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Characterization and Comparison of Soluble and Immobilized Pig Muscle Aldolases

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ABSTRACT

Pig muscle aldolase was insolubilized by covalent attachment to a polyacrylamide matrix containing carboxylic functional groups. The catalytic activity of the Akrilex C-aldolase was 2014 units/g solid, i.e., an activity loss of only about 5% relative to the initial activity. The pH optimum for catalytic activity shifted form 7.25 to 7.5 and the apparent temperature optimum from 313 to 318 K. The Michaelis constant of the insolubilized enzyme was significantly higher than that of the soluble aldolase. Heat- and urea-inactivation experiments revealed that the immobilization increased the stability of the enzyme.

Index Entries: Immobilized aldolase; pig muscle aldolase; covalent attachment; polyacrylamide matrix, containing carboxylic functional groups; Akrilex C-100; gel, with high aldolase activity; aldolase, with high stability.

INTRODUCTION

Different enzymes have recently been immobilized for the continuous production of metabolites or determination of the concentrations of their substrates. Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-

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phosphate lyase, EC 4.1.2.13), a glycolytic enzyme, was entrapped in polyacrylamide gel (*1–4*) or was covalently bound to various polymeric supports, such as Sepharose 4B, aminoethyl cellulose, poly(maleic anhydride), *p*-aminobenzyl cellulose, CM-agarose, and polyacrylamide gels (*5–8*).

We have attempted to find a support suitable for the covalent binding of aldolase with high efficiency, with or without only slight loss of activity. Akrilex C-100, a polyacrylamide support having carboxylic functional groups, proved to be appropriate for this purpose. A comparative study on the catalytic properties and stabilities of soluble and immobilized aldolases is reported in this paper.

MATERIALS AND METHODS

Aldolase was isolated from pig muscle (*9*). Its specific activity was 8.2 U/mg. Akrilex C-100, a polyacrylamide-based material (100–320 μm) containing carboxylic functional groups (6.2 ± 0.3 mEq/g), was a product of Reanal Laboratory Chemicals (Budapest, Hungary). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) was purchased from Sigma (St. Louis, USA) and ethylene glycol monomethyl ether from Merck (Darmstadt, FRG). All other chemicals were reagent-grade commercial preparations.

Immobilization of Aldolase

Immobilized aldolase was prepared by the method previously described for aminoacylase and cholinesterase (*10–12*). One gram of xerogel was suspended and swollen in 50 mL of 0.1*M* potassium phosphate buffer (pH 7.0) and 2 g CMC, dissolved in 25 mL of cold buffer, was added under stirring and cooling in an ice bath. After 10 min mixing, 0.4 g pig muscle aldolase in 25 mL buffer was added to the suspension. The mixture was stirred at 4°C for 48 h. Then the gel was washed three times with 100 mL of 0.1*M* potassium phosphate buffer (pH 7.0), three times with 100 mL of the same buffer containing 0.5*M* sodium chloride, three times with 100 mL of buffer once again to remove the unbound proteins. Akrilex C-100, with its carboxylic functional groups activated by CMC, was found to be suitable for the covalent attachment of pig muscle aldolase. The volume of swollen, sedimented gel was 120 mL. This gel suspension was stored at 283 K and used for experiments.

Protein was measured according to Lowry et al. (*13*). The amount of bound protein was calculated as the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washings after immobilization.

Assay of Aldolase Activity

Aldolase activity was assayed in some experiments with dinitrophenylhydrazine (*14*) and in other experiments by the use of a second enzyme. In the latter case, the reaction mixture contained 0.8 m*M* FDP, 1.7 m*M* NAD, 33 U glyceraldehyde-3-phosphate dehydrogenase (GAPD), and 0.8 m*M* Na_2HAsO_4 in 0.1*M* Tris/HCl buffer, pH 7.5. The reaction was started with 50 μL soluble aldolase and was followed via the production of NADH + H^+ at 340 nm. Immobilized enzyme was assayed in the same mixture with continuous stirring. The reaction was started with 0.1 mg immobilized enzyme (i.e., 50 μL suspension contained 0.5 mg gel–enzyme) and stopped by filtration of the gel. The activity was calculated from the difference in extinction of the starting and the filtered reaction mixtures at 340 nm. The activity values measured in the two ways were in good agreement.

RESULTS

Immobilization of Aldolase

The covalent binding of aldolase on the Akrilex C-100 support occurred with high efficiency. The activity and the recovery in the immobilization experiment are shown in Table 1.

Storage Stability

The insolubilized enzyme had a much higher storage stability than that of the soluble enzyme. It lost only 48% of its activity during 8 wk at 283 K in 0.1*M* Tris/HCl buffer, pH 7.6, whereas the soluble enzyme lost nearly its total activity during 11 d (Table 2).

TABLE 1
Data on Immobilization of Pig Muscle Aldolase on Akrilex C-100 Gel

Protein concentration in the reaction mixture	400 mg	100%
Total activity at the beginning	3272 units[a]	100%
Immobilized protein, 1 g gel[b]	270 mg	67.75%
Immobilized activity, 1 g gel[b]	2014 units[a]	61.6%
Activity recovered in filtrate and washings	1084 units[a]	33.1%
Activity loss	174 units[a]	5.3%

[a]One enzyme unit corresponds to 1 μmol substrate conversion to product during 1 min.
[b]Calculated for 1 g xerogel matrix.

TABLE 2
Stability of Immobilized Aldolase in 0.1*M* Tris/HCl Buffer[a]

Duration of incubation, d	Remaining activity, %	
	Immobilized aldolase	Soluble aldolase
0	100	100
1	99.8	79.0
3	98.2	48.0
7	96.1	21.3
11	95.0	3.9
14	93.8	0.0
21	89.0	—
28	77.0	—
42	63.1	—
56	52.1	—

[a]pH 7.6, at 283 K. The concentration of soluble enzyme was 0.1 mg/mL (the same results were obtained using 0.01 or 1.0 mg/mL enzyme solution).

Comparison of the Catalytic Properties of Soluble and Immobilized Aldolases

Catalytic Activity

With FDP as substrate, the catalytic activity of the immobilized enzyme was found to be 2014 U/g solid. Its specific activity was 7.5 U/mg. The enzyme lost only about 5% of its original activity during the procedure of immobilization.

pH-Dependence of Activity of Insolubilized Aldolase

The pH-dependences of activities of the soluble and immobilized aldolases were measured in the pH range 6.0–8.0 with 0.05*M* phosphate buffer, and at pH 7.0–9.0 with 0.05*M* Tris/HCl buffer (Fig. 1). The pH optimum of the immobilized aldolase activity was at 7.5, and that of the soluble enzyme was at 7.25.

Temperature Dependence of Enzyme Activity

This was measured in the temperature range 298–333 K, the enzyme activity being determined by the dinitrophenylhydrazine method. The apparent temperature optimum of Akrilex–aldolase lay at 318 K, compared with 313 K for the soluble enzyme at the pH optimum (Fig. 2).

Michaelis Constant

The value of K_{mapp} for the immobilized aldolase was $1.5 \times 10^{-4}M$ for FDP in 0.05*M* Tris/HCl buffer, pH 7.6, the activity being determined by the enzymatic method. This value was five times that of the soluble enzyme (Fig. 3).

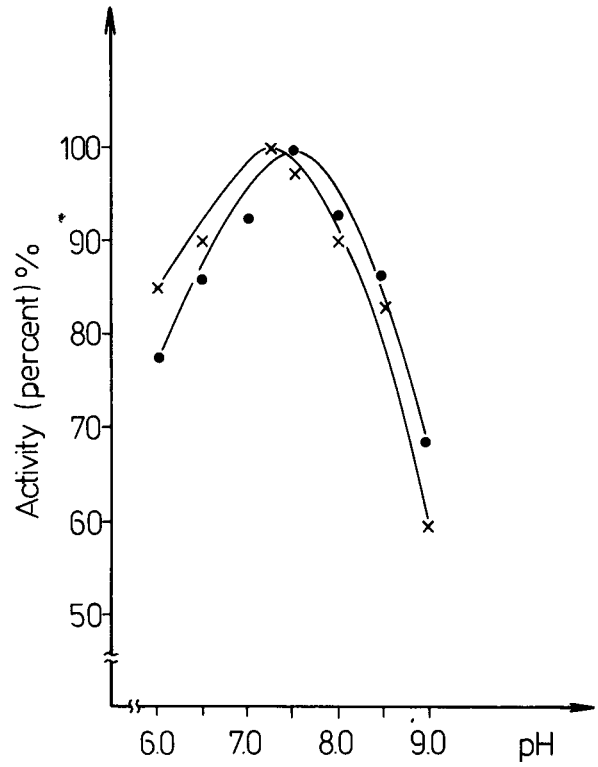

Fig. 1. pH dependences of activities of soluble and immobilized aldolases. The experiments were carried out in 0.05*M* phosphate buffer (pH 6.0–8.0) and in 0.05*M* Tris/HCl buffer (pH 7.0–9.0) Symbols: ×, soluble enzyme; •, immobilized enzyme. For both soluble and immobilized enzyme, the maximum activity measured was taken as 100%.

Comparison of Thermal Stabilities of Soluble and Immobilized Aldolases

The rates of heat-inactivation of immobilized and soluble aldolases were measured in the temperature range 323–338 K at the pH optimum of enzyme activity (Fig. 4). A slight activating effect of the elevated temperature was observed at the beginning of incubation at 323 and 328 K; this was followed by a fast decrease in activity. Subsequently, slow inactivation occurred. At higher temperatures the rate of inactivation of Akrilex C-aldolase was markedly increased, and at 338 K 30 min was sufficient for total inactivation of the enzyme. The rate of heat inactivation of the insolubilized enzyme was lower than that of soluble aldolase (Fig. 4).

pH-Dependence of Heat Inactivation

The effects of pH on the rates of heat inactivation of immobilized and soluble aldolases are shown in Fig. 5; the enzyme was incubated at 328 K. The thermal stability of the insolubilized enzyme was maximum in

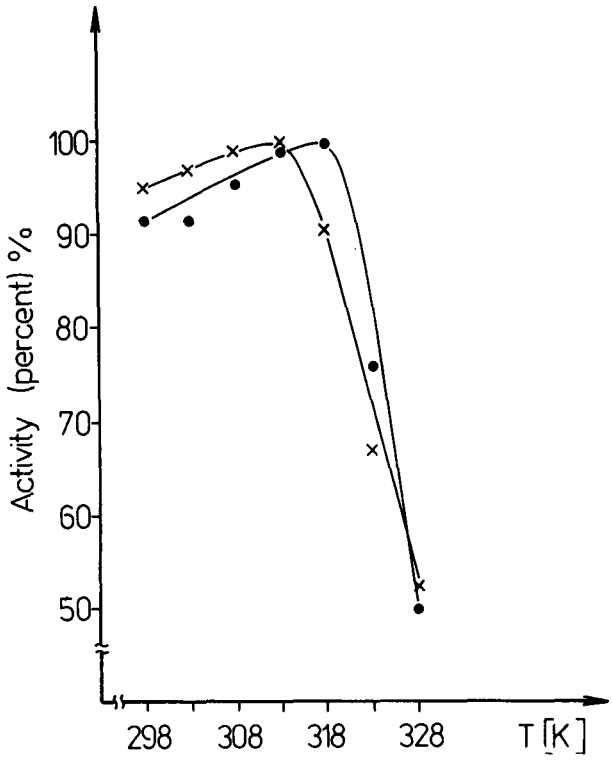

Fig. 2. Effects of temperature on activities of soluble and immobilized aldolases. The experiments were carried out in 0.05*M* Tris/HCl buffer, pH 7.5. The enzyme activity was measured by the method of Sibley and Lehninger. Symbols: ×, soluble enzyme; •, immobilized enzyme.

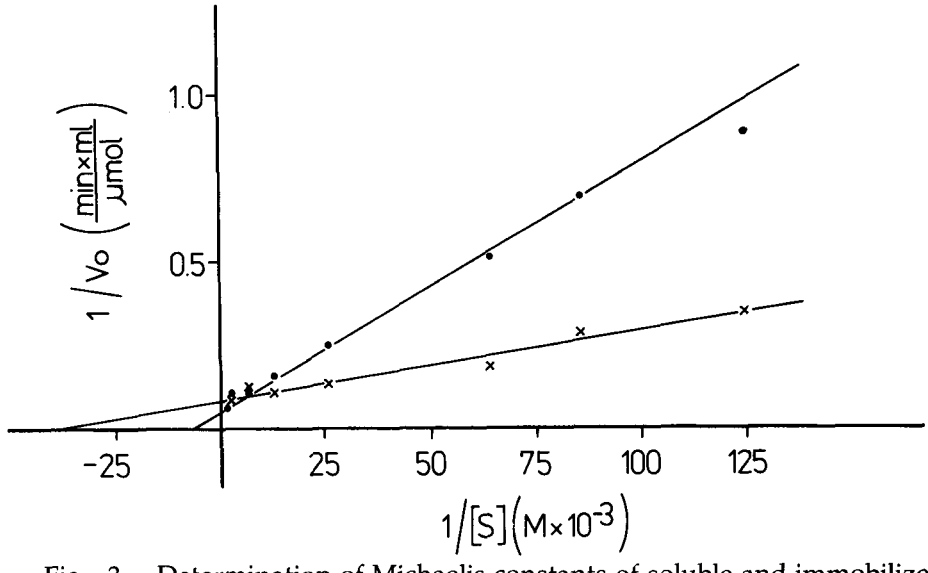

Fig. 3. Determination of Michaelis constants of soluble and immobilized aldolases. The experiments were carried out in 0.05*M* Tris/HCl buffer, pH 7.6, at 298 K, with FDP as substrate. Symbols: ×, soluble enzyme; •, immobilized enzyme.

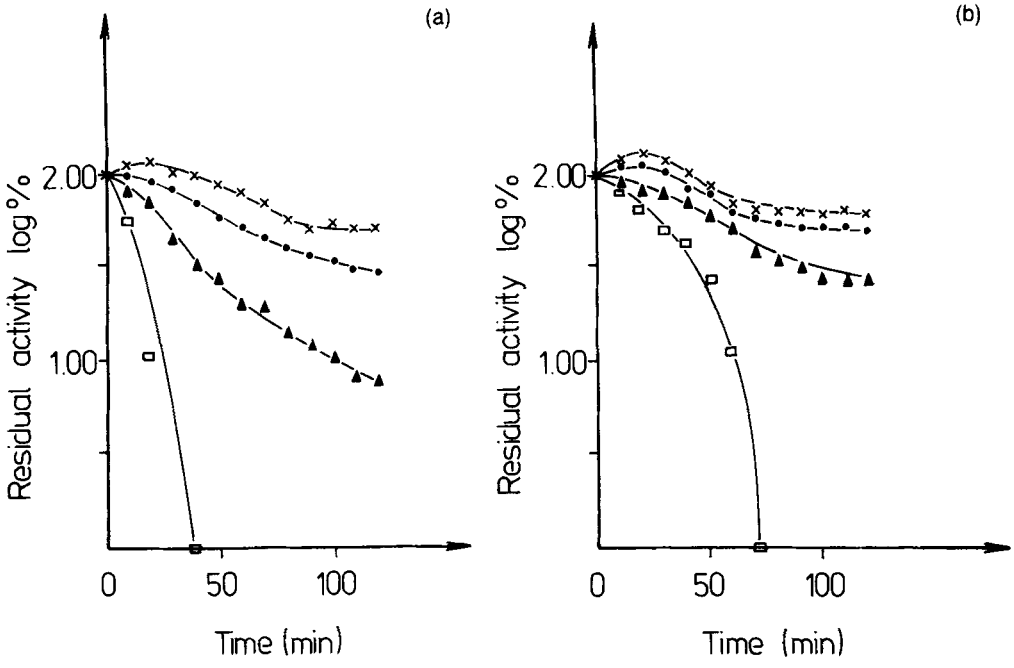

Fig. 4. Heat-inactivation of soluble (a) and immobilized (b) aldolases at various temperatures. Experiments were carried out in 0.05*M* Tris/HCl buffer, pH 7.5. Concentrations used: immobilized enzyme 0.833 mg solid/mL, 0.01 mg/mL soluble enzyme. Heat-inactivation progress curves are in a semi-logarithmic representation. Symbols: ×, 323 K; ●, 328 K; ▲, 333 K; □, 338 K. The starting activity was taken as log 100%.

the pH range 7.0–7.5, compared with pH 6.0–7.5 for the soluble enzyme. The thermal stability of Akrilex–enzyme was lower than that of the soluble aldolase at pH 6.0.

Comparison of the Effects of Urea on the Activities of Immobilized and Soluble Enzyme

The degrees of unfolding and dissociation of aldolase were investigated in the urea concentration range 1.0–5.0*M*. Elevation of the urea concentration from 1.0 to 4.0*M* caused only a slight decrease in the catalytic activity of Akrilex C-aldolase, but at 5.0*M* urea, the enzyme activity decreased significantly, and during 2 h it lost nearly its total activity. Rapid inactivation of the soluble enzyme was observed on increase of the urea concentration, and in 3.0*M* urea the aldolase unfolded and dissocaited totally during 2 h.

DISCUSSION

The covalent attachment of aldolase to a polyacrylamide support containing carboxylic functional groups activated by water-soluble

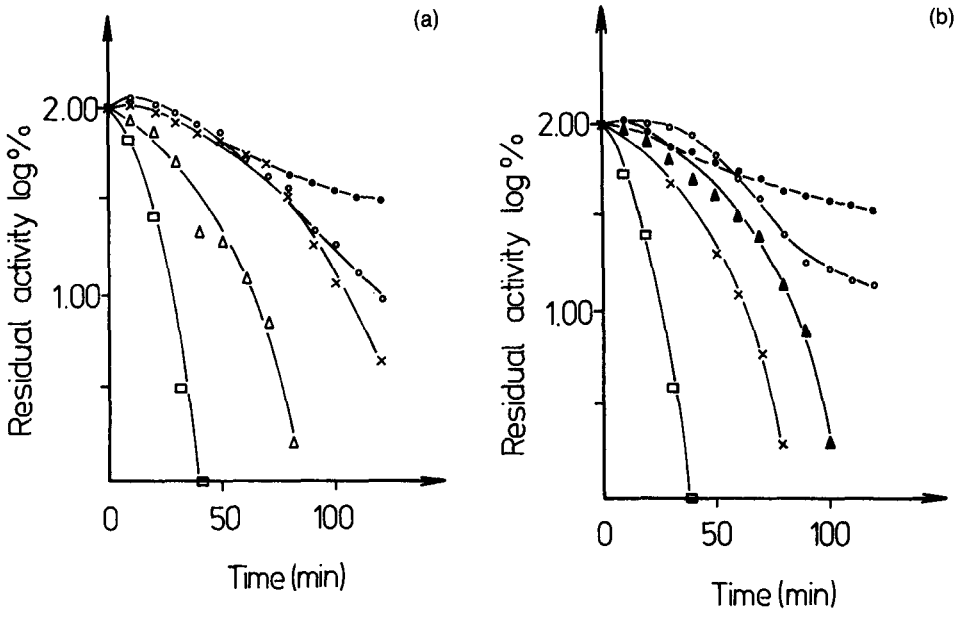

Fig. 5. pH dependences of heat-inactivation of soluble (a) and immobilized (b) aldolases at 328 K. Experiments were carried out in 0.05*M* acetate buffer (pH 5.0), 0.05*M* phosphate buffer (pH 6.0–8.0) and 0.05*M* Tris/HCl buffer (pH 7.0–8.5). Concentrations used: immobilized enzyme 0.833 mg solid/mL, soluble enzyme 0.01 mg/mL. Heat-inactivation progress curves at 328 K are in a semilogarithmic representation. A value of log 100% was assigned to the starting activity. Symbols: □, pH 5.0; ×, pH 6.0; ○, pH 7.0; ●, pH 7.5; ▲, pH 8.5.

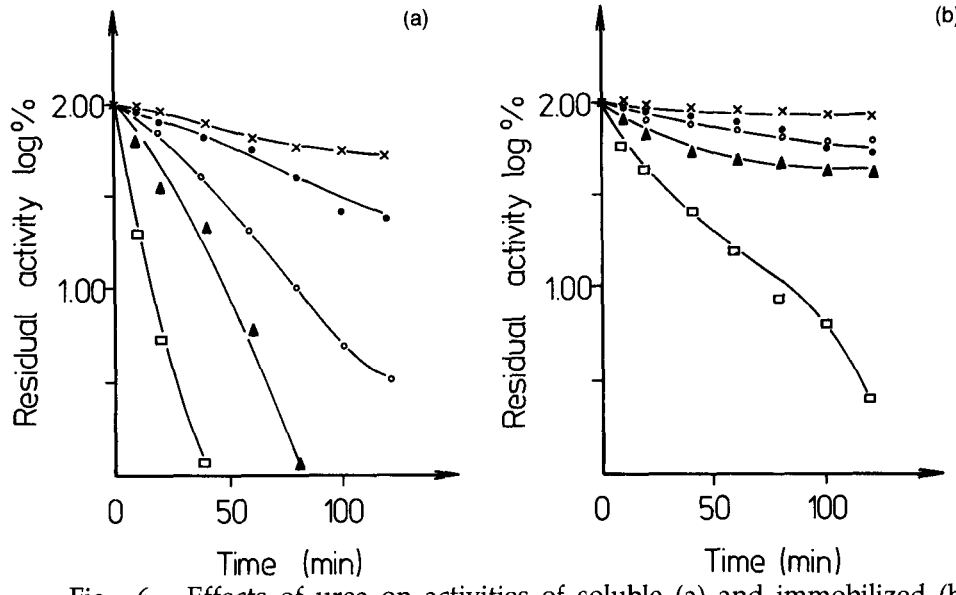

Fig. 6. Effects of urea on activities of soluble (a) and immobilized (b) aldolases. Experiments were carried out in 0.05*M* Tris/HCl buffer, pH 7.5, at 298 K. Concentrations used: immobilized enzyme, 0.833 mg solid/mL; soluble enzyme, 0.01 mg/mL. The starting activity was taken as log 100%. Symbols: ×, 1*M* urea; ●, 2*M* urea; ○, 3*M* urea; ▲, 4*M* urea; □, 5*M* urea.

carbodiimide was found to be the most favorable method for the preparation of a matrix-bound enzyme with high activity and stability. The catalytic activity of the insolubilized aldolase was 2014 U/g solid and the activity loss during preparation was only about 5%. The bound activity and protein were higher in our experiments than in other aldolase immobilization procedures (*4,6–8*). Further, only a slight loss in activity was observed during the immobilization. This latter finding was surprizing, since the covalent immobilization involved the attachment of the amino groups to the activated carboxylic groups of the support; aldolase contains a lysyl residue at the active site and its amino functional group is essential for the catalytic activity.

The immobilized aldolase had a pH optimum shifted towards more alkaline values. A similar effect was observed for aminoacylase and cholinesterase immobilized on Akrilex C-100 gel (*11,12*). This phenomenon might be caused by the polyanionic microenvironment surrounding the enzyme. Another possible explanation of the pH shift is the modification of some basic amino acid side-chain during the immobilization, as suggested earlier for pig kidney aminoacylase (*11*).

The value of $K_{m\ app}$ for the immobilized aldolase was five times that of the soluble enzyme. A possible explanation is that, in the case of the insolubilized enzyme, both the matrix and the substrate are negatively charged, as was found for aldolase–ethylene maleic anhydride copolymer (*6*).

The heat inactivation of the insolubilized and soluble enzymes was carried out in the temperature range 323–338 K. The fixation of the enzyme is presumed to stabilize the conformation of the soluble aldolase. This finding is supported by the effect of urea. The matrix-bound enzyme showed a high stability against 1–4*M* urea, whereas the soluble enzyme quickly underwent denaturing.

The heat inactivation of aldolase at 328 K showed a significant pH dependence. The rate of inactivation of the immobilized enzyme was nearly constant in the pH range 7.0–7.5, but was higher at pH 6.0. The soluble enzyme was stable in the pH range 6.0–7.5. The shift in pH relating to the maximum thermal stability of the immobilized enzyme had the same direction as in the case of the pH shift for the maximum activity.

Our experiments indicated that the heat-inactivation process is a complex, multistep one. This might be a result of the quaternary structure or the heterogeneity of the molecules. Use of aldolase immobilized on Akrilex C-100 gel can be suggested for the continuous production of triose–phosphates or the detection of fructose-1,6-diphosphate.

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