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Comparative Studies on Soluble and Immobilized Rabbit Muscle Pyruvate Kinase

L. M. SIMON,* M. KOTORMÁN, B. SZAJÁNI, AND L. BOROSS

Department of Biochemistry, Attila József University, Szeged, PO Box 533, Hungary

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ABSTRACT

Rabbit muscle pyruvate kinase was immobilized by covalent attachment to a polyacrylamide support (Akrilex C) containing carboxylic functional groups. As a result of immobilization, the pH optimum for catalytic activity shifted into a more alkaline direction. The apparent K_m value with phosphoenolpyruvate increased, and that with ADP slightly decreased. With respect to the stability against urea and thermal inactivation, the immobilized pyruvate kinase seemed to be the more stable at lower urea concentrations and between 45 and 55°C. At 1.5 and 2.5*M* urea and at higher temperature, there were no marked differences between the soluble and the immobilized enzyme.

Index Entries: Pyruvate kinase, soluble and immobilized; covalent coupling, of pyruvate kinase to Akrilex C; catalytic properties, and stability of immobilized pyruvate kinase; kinase, soluble and immobilized pyruvate; rabbit muscle, soluble and immobilized rabbit muscle from; muscle pyruvate kinase, soluble and immobilized.

INTRODUCTION

A number of workers have described the preparation of enzymes attached to a water-insoluble support by a variety of methods (*1*,*2*). These solid-supported catalysts are of interest for both theoretical and practical reasons. First, they may form a model for enzymic reactions performed in an intracellular membrane; secondly, they may be used as recoverable

*Author to whom all correspondence and repair requests should be addressed.

catalysts in certain biochemical conversions on a laboratory or even an industrial scale.

Wilson et al. (3) attached pyruvate kinase to filter-paper disks, and described the operation of two enzyme reactors formed by coupling the reactions of pyruvate kinase and lactate dehydrogenase.

Campbell and Chang (4) coimmobilized pyruvate kinase and hexokinase within semipermeable collodion microcapsules; this system was capable of recycling both ATP and ADP. Pyruvate kinase has also been attached to aminosilanized porous glass and dichloro-*S*-triazinyl cellulose (5,6).

The present paper describes the preparation and properties of pyruvate kinase (EC 2.7.1.40) attached to Akrilex C, and the comparison of the soluble and immobilized forms.

MATERIALS AND METHODS

Pyruvate kinase was isolated from rabbit muscle according to Beisenherz et al. (7) and was stored in 3.2*M* ammonium sulfate solution until use. The specific activity of the enzyme was 60–100 U/mg protein and its molecular purity was about 30% as judged from polyacrylamide gel electrophoresis. Akrilex C, a polyacrylamide-type bead (100–320 μm) polymer containing carboxylic functional groups (6.4 mEq/g xerogel) was purchased from Reanal Factory of Laboratory Chemicals, Budapest, Hungary. The swollen gel excludes the molecules having molecular weight larger than about 100,000 daltons (8). The 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate was a Merck-Schuchardt product. Lactate dehydrogenase was obtained from Reanal and had a specific activity of 200 U/mg protein. All other chemicals were reagent-grade, commercial preparations (Reanal).

Preparation of Immobilized Pyruvate Kinase

A 0.5 g quantity of xerogel was swollen in 25 mL of 0.1*M* potassium phosphate buffer, pH 7.0, and 0.5 g of carbodiimide was added. The mixture was stirred for 20 min and 1–5 mL of the enzyme was added (50–70 mg/protein). After 24 h of stirring at 4°C the gel was successively washed three times with 25 mL of 0.1*M* potassium phosphate buffer (pH 7.0), three times with the same buffer containing 0.5*M* potassium chloride, and three times with buffer again to remove the noncovalently bound proteins. The activity and the protein were determined in the gel and in the washing solutions.

Measurement of Protein

Protein determinations were performed according to Lowry et al. (9). The amount of immobilized protein was determined indirectly from the difference between the amount of protein introduced into the reac-

tion mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Pyruvate Kinase Activity

Pyruvate kinase activity was determined spectrophotometrically by following the change in absorbance at 340 nm in a coupled assay with lactate dehydrogenase at 25°C. At the activity assay of the soluble enzyme, the reaction mixture (3.0 mL) contained 87 m*M* triethanolamine, 0.53 m*M* phosphoenolypyruvate, 2.5 *mM* $MgCl_2$, 10 m*M* KCl, 4.7 m*M* ADP, 0.2 m*M* NADH, and 10 U lactate dehydrogenase. The reaction was initiated by the addition of 100 μg of pyruvate kinase. The pH of the reaction mixture was 7.0. The rate of reaction was calculated from the disappearance of NADH, i.e., from the secondary reaction of the pyruvate producing lactate.

In the case of immobilized pyruvate kinase 100 mg of immobilized enzyme was suspended and swollen in 4.0 mL of triethanolamine (pH 7.6) containing 0.53 m*M* phosphoenolpyruvate, 2.5 m*M* $MgCl_2$, 10 m*M* KCl, and 4.7 m*M* ADP and was stirred for an appropriate period of time (1–10 min) at 25°C. Then the enzyme was filtered off quickly (a few seonds) and the pyruvate produced was determined in the filtrate using lactate dehydrogenase. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of pyruvate from phosphoenolpyruvate per minute at 25°C. In some experiments pyruvate kinase was assayed directly by determining the disappearance of phosphoenolpyruvate at 230 nm (*10*). This procedure, which yields the same value as does the coupled reaction with lactate dehydrogenase, was found to be particularly useful for measuring pyruvate kinase activity in the presence of urea.

The Michaelis constants were determined by varying the concentration of the substrates. The concentration of phosphoenolpyruvate was varied from 0.1 to 0.7 m*M*, and that of ADP from 0.1 to 1.5 m*M*.

The stability of pyruvate kinase was determined in 0.1*M* triethanolamine buffer. Inactivation was monitored by assaying the residual enzyme activity at appropriate time intervals.

RESULTS AND DISCUSSION

Pyruvate kinase one of the enzymes of glycolytic pathway catalyzing the dephosphorylation of phosphoenolpyruvate to pyruvate. Its immobilized form might be applied in packed bed reactors for the determination of phosphoenolpyruvate concentration in fluids of different origin using together with lactate dehydrogenase in a coupled-enzyme reaction (cf. 5). To produce immobilized pyruvate kinase the enzyme was covalently attached to a polyacrylamide-type bead polymer containing carboxylic functional groups activated by water-soluble carbodiimide. The results

are summarized in Table 1. As it can be seen from the table, about 30% of protein introduced into the reaction mixture bound to the support. On the basis of this observation, we suppose that only the carboxylic groups located on the outer surface of the support participated in the coupling reaction, since the swollen gel excluded a significant part of the enzyme molecules.

The catalytic activity of the immobilized pyruvate kinase was 190 U/g solid and about 20% of the original activity was lost during the immobilization. Probably, many enzyme molecules denatured during the coupling procedure and/or were sterically hindered by the structural parts of support or other immobilized protein molecules. Some inhibition could be caused by supposed multipoint connections between the enzyme and the polymer matrix.

pH Dependence of Activity

A change in pH optimum normally accompanies with the insolubilization of enzymes, depending upon the polymer used as the support (*11*). Since enzyme activity is markedly influenced by environmental conditions, and especially by the pH, the altered behavior of the enzyme caused by the immobilization is useful for understanding the structure–function relationship of enzyme proteins. Therefore, it is very useful to compare the activities of the soluble and immobilized enzymes as a function of pH. The pH dependences of the activities of soluble and immobilized pyruvate kinase are shown in Fig. 1 at the same ionic strength. The optimum pH was at about 7.0 for the soluble enzyme, and 7.6 for the immobilized pyruvate kinase. This shift in pH optimum might be due to the polyanionic microenvironment surrounding the enzyme molecule, or to the decrease in the number of positive side chains of the enzyme molecule as a result of immobilization (*12*). Therefore the number of residual free carboxyl groups was determined after the coupling reaction by titration with 0.001*M* NaOH and found to be 0.7 mEq/g solid. On the basis of this results the immobilized enzyme was localized in slightly anionic microenvironment which might cause an alkaline shift in pH optimum value.

TABLE 1
Balance Sheet of Immobilization of Pyruvate Kinase[a]

Protein content of reaction mixture	108 mg	100%
Total activity of mixture initially	5168 U	100%
Immobilized activity (per g dry gel)	190 U	3.6%
Immobilized protein (1 g dry gel)	26.9 mg	27.4%
Activity recovered in filtrate and washing solutions	3834 U	74.18%
Activity loss	1144 U	22.1%

[a]The experiment was carried out in 0.1*M* potassium phosphate buffer, pH 7.0, at 4°C. For experimental details, see text.

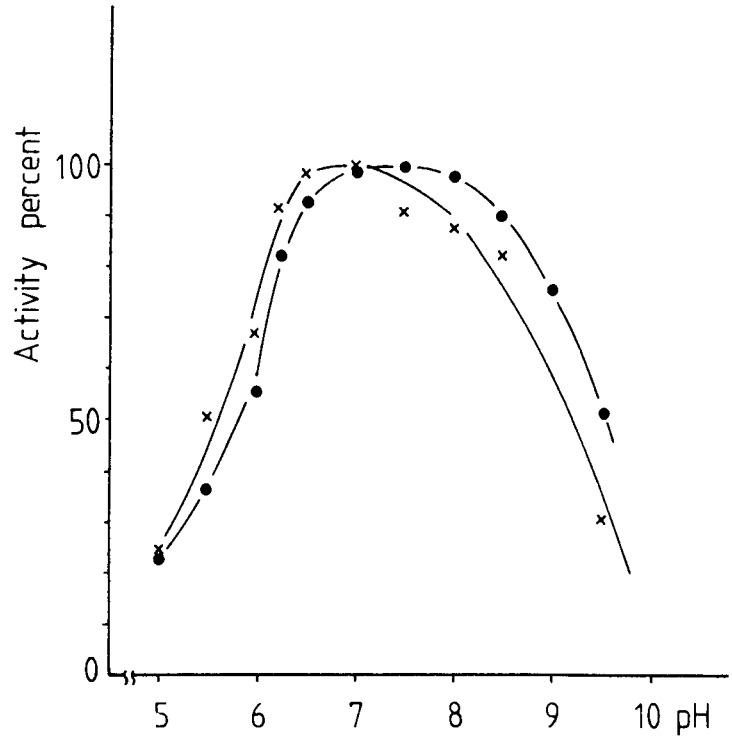

Fig. 1. Effect of pH on activity of soluble and immobilized pyruvate kinase. Experiments were carried out in 0.1*M* potassium phosphate buffer, pH 5–8, and 0.1*M* triethanolamine buffer, pH 7.5–9.5, at 25°C. For both soluble and immobilized enzyme, the maximum activity measured was taken as 100%. (The maximum activities soluble enzyme 6.0 U/mL; immobilized enzyme 3.0 U/mL). Symbols: (×) soluble enzyme, (●) immobilized enzyme.

Temperature Dependence of Activity

The temperature dependence of pyruvate kinase activity was determined in 0.1*M* triethanolamine buffer at the optimum pH for the catalytic activity (soluble enzyme, pH 7.0; immobilized enzyme, pH 7.6) in the temperature range 15–55°C. Reaction time was 5 min. Figure 2 indicates that the temperature optimum lies at about 40°C for the soluble enzyme and between 35 and 42°C for the immobilized one.

Michaelis Constants

Lineweaver-Burk plots were used to determine the Michaelis constants (K_m) of the soluble and the immobilized enzyme. The results are presented in Table 2.

In the case of the pyruvate kinase both the substrate (phosphoenolypyruvate) and the cosubstrate (ADP) has negative charges. It would be expected that both substrates are repulsed by the negative charges of support resulting in increased $K_{m,app}$ values. The opposite direction in the changes of apparent K_m values for the phosphoenoly-

TABLE 2
Michaelis Constants of Soluble and Immobilized Pyruvate Kinase[a]

Substrate	Michaelis constant, K_m, mM	
	Soluble enzyme	Immobilized enzyme
Phosphoenolpyruvate	0.52 ± 0.03	0.80 ± 0.06
ADP	0.54 ± 0.02	0.42 ± 0.04

[a]Experiments were carried out in 0.1*M* triethanolamine buffer at the optimal pH for catalytic activity (soluble enzyme, pH 7.0; immobilized enzyme pH 7.6).

pyruvate and ADP reveal the complex character of the forces operating in the formation of the enzyme–substrate complex. It is suggested that at the formation of the enzyme–phosphoenolpyruvate complex dominate the repulsive forces between the negative charges surrounding the enzyme and the negatively charged substrate. For ADP the repulsive forces are overcome by advantageous interactions existing between the matrix and the cosubstrate. The direction of the changes in $K_{m,app}$ values might be influenced by the altered structure of the immobilized enzyme molecules too.

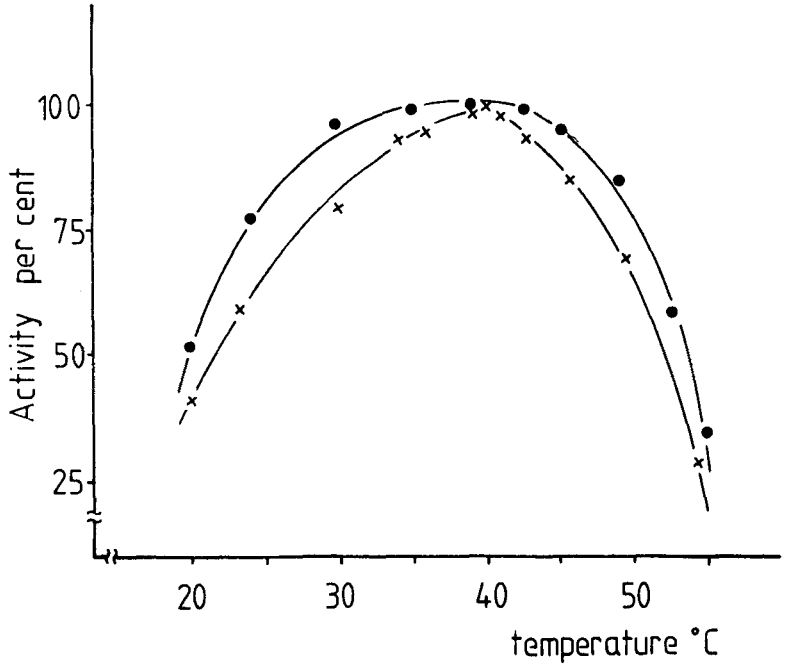

Fig. 2. Effect of temperature on activity of soluble and immobilized pyruvate kinase. Experiments were carried out in 0.1*M* triethanolamine buffer. The maximum activities (soluble enzyme: 9.0 U/mL; immobilized enzyme, 5.4 U/mL) were taken as 100%. Symbols: (×) soluble enzyme, (●) immobilized enzyme.

Wilson et al. (3) determined the K_m values of soluble and immobilized pyruvate kinase (covalently attached to cellulose paper) for ADP and found to be 0.52 and 0.48 m*M*.

Thermal Stability

The rate of thermal inactivation of pyruvate kinase was studied in the temperature range 45–65°C at the optimal pH for the catalytic activity (soluble enzyme, pH 7.0; immobilized enzyme, pH 7.6) in 0.1*M* triethanolamine buffer. The pH of buffer was monitored during the experiments and it did not change at the elevated temperatures. It was found that both the soluble and the immobilized enzyme was stable at 45°C, and at 50°C their heat inactivation proceed with measurable velocity. The time curves of inactivation fitted well to the theoretical curve of first-order reactions. The calculated half-life values, summarized in Table 3, show that the immobilized enzyme was apparently more stable at 50 and 55°C. After a 3-h incubation at 50°C, more than 60% of the activity of the immobilized enzyme remained, compared with only about 30% of the original activity of the soluble enzyme. At 60 and 65°C both the soluble and the immobilized enzyme were inactivated quickly.

Stability to Change in pH

The stability of pyruvate kinase was investigated at pHs 7, 8, and 9, respectively, in 0.1*M* triethanolamine buffer at 25°C. The results are presented in Fig. 3. Like the soluble enzyme, the immobilized pyruvate kinase was most stable at pH 7. In the case of the soluble pyruvate kinase the fast activity loss in the initial period of the heat treatment was probably caused by the dissociation of the oligomer to less stable dimeric or monomeric form.

Stability Against Urea

The immobilization of enzymes induces changes in their stability toward protein-denaturing agents. The effect of urea on the stability of pyruvate kinase was studied in the concentration range 0.1–3.0*M* in 0.1*M*

TABLE 3
Comparison of Heat Stability of Soluble and Immobilized Pyruvate Kinase[a]

Temperature, °C	Half-life, h	
	Soluble enzyme	Immobilized enzyme
50	6.55	15.73
55	3.36	7.05
60	1.05	0.74

[a]Experiments were carried out in 0.1*M* triethanolamine buffer at the optimal pH for catalytic activity (soluble enzyme, pH 7.0; immobilized enzyme pH 7.6).

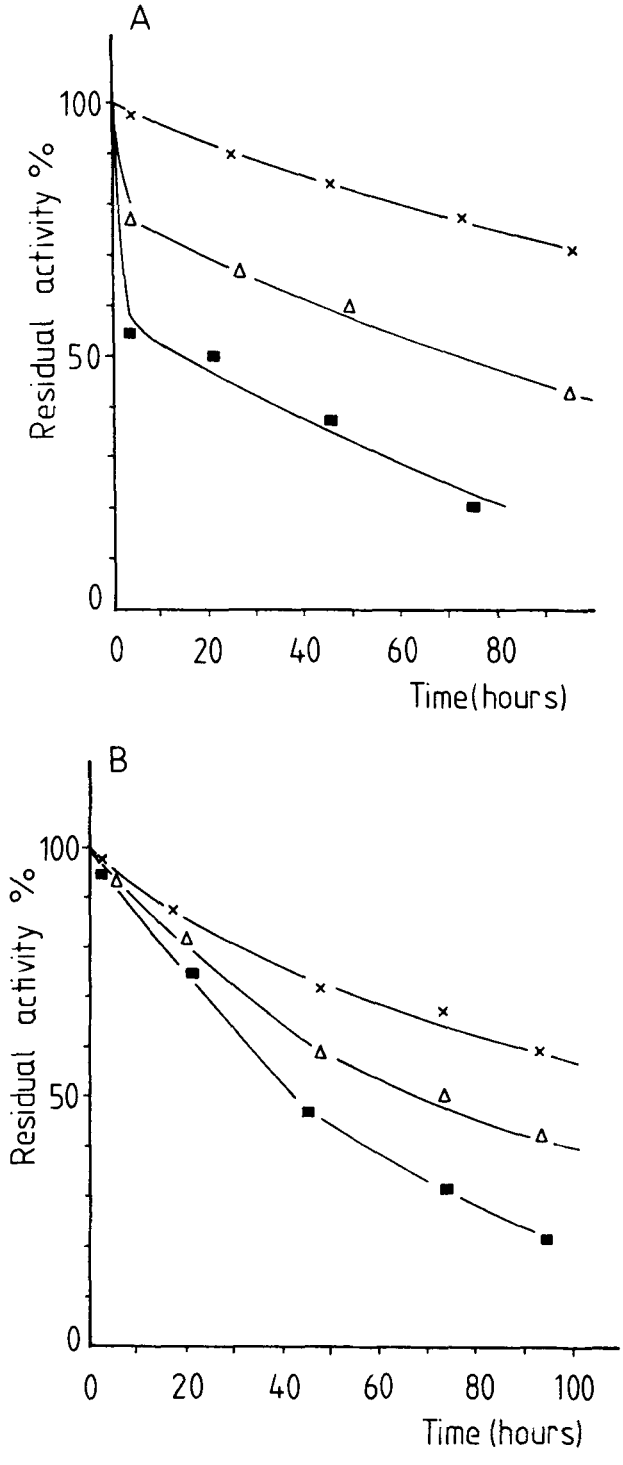

Fig. 3. Stability of soluble (A) and immobilized (B) pyruvate kinase at various pH values at 25°C: (×) pH 7.0; (△) pH 8.0; and (■) pH 9.0.

triethanolamine buffer, pH 7.6. The progress curves for inactivation of the soluble and immobilized enzymes are shown in Fig. 4. A fast loss of activity was observed after some minutes of urea treatment, followed by a slower inactivation period. The loss of activity in the fast period depended on the concentration of urea. In the presence of a low concentration of urea the immobilized enzyme proved to be the more stable. It is

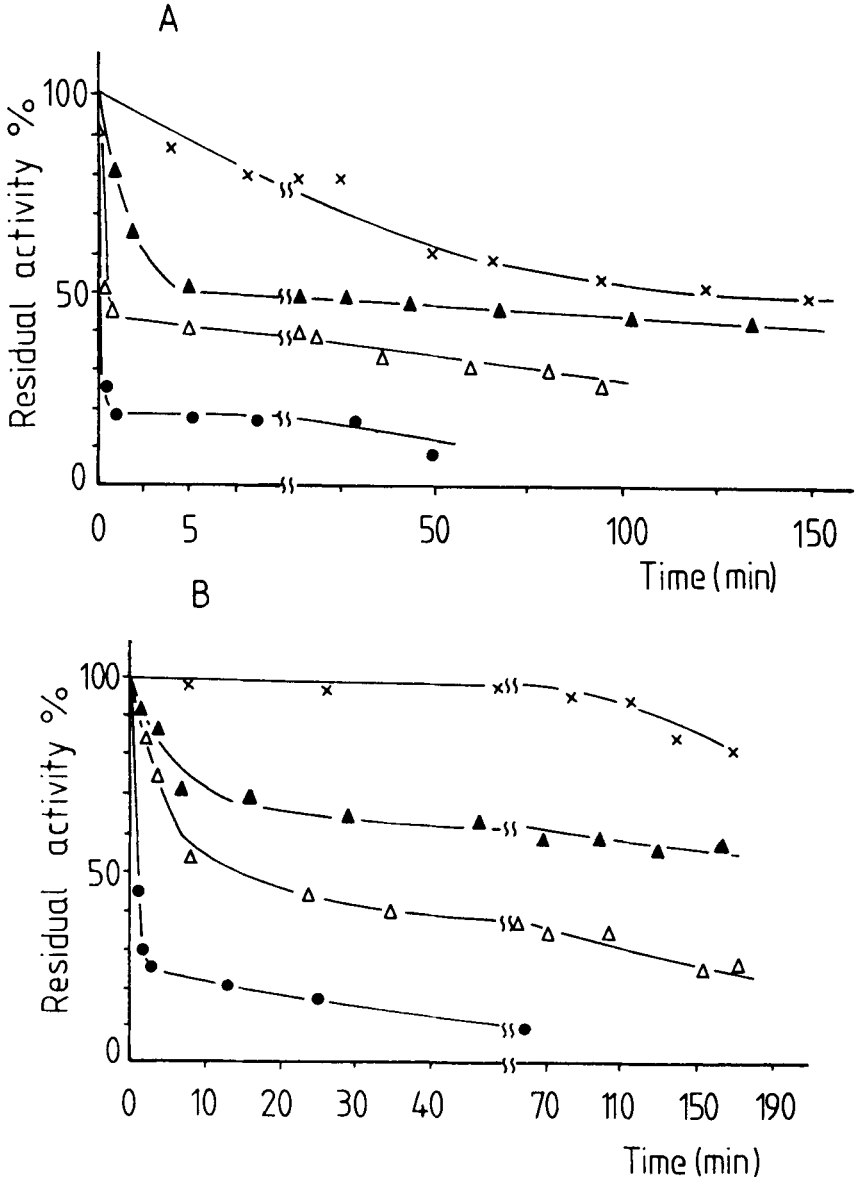

Fig. 4. Effect of urea on activity of soluble (A) and immobilized (B) pyruvate kinase. Enzyme concentrations used were soluble enzyme, 0.1 mg protein/mL; immobilized enzyme, 10.0 mg solid/mL. The starting activities (soluble enzyme 6.0 U/mL; immobilized enzyme, 2.0 U/mL) were taken as 100%. Experiments were carried out in the presence of urea: (×) 0.18*M*; (▲) 1*M*; (△) 1.5*M*; (●) 2.5*M*.

suggested that in the case of the soluble enzyme the initial fast activity loss might be caused by the dissociation of the tetrameric enzyme and a faster denaturation of the dimeric and monomeric forms. This idea is based on the data of literature (*13–17*). Steinmetz and Deal (*13*) pointed out that the native tetramer dissociated first into active dimers and then into monomers. Doster and Hess (*16*) reported that the native tetramer was transformed into a less compact tetramer, and that this was followed by dissociation into partially folded dimers and disordered monomers.

In the case of the immobilized enzyme the phenomenon seems to be more complicated. It should be considered the existence of the enzyme molecules differently bound on the support. The steric position of the molecules and/or their single point or multipoint attachment influences their stabilities against heat treatment, changes in pH and denaturing agents, respectively. The dissociation of noncovalently bound subunits might also altered the stabilities.

We investigated in more details the effect of 4*M* urea on the immobilized pyruvate kinase. About 3% of the bound protein released from the support, but the recovery of activity in the solution—after removal of denaturing agent—was not measurable. This might mean that protein contamination released or the dissolved subunits associated to inactive tetramers.

Storage Stability

The storage stability of Akrilex pyruvate kinase was studied at 8°C. The results are shown in Fig. 5. It can be seen that after about 15 d half of the activity was lost, but about 40% of the original activity was retained during the experiments over 40 d.

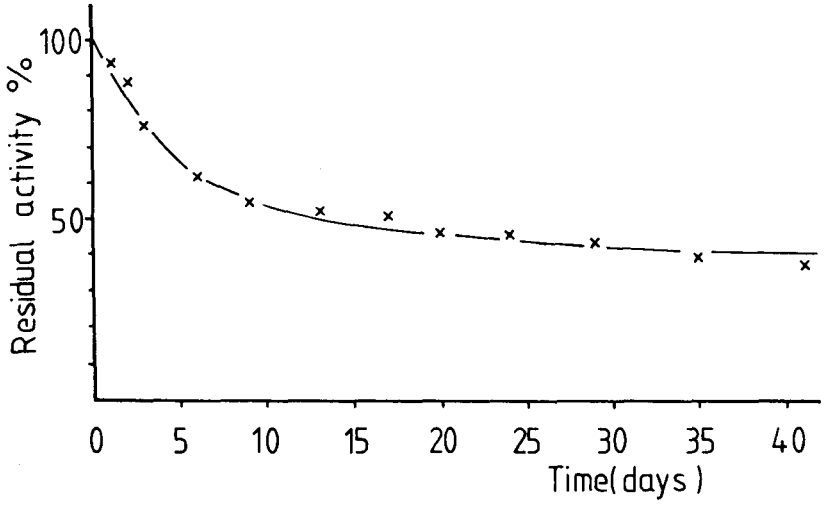

Fig. 5. Storage stability of immobilized pyruvate kinase at 8°C.

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