2}$ value increased only slightly for glucose oxidase and was constant at all gel concentrations. The value increased about 7-fold for *T. lanuginosus* glucoamylase, whereas *Rhizopus* glucoamylase exhibited a decrease in the $t_{1/2}$ value when compared with the native soluble enzyme. The gel concentration required for the maximum thermal

stabilization was 20% for glucose oxidase and 40% for the glucoamylases.

The Arrhenius plots for the thermoinactivation of glucose oxidase and *Rhizopus* glucoamylase are given in Fig. 3. The insets to Fig. 3 show the thermostability curves of these enzymes in their native and entrapped forms. The activation energies calculated for the thermoinactivation of these enzymes in their native and entrapped forms remained more or less constant in the temperature range tested. Thus the activation energies for the thermoinactivation of glucose oxidase entrapped in 20% polyacrylamide and polyacrylate gels and for the native enzyme are 188, 222 and 201 kJ/mol respectively (Fig. 3a). The corresponding values for the *Rhizopus* glucoamylase entrapped in 40% polyacrylamide and polyacrylate gels and for the native enzyme were 406, 297, 293 kJ/mol respectively (Fig. 3b). These values indicated that the fold increase (or decrease) in the thermal stabilities of glucose oxidase and *Rhizopus* glucoamylase were independent of temperature.

Discussion

The percentage recovery of trapped glucose oxidase and glucoamylase activities increased with increasing gel concentration. This is because of the decrease in the gel porosities at high gel concentrations resulting in the enhancement of the trapping efficiency (Fawcett & Morris, 1966). The sharp decrease in the yield of trapped activity of acrylamide concentrations higher than 30% was most probably due to the inactivation of the

Fig. 3. Arrhenius plots for the thermostabilization of enzymes

Experimental details are given in the text. (a) Thermostabilization of glucose oxidase entrapped in 20% polyacrylamide (O) or polyacrylate gels (●) or in its native form (Δ). (b) Thermostabilization of *Rhizopus* glucoamylase entrapped in 40% polyacrylamide (O) or polyacrylate gels (●) or in its native form (Δ). Insets represent the thermostability curves of the corresponding enzyme preparations at different temperatures in the range of 40–75°C for 1 h.

enzymes in the polymerization mixtures. This is supported by the fact that inactivation of the enzymes was observed on their incubation with acrylamide at concentrations greater than 20%. Acrylamide apparently acted as a denaturing agent in a way similar to the well-known amidic compounds such as urea.

The thermal stability of glucose oxidase and the glucoamylases was independent of their initial concentrations and followed first-order kinetics.

The thermal stability of these enzymes was increased several-fold when they were entrapped in polyacrylamide gels. This is explained in the following way. When the enzymes were entrapped in the interstitial spaces provided by the three-dimensional lattice of the gel matrix, at high gel concentrations, their translational and rotational diffusions are obstructed (Martinek *et al.*, 1977b). The retardation of this molecular mobility favours the formation of weak non-covalent interactions between the enzyme and the gel matrix. As a consequence of this, the enzymes attain conformational rigidity, resulting in decreased thermal denaturation.

On the other hand, for the polyacrylate-gel-entrapped enzymes, the $t_{1/2}$ value increased slightly for glucose oxidase, increased 7-fold for *T. lanuginosus* glucoamylase and decreased considerably for *Rhizopus* glucoamylase, as compared with their soluble counterparts. The reason for this behaviour may be explained on the basis of the microenvironmental effects arising from the polyelectrolyte nature of the gel (Katchalski *et al.*, 1971). The enzymes when entrapped in the polyanionic polyacrylate gels may be exposed to an entirely different microenvironment, resulting from a shift in the pH of the surrounding medium, from that when they are entrapped in the polyacrylamide gels, which is electrostatically neutral. This may affect the thermal inactivation of the individual enzymes to different extents, as pH is known to have a profound effect on the thermal denaturation of enzymes (Dixon & Webb, 1967; Segel, 1975). Moreover, the formation of electrostatic interactions between the enzymes and the support might affect the integrity of the active site to a different extent, resulting in the denaturation of the enzymes at different rates.

The t_4 value reached the maximum at 20% gel concentration for glucose oxidase and 40% or more for the glucoamylases. These results are consistent with the hypothesis that the optimal concentration of the gel required for stabilization of enzymes resulting from the retardation of their molecular mobilities may depend on their molecular weights. This is substantiated by the fact that 20% gel concentration was required to retard the mobility of glucose oxidase, whose molecular weight is 150 000, whereas for the glucoamylases, whose molecular weight is about 60 000, 40% or greater gel concentration was required.

The reason for the decrease in the thermal stability of the enzymes in highly concentrated polyacrylamide gels is not clear, and can probably be attributed to the mechanical compression of the enzyme molecules by the polymer chains.

In conclusion, the results presented above demonstrated that thermal stabilization can be achieved by physically trapping the enzymes in polyacrylamide gels. This phenomenon may be generalized to other enzymes, and may therefore serve as a valuable technique in stabilizing a wide variety of enzymes and for studying the physicochemical factors governing the thermal denaturation processes.

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