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Immobilized Triosephosphate Isomerases

A Comparative Study

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ABSTRACT

Pig muscle triosephosphate isomerase was covalently attached to polyacrylamide and silica-based supports possessing carboxylic or aldehyde functional groups or activated with *p*-benzoquinone. A silica-based support activated with *p*-benzoquinone proved to be the most advantageous. There were no profound alterations in the catalytic properties as a result of the immobilization. The immobilization enhanced the resistance against urea and heat treatment. At the start of the treatments, the enzyme was activated. The extent of activation depended on the pH, and on the buffer and salt concentrations. Increase of the ionic strength decreased or eliminated the activation. The phosphate ion had a specific effect on the thermal inactivation.

Index Entries: Triosephosphate isomerase, immobilized; support, silica-based; support, polyacrylamide-type; catalytic properties, immobilized triosephosphate isomerase; stability tests, immobilized triosephosphate isomerase.

INTRODUCTION

One of the most important requirements for the practical application of enzymes is their conformational stability, which depends on the molecular microheterogeneity and the presence of izoenzymes or other proteins in the technical preparations (*1*). Covalent attachment to solid-phase

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supports generally enhances the stability, but the chemical modification of the amino acid side-chains and/or the microenvironmental effects might lead to activity loss during the immobilization (2).

Triosephosphate isomerase is of practical importance in the preparation of dihydroxyacetone phosphate and in the determination of glycolytic intermediates. The pig muscle enzyme was immobilized by covalent attachment on both inorganic and organic supports with various properties. The catalytic properties and stabilities of the immobilized triosephosphate isomerases were compared. The influences of pH, ion concentration, and phosphate ions on the heat stability were also investigated.

MATERIALS AND METHODS

Triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase; EC 5.3.1.1) was isolated from pig skeletal muscle as reported earlier (3). The specific activity of the twice crystallized enzyme was 3,045 U/mg. One unit of activity is defined as the amount of enzyme catalyzing the formaton of 1 μmol of glycerol-3-phosphate/min at pH 8.0 and 25°C in a coupled enzyme reaction using glycerol-3-phosphate dehydrogenase. The enzyme preparation was gel-filtered on a Sephadex G-25 column (0.9×7.5 cm) at 4°C with 0.02*M* phosphate buffer (pH 7.0) as eluent. Glycerol-3-phosphate dehydrogenase (NAD^+) (*sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase; EC 1.1.1.8) isolated from rabbit skeletal muscle was a Reanal Factory of Laboratory Chemicals (Budapest, Hungary) product with a specific activity of 40 U/mg protein. One unit of activity is defined as the amount of enzyme catalyzing the conversion of 1.0 μmol of dihydroxyacetone phosphate to α-glycerophosphate/min at pH 7.4 at 25°C.

Akrilex C-100, a polyacrylamide-type bead (100–320 μm) polymer possessing carboxylic functional groups (6.4 meq/g dry wt) and Akrilex P-100, a polyacrylamide-bead polymer (particle size 40–120 μm; pore size, max 12 nm), were commercial products of Reanal. Silochrome-aldehyde and Silochrome *p*-benzoquinone supports were obtained from NPO Biolar (Riga-Olaine, Latvia). The functional group densities were 27 μmol/g and 37 μmol/g dry wt, respectively, the pore size was 50 nm and their particle size was 0.3–0.5 mm.

1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluene sulfonate was purchased from Fluka AG, Buchs, Switzerland. All other chemicals were reagent grade commercial preparations of Reanal.

Methods of Immobilization

Immobilization on Akrilex C

Triosephosphate isomerase was covalently attached to the support by using a method successfully applied for the immobilization of other en-

zymes (*4–15*). One g of Akrilex C-100 xerogel was suspended and swollen in 50 mL of 0.1*M* potassium phosphate buffer (pH 7.0), and 1 g of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluene sulfonate dissolved in 50 mL of cold (0°C) 0.1*M* potassium phosphate buffer, was added under continuous stirring and cooling in an ice-bath. After 10 min, the activated support was washed quickly with 50 mL of ice-cold 0.1*M* phosphate buffer (pH 7.0) to remove the excess of carbodiimide. Then, 50 mL of triosephosphate isomerase solution (41 mg in buffer) was added, and the pH was adjusted to 7.0. The mixture was incubated at 0–4°C during 48 h with agitation. The gel was filtered by suction and successively washed 3 times with 50 mL of 0.1*M* phosphate buffer (pH 7.0), 3 times with 100 mL of the same buffer containing 1*M* sodium chloride, and again 3 times with 100 mL of buffer to remove the unbound proteins.

Immobilization on Akrilex P

Activated with p-Benzoquinone

Akrilex P-100 xerogel was activated with *p*-benzoquinone and used as an enzyme support according to Kálmán et al. (*16,17*).

One hundred mg of dry beads was suspended and swollen in 4 mL of 0.1*M* potassium phosphate (pH 8.0), and 1 mL of 0.25*M* *p*-benzoquinone dissolved in 20% dioxane was added. During a 24 h incubation at 50°C, the originally colorless beads became brownish, indicating the activated state. After activation, the suspension was filtered by suction and washed 10 times with 20 mL of 20% dioxane and 10 times with water. Then 34 mg of triosephosphate isomerase dissolved in 2 mL of 0.1*M* phosphate buffer (pH 7.5) was added to the activated support. The mixture was stirred for 24 h at 4°C. The gel was filtered by suction and successively washed 3 times with 10 mL of 0.1*M* phosphate buffer (pH 7.5), 3 times with 10 mL of the same buffer containing 1.0*M* sodium chloride, and 3 times again with 10 mL of buffer.

Immobilization on Silochrome-Aldehyde

The attachment of the enzyme was carried out according to the method previously described for the immobilization of aldolase (*18*).

One g of support was washed with water and suspended in 8 mL of triosephosphate isomerase solution containing 76.8 mg of protein dissolved in 0.02*M* phosphate buffer (pH 8.0). The suspension was stirred for 2 h at 4°C. Then, the suspension was filtered by suction and washed with 5×40 mL of water to remove unbound proteins. After washing, the conjugate was stirred with 8 mL of 1% $NaBH_4$ for 15 min at 4°C, then filtered off and washed again with 5×40 mL of water. For the blocking of unreacted aldehyde groups, 8 mL of hydroxylamine (1 mg/mL, pH 8.0) was added and the suspension was stirred for 1 h at 4°C. The suspension was then filtered and washed with 5×40 mL of water, and the $NaBH_4$ treatment was repeated. Finally, the immobilized enzyme was washed with 3×40 mL of 0.02*M* phosphate buffer (pH 7.0).

Immobilization on Silochrome Activated with p-Benzoquinone

One g of Silochrome xerogel activated with *p*-benzoquinone was successively washed with 1*M* sodium chloride and with distilled water, and equilibrated with 0.02*M* phosphate buffer (pH 7.0). Fifty-five mg of triosephosphate isomerase in 6 mL of 0.02*M* phosphate buffer (pH 7.0) was added to the support. Coupling was performed at 4°C for 2 h with gentle shaking. The gel was filtered by suction and successively washed with 0.02*M* phosphate buffer (pH 7.0), with the same buffer containing 1.0*M* sodium chloride, and again with the buffer to remove unbound proteins.

Measurement of Protein

Protein determinations were performed according to the method of Bradford (*19*). The amount of bound protein was calculated as the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Triosephosphate Isomerase Activity

Triosephosphate isomerase activity was determined spectrophotometrically in a reaction mixture (3 mL) containing 94.9 m*M* triethanolamine buffer (pH 8.0), 0.29 m*M* D-glyceraldehyde-3-phosphate, 0.25 U/mL glycerol-3-phosphate dehydrogenase and 0.2 m*M* NADH. The reaction was started by addition of the enzyme solution, and the reaction rate was calculated from the decrease in NADH concentration.

In the case of immobilized enzyme, the reaction was started with 10–50 μg (wet wt) of immobilized enzyme. The reaction mixture was stirred for an appropriate time (1–5 min). The enzyme was then filtered off quickly (a few seconds), and the NADH was determined spectrophotometrically, at 340 nm.

Heat Stability Tests

Thermal inactivation experiments were carried out in 0.05 or 0.1*M* triethanolamine buffer in an incubation mixture of 1.0 mL. The concentration of the immobilized triosephosphate isomerase was 0.34 mg/mL and that of the soluble enzyme was 0.3 mg/mL. After appropriate incubation times, the samples were rapidly cooled in an ice-bath and, after dilution with buffer, the residual activities were determined at 25°C.

Urea Treatments

Urea treatment was performed in 0.05*M* triethanolamine buffer at pH 7.5 and 25°C in the absence of substrate. The denaturation was stopped by dilution with buffer and the residual activities were determined at 25°C.

Table 1
Immobilization of Triosephosphate Isomerase
by Covalent Bonding on Various Supports[a]

Support	Immobilized protein		Immobilized activity, %	Activity loss, %	Activity of immobilized enzyme	
	mg/g solid	%			U/g solid	U/mL wet gel
Akrilex C-100	28.3	68.5	4.7	71.6	387.2	5.5
Akrilex P-100 activated with *p*-benzoquinone	16.8	50.0	0.1	79.5	8.7	0.5
Silochrome activated with *p*-benzoquinone	11.1	40.9	3.3	79.2	182.0	67.4
Silochrome aldehyde	42.0	54.7	0.5	68.3	73.2	18.3

[a]For experimental details, see text.

RESULTS

Immobilization of Triosephosphate Isomerase

Triosephosphate isomerase was covalently attached to inorganic or organic supports. The experimental data are summarized in Table 1. Most protein was bound to Silochrome-aldehyde, but the bound enzyme activity was more than 5 times higher on one gram of Akrilex C xerogel and about 2.5 times higher on Silochrome activated with *p*-benzoquinone. A detailed comparison was made of the activities of the enzyme immobilized on Akrilex and Silochrome, both activated with *p*-benzoquinone. Surprisingly, the specific activity of the Akrilex-bound enzyme on a protein basis was only 3.1% of that for the Silochrome-bound enzyme. As regards the volume activity of swollen supports, much higher activities could be achieved with the application of an inorganic support than those with polyacrylamide beads. The activity loss was relatively high in all cases.

pH Dependence of Catalytic Activity

The experiments were performed in 0.1*M* triethanolamine buffer (Fig. 1). The optimum pH for the catalytic activity of the triosephosphate isomerase attached to Silochrome activated with *p*-benzoquinone was pH 8, which was practically identical with that of the soluble enzyme (pH 8.0–8.1), whereas the pH optimum of the Akrilex C-100-bound enzyme was shifted slightly in the alkaline direction, to pH 8.4–8.5.

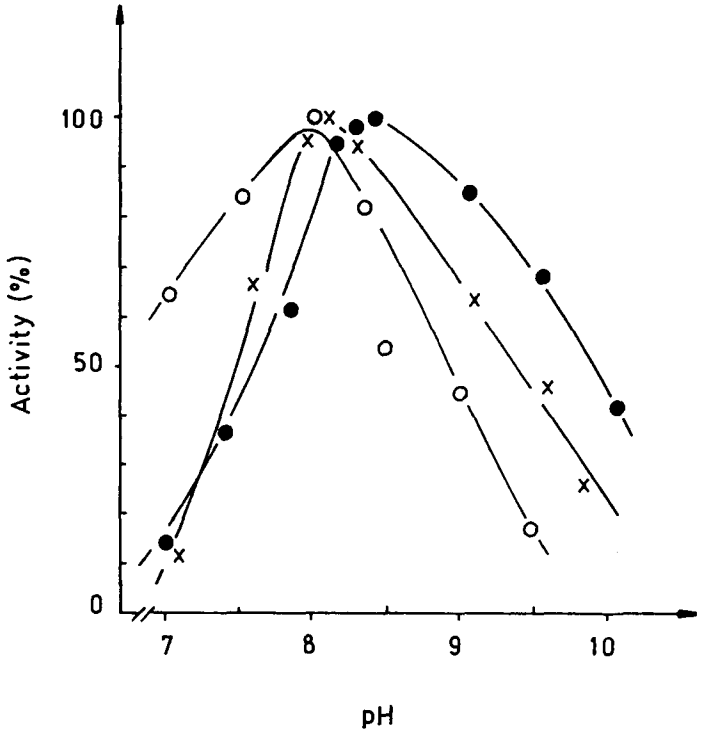

Fig. 1. Effect of pH on catalytic activities of soluble and immobilized triosephosphate isomerases. Experiments were performed in 0.1*M* triethanolamine buffer at 25°C. X, Soluble enzyme; ●, enzyme bound on Akrilex C-100; ○, enzyme bound on Silochrome activated with *p*-benzoquinone. For both soluble and immobilized enzymes, the maximum activity was taken as 100%.

Effect of Substrate Concentration on the Catalytic Reaction

The effect of the D-glyceraldehyde-3-phosphate concentration on the initial rate of the catalyzed reaction was investigated in the concentration range 0.5–3.0 m*M* at the optimum pH for catalytic activity. Experiments were carried out in 0.1*M* triethanolamine buffer (pH 8.0) at 25°C. For calculation of K_m values, kinetic plots according to Lineweaver and Burk (*20*) were used. From the plots, K_m of soluble triosephosphate isomerase was calculated to be $3.83 \times 10^{-5} M$. $K_{m\,app}$ for the Akrilex C-bound enzyme was $4.72 \times 10^{-5} M$, and that for the triosephosphate isomerase attached to Silochrome activated with *p*-benzoquinone was $2.14 \times 10^{-5} M$.

Resistance Against Urea

The denaturing effect of urea in a concentration of 8*M* in 0.05*M* triethanolamine buffer (pH 7.5) was tested at 25°C. Treatments were performed in the absence of substrate. Both the soluble and the immobilized

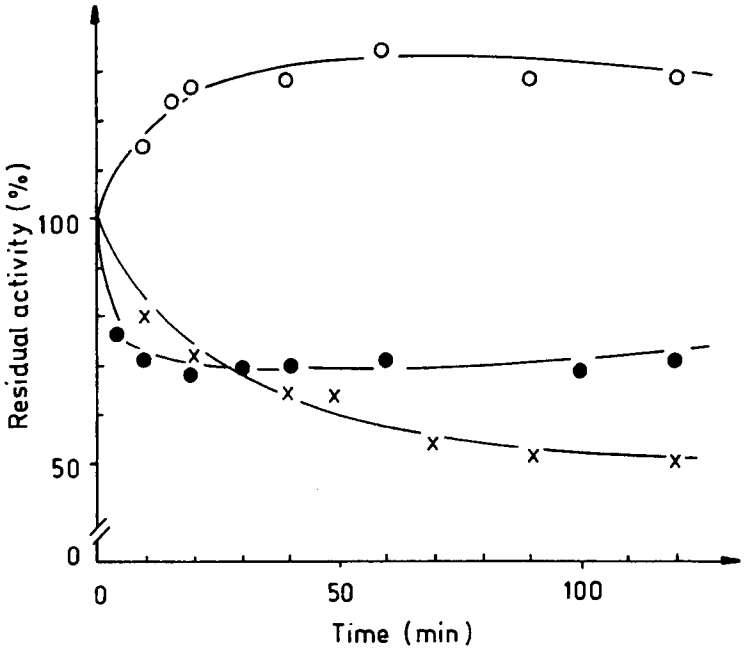

Fig. 2. Effects of urea on activities of soluble and immobilized triosephosphate isomerases. Incubation was carried out in 0.05*M* triethanolamine buffer (pH 7.5) at 25°C in the absence of substrate. Enzyme concentrations used: soluble enzyme, 0.68 mg protein/mL; immobilized enzymes, 0.65 mg protein/mL. Urea concentration: 8*M*. X, Soluble enzyme; ●, Akrilex C-100-bound enzyme; ○, enzyme bound on Silochrome activated with *p*-benzoquinone. For both soluble and immobilized enzymes, the starting activity was taken as 100%.

triosephosphate isomerase attached to Silochrome activated with *p*-benzoquinone were activated by urea, and the enhanced activity was maintained during the 2 h incubation period. The Akrilex C-100-immobilized enzyme was more resistant than the soluble enzyme against urea.

Thermal Stability

The rates of thermal inactivation of the soluble and immobilized enzymes were compared in 0.1*M* triethanolamine buffer (pH 8.0) at 50°C (Fig. 3). The triosephosphate isomerase immobilized on Silochrome showed a transient activation, whereas the Akrilex-bound enzyme was found to be less stable than the enzyme coupled to the inorganic support.

Ion Effects on Thermal Inactivation of Immobilized Triosephosphate Isomerase

The ion effects on the thermal inactivation were tested in the case of triosephosphate isomerase attached to Silochrome activated with *p*-benzoquinone. The effect of the hydrogen ion concentration on the thermal

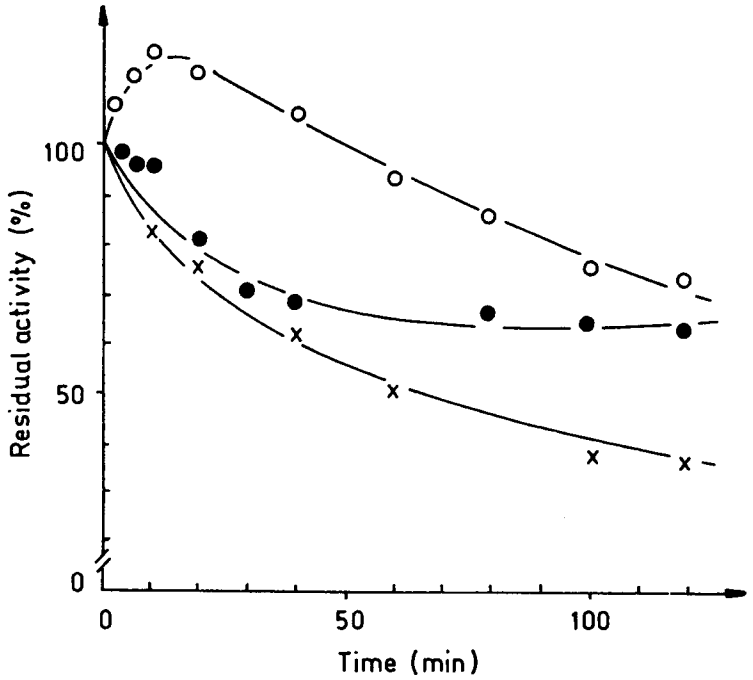

Fig. 3. Thermal inactivation of soluble and immobilized triosephosphate isomerases. Experiments were carried out in 0.01*M* triethanolamine buffer (pH 8.0) at 50°C. Enzyme concentrations used: soluble enzyme, 0.3 mg/mL protein; immobilized enzymes, 0.34–0.36 mg/mL protein. X, Soluble enzyme; ●, Akrilex C-100-bound enzyme; ○, enzyme bound on Silochrome activated with *p*-benzoquinone. For both soluble and immobilized enzymes, the starting activity was taken as 100%.

inactivation process was studied in 0.05*M* triethanolamine buffer at 50°C (Fig. 4). The activation was higher at pH 9.0 than at pH 8.0. No activation was observed at pH 7.0.

The effects of buffer and salt concentrations on the thermal inactivation process are presented in Fig. 5. The immobilized enzyme appeared to be more stable in 0.05*M* triethanolamine buffer than in 0.1*M* buffer because of the higher degree of activation. An increase of the ionic strength decreased or eliminated the activation. At high ionic strength the thermal inactivation of the immobilized triosephosphate isomerase was relatively fast. Phosphate ions in low concentration (0.05*M*) caused a marked activation (Fig. 6). The rate of activation was lower than that in 0.05*M* triethanolamine buffer.

Storage Stability

The storage stabilities of the soluble and the Silochrome-bound triosephosphate isomerase were compared in 0.1*M* phosphate (pH 7.0) at

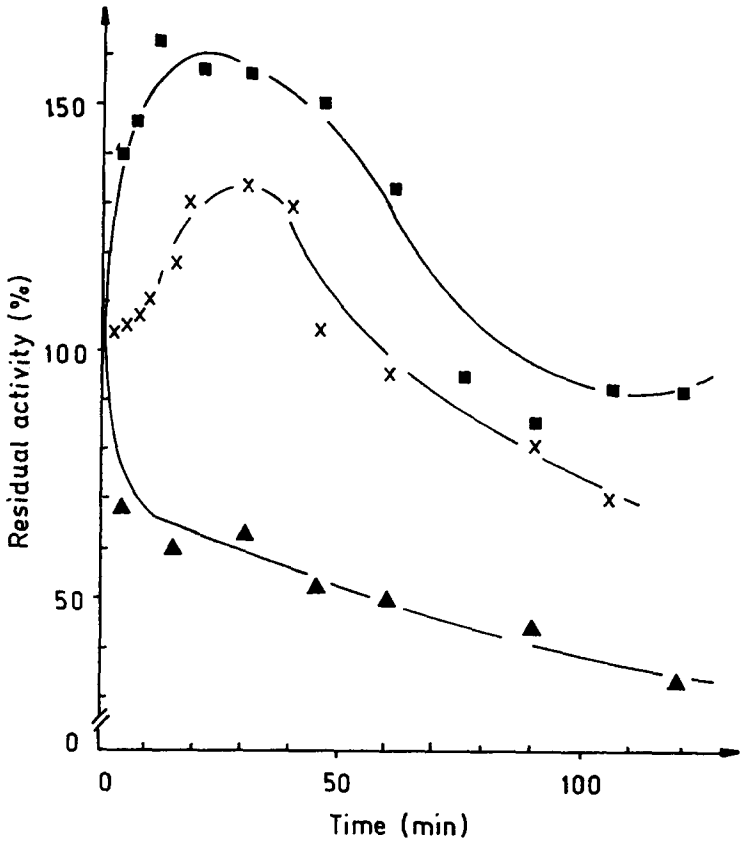

Fig. 4. pH dependence of thermal stability of triosephosphate isomerase attached to Silochrome activated with *p*-benzoquinone. Experiments were carried out in 0.05*M* triethanolamine buffer at 50°C. Enzyme concentration: 0.3 mg/mL protein. ▲, pH 7.0; X, pH 8.0; ■, pH 9.0. In all cases, the starting activity was taken as 100%.

4°C. Under such conditions, the immobilized enzyme proved to be more stable than the soluble one. During storage for 10 wk, the immobilized enzyme lost only 5% of its original activity, whereas the soluble enzyme was totally inactivated.

DISCUSSION

Triosephosphate isomerase, a glycolytic enzyme, was covalently attached to both inorganic and organic supports possessing different functional groups. A silica-based inorganic support, Silochrome, activated with *p*-benzoquinone proved to be the most advantageous.

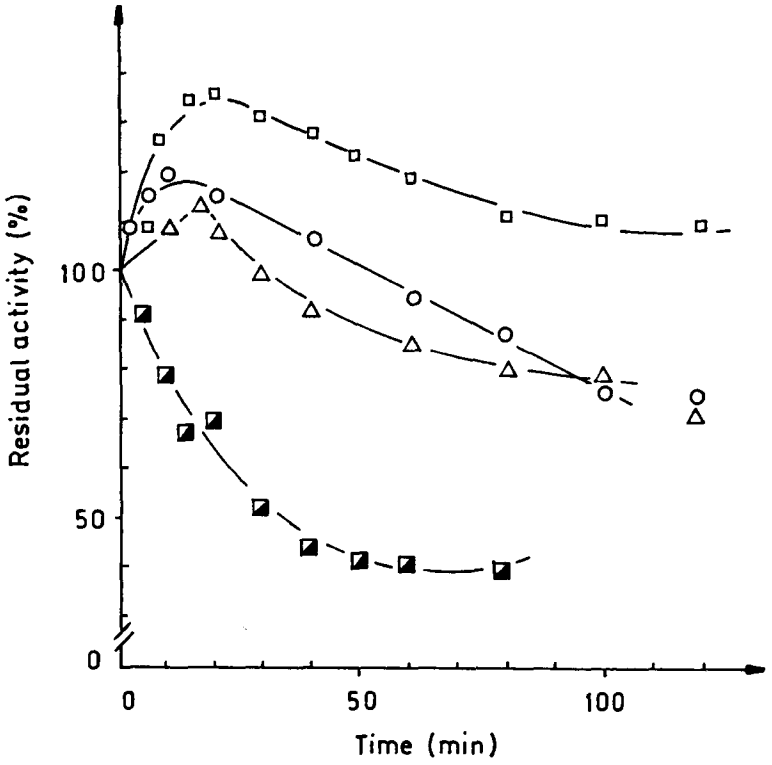

Fig. 5. Effects of buffer and salt concentrations on thermal inactivation of immobilized triosephosphate isomerase at pH 8.0 and 50°C. Enzyme concentration: 0.3 mg/mL protein. □, 0.05*M* triethanolamine; ○, 0.1*M* triethanolamine; △, 0.05*M* KCl in 0.05*M* triethanolamine buffer (pH 8.0); ◪, 0.5*M* KCl in 0.05*M* triethanolamine buffer (pH 8.0). In all cases, the starting activity was taken as 100%.

The catalytic properties of the immobilized triosephosphate isomerase were not profoundly changed compared with those of the soluble enzyme. Only in the case of the enzyme immobilized on the polyanionic Akrilex C-100 was an alkaline shift found in the optimal pH for the catalytic activity. The Michaelis constants were of the same order of magnitude.

The immobilization induced characteristic changes in the stability of triosephosphate isomerase. The immobilization enhanced the resistance against urea and heat treatment. At the start of the treatments, the enzyme attached to *p*-benzoquinone-Silochrome was activated. The extent of activation depended on the pH, and on the buffer and salt concentrations. An increase of the ionic strength decreased or eliminated the activation. It is supposed that secondary ionic interactions could limit or hinder the conformational changes resulting in the activation. It appears that the phosphate ion as substrate analog has a specific effect on the thermal inactivation process of the immobilized triosephosphate isomerase.

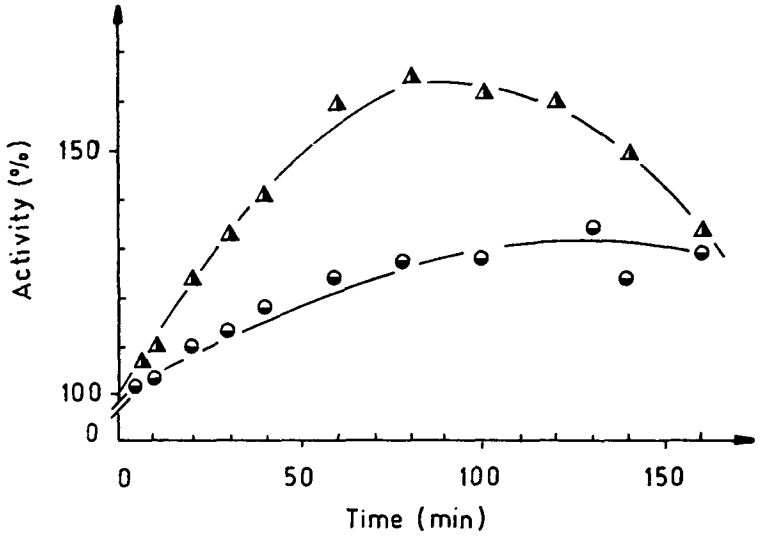

Fig. 6. Effect of phosphate ions on thermal stability of immobilized triosephosphate isomerase at 50°C. Experiments were carried out in 0.05*M* triethanolamine buffer (pH 8.0). Enzyme concentration: 0.3 mg/mL protein. ◭, 0.05*M* K_2HPO_4; ◒, 0.5*M* K_2HPO_4. In both cases, the starting activity was taken as 100%.

A detailed analysis of the complex process of inactivation of the immobilized triosephosphate isomerases is a further task.

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