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Immobilization of Pig Muscle Aldolase on a Silica-Based Support

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ABSTRACT

Pig muscle aldolase was covalently attached to a silica-based support possessing aldehyde functional groups. The activity of the immobilized enzyme was 37 U/g solid, and the specific activity calculated on a bound protein basis was 1.9 U/mg protein. The optimum pH for the catalytic activity was pH 7.5. The apparent optimum temperature was found to be 45°C. The K_m app value of the immobilized aldolase with D-fructose 1,6-diphosphate as substrate was 1.25×10^{-4} *M*. The conformational stability was improved by the immobilization. The immobilized aldolase was used for the continuous splitting of D-fructose 1,6-diphosphate.

Index Entries: Aldolase, immobilized; pig muscle aldolase; support, silica-based; catalytic properties, immobilized aldolase; stability tests, immobilized aldolase; D-fructose 1,6-diphosphate splitting; enzyme reactor.

INTRODUCTION

Sugar phosphates are important substrates in clinical chemistry and enzymatic analysis. For their continuous production, immobilized glycolytic enzymes may be applied. One of the glycolytic enzymes, aldolase (D-fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC

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4.1.2.13), isolated from rabbit muscle, has been entrapped in gels (*1–4*) and also covalently attached to different supports (*5–8*). Pig muscle aldolase is cheaper and more stable than the rabbit enzyme (*9*) and is therefore more favorable for practical purposes. In our experiments, a silica-based support possessing aldehyde functional groups was used. The catalytic properties and stabilities of the immobilized pig muscle aldolase were studied and compared with the corresponding properties of the soluble enzyme. The practical use was tested in the case of D-fructose 1,6-diphosphate splitting.

MATERIALS AND METHODS

Aldolase was isolated from pig muscle (*9*). Its specific activity was 8.7 U/mg protein. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the splitting of one micromol of D-fructose 1,6-diphosphate per minute at pH 7.5 and 25°C. The Silochrome aldehyde support was obtained from NPO Biolar (Riga-Olaine, USSR). The binding capacity (aldehyde content) was 13 μmol/g solid, the average pore size was 400–600 Å and the specific surface was 80 m^2/g solid. All other chemicals were reagent grade commercial products of Reanal.

Immobilization of Aldolase

Aldolase was covalently attached to a silica-based support (Silochrome) possessing aldehyde functional groups. The procedure was based on the method of Ryan and his coworkers (*10*) relating to the binding of pyridoxal phosphate on Sepharose 4B containing amino groups.

One g of support was washed with water and suspended in 5 mL of pig muscle aldolase solution containing 32 mg of protein dissolved in 0.02 *M* phosphate buffer (pH 8.0). The suspension was stirred for 2 h at 4°C. After this procedure, 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 1 h at 4°C, then filtered off and washed again with 5×40 mL of water. To block unreacted aldehyde groups, 8 mL of hydroxylamine (1 mg/mL) 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 0.1 *M* phosphate buffer (pH 7.0) and was stored in the same buffer at 4°C.

Assay of Aldolase Activity

The aldolase activity was determined in a coupled-enzyme reaction, using glyceraldehyde-3-phosphate dehydrogenase. The reaction mixture (1.8 mL) contained 0.8 m*M* D-fructose 1,6-diphosphate, 1.7 m*M* NAD, 0.8

mM Na_2HAsO_4 and 33 U glyceraldehyde-3-phosphate dehydrogenase in 0.1 *M* Tris-HCl or 0.1 *M* phosphate buffer. The reaction was started with 50 μL of aldolase (2 mg/mL) and the NADH formation was registered at 340 nm. The temperature was 25°C.

In the case of the immobilized aldolase, 0.1 mg was suspended in the reaction mixture. The suspension was stirred for 1 min at 25°C, the immobilized enzyme was then filtered off quickly (a few seconds) and the absorbance of the filtrate was measured at 340 nm. The activity of the immobilized aldolase was calculated from the difference between the absorbance of the starting reaction mixture and that of the filtrate after the reaction.

Owing to the possible denaturation of the coupled enzyme, the method of Sibley and Lehninger (*11*) was used to study the temperature-dependence of the catalytic activity.

Measurements of Protein

Protein determinations were performed according to the method of Lowry and coworkers (*12*). The amount of immobilized 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 washing solutions after immobilization.

Stability Tests on Soluble and Immobilized Aldolase

The heat treatments were performed in 0.1 *M* phosphate, with reaction mixtures of 1 mL volume. After appropriate periods of incubation, the samples were rapidly cooled in an ice bath and the residual activities were assayed at 25°C.

Urea treatments were performed in 0.1 *M* phosphate 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.

Assays of D-Fructose 1,6-Diphosphate Splitting

For determination of the D-fructose 1,6-diphosphate split in the continuous enzyme reaction, a calibration curve was made. To 150 μL of D-fructose 1,6-diphosphate solution (0-7.0 mM) dissolved in 0.1 *M* phosphate buffer (pH 7.5), 100 μL of aldolase (170 units/mL) was added. After a 30-min incubation at 25°C, 100 μL of 30% TCA solution and then 500 μL of 0.6% NaOH solution was added. The mixture was treated with 250 μL of 0.075% 2.4-dinitrophenylhydrazine dissolved in 1.5 *N* HCl, and finally with 5 mL of an alkaline solution of methyl cellosolve (a 1:1 v/v mixture of methyl cellosolve and 1 *N* NaOH). The absorbances were measured at 570 nm. Samples (250 μL) from the enzyme reactor were treated in the same way as the reaction mixtures containing soluble aldolase.

RESULTS

Catalytic Activity

Under the applied conditions, the highest activity achieved was 37 U/g solid, the specific activity calculated on a bound protein basis was 1.9 U/mg protein, i.e., 21.3% of that of the soluble enzyme.

pH-Dependence of Catalytic Activity

The pH-dependence of the initial rate of D-fructose 1,6-diphosphate splitting was studied at constant ionic strength in the pH range 5.0–9.0. After the incubation, the pH was checked again, but changes were not found. At the different pH values, the maximum rates (V_{max}) were determined. The optimum pH for the catalytic activity (pH 7.5) was not altered by the immobilization of the enzyme, but the activity of the immobilized aldolase showed a sharper pH-dependence than that of the soluble enzyme (Fig. 1).

Temperature-Dependence of Catalytic Activity

The temperature-dependences of the activities of the soluble and immobilized aldolase were studied in 0.1 *M* phosphate buffer at the optimum pH for the catalytic activity (pH 7.5), with D-fructose 1,6-diphosphate as substrate, in the temperature range 25–60°C. Initial rates were found by measuring the activity for 30 min at selected temperatures. The apparent optimum temperature for the catalytic activity of immobilized aldolase was 45°C, i.e., somewhat higher than that of the soluble enzyme (40°C) (Fig. 2).

Effect of Substrate Concentration on Catalytic Reaction

The effect of the D-fructose 1,6-diphosphate concentration on the initial rate of the catalyzed reaction was investigated in the concentration range from 1.4×10^{-5} *M* to 1.4×10^{-4} *M* at the optimum pH for catalytic activity (pH 7.5). Experiments were carried out in 0.1 *M* phosphate buffer at 25°C. The K_m value for the soluble enzyme was found to be 2.7×10^{-5} *M*, whereas the $K_{m\,app}$ value for the immobilized form was 1.25×10^{-4} *M*. The pH-dependence of the Michaelis constant was only slightly affected by the immobilization of the enzyme.

Thermal Stability

The rates of heat inactivation of the soluble and immobilized aldolases were compared in 0.1 *M* phosphate buffer (pH 7.5) in the temperature range 50–65°C. At 50°C, the soluble enzyme showed a characteristic activation. The activation could not be detected above 55°C, and the aldolase

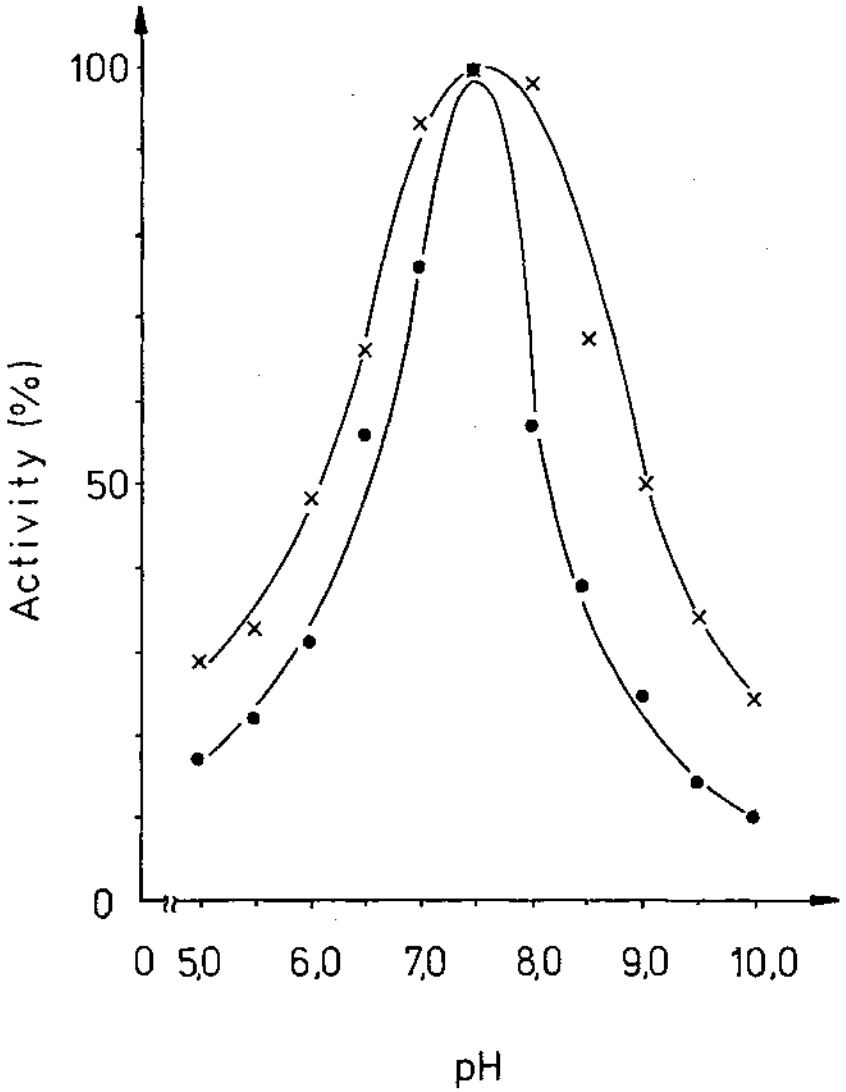

Fig. 1. Effects of pH on activities on soluble and immobilized aldolase. Experiments were carried out in 0.1 *M* phosphate buffer at 25°C, with D-fructose 1,6-diphosphate as substrate. x, Soluble enzyme, ●, immobilized enzyme. For both soluble and immobilized enzyme, the maximum activity was taken as 100%.

was quickly inactivated (Fig. 3A). The immobilized enzyme was not activated by the heat treatment. At 65°C, the heat-resistance of the immobilized aldolase surpassed that of the soluble enzyme (Fig. 3B).

pH-Dependence of Thermal Inactivation ("pH Stability")

The effect of the hydrogen ion concentration on the thermal inactivation process was studied in 0.1 *M* phosphate buffer in the pH range pH 6.0–8.5 at 55°C (Fig. 4). In the case of the soluble aldolase pH 6.0–7.5, a

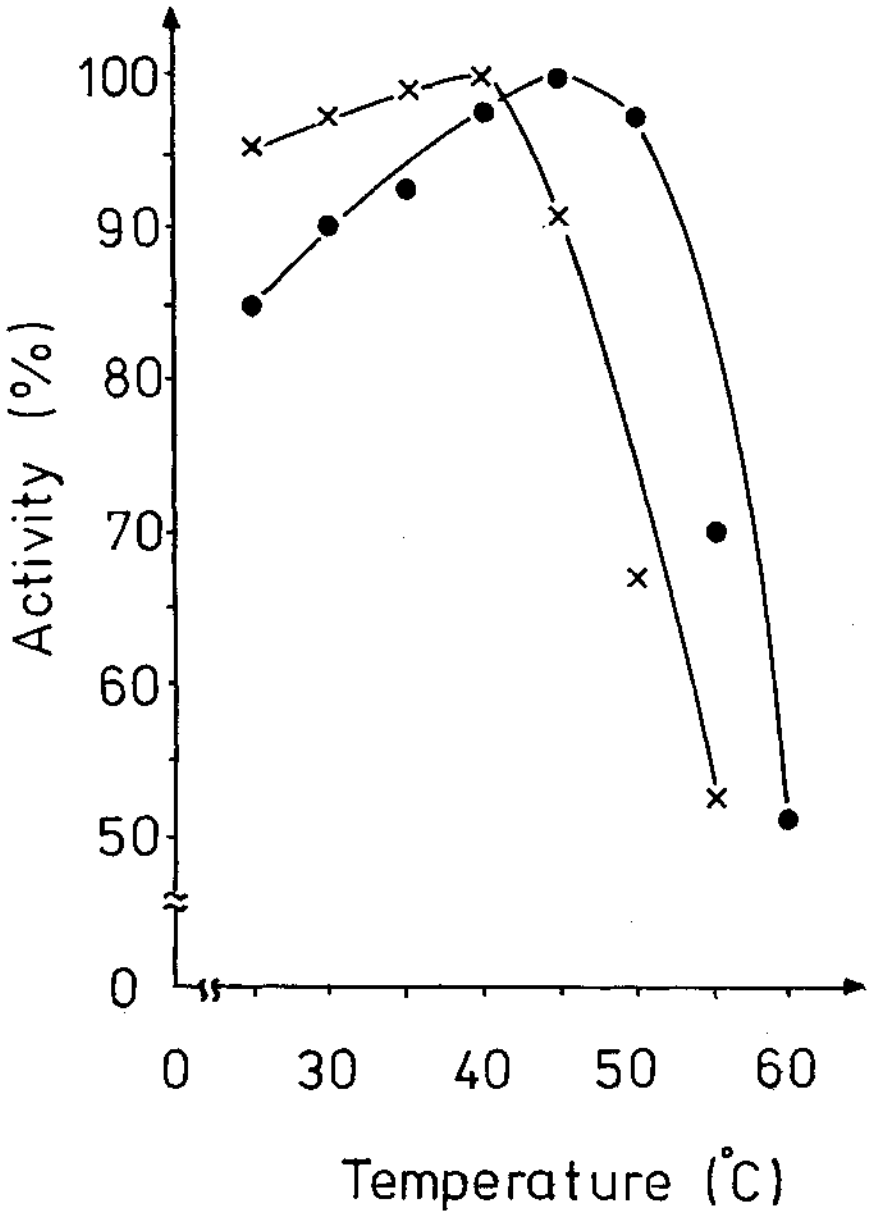

Fig. 2. Effects of temperature on activities of soluble and immobilized aldolase. Experiments were carried out in 0.1 *M* phosphate buffer at pH 7.5, with D-fructose 1,6-diphosphate as substrate. x, Soluble enzyme, ●, immobilized enzyme. For both soluble and immobilized enzyme, the maximum activity was taken as 100%.

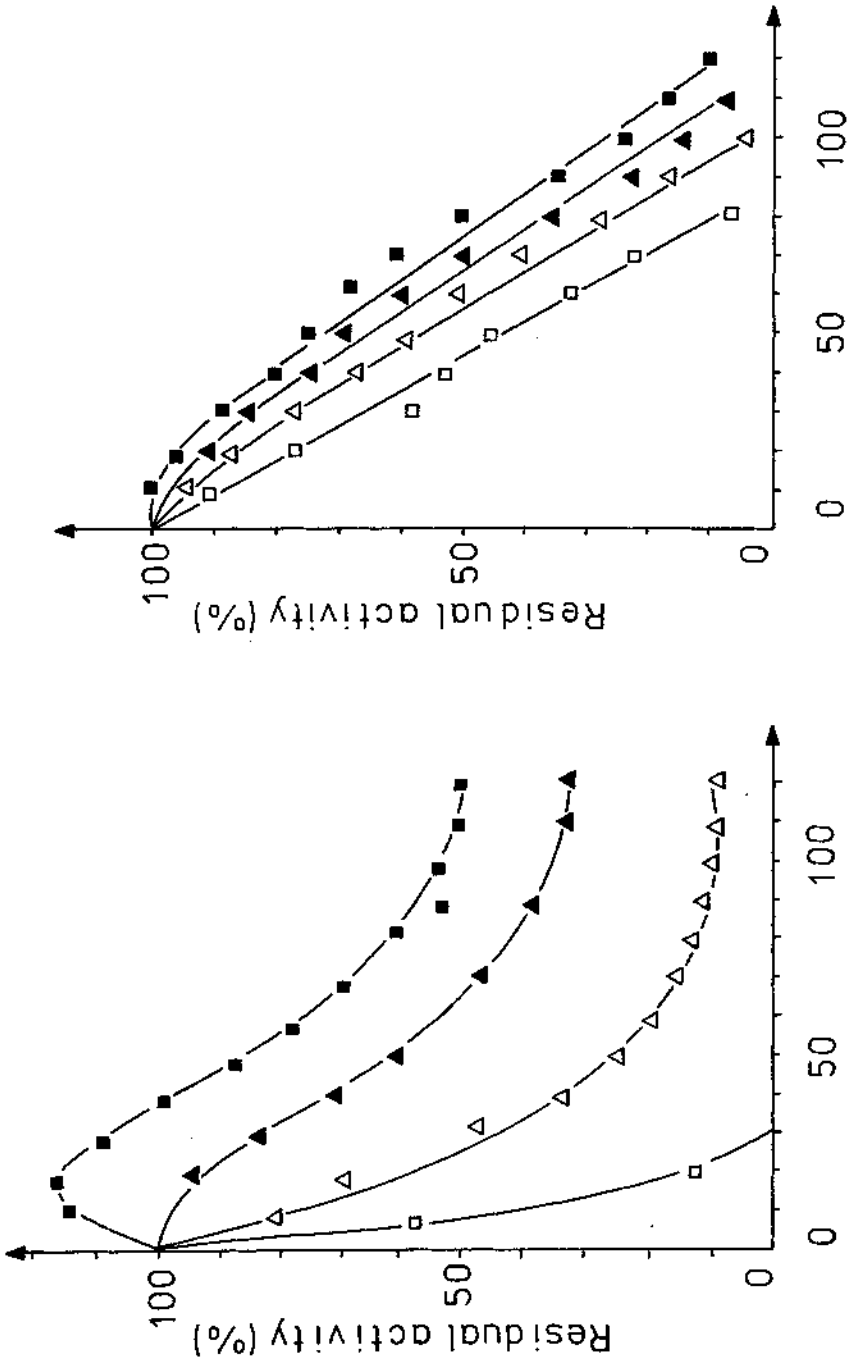

Fig. 3. Thermal inactivation of soluble (A) and immobilized (B) aldolase in absence of substrate. Experiments were carried out in 0.1 *M* phosphate buffer at pH 7.5. Enzyme concentrations used: soluble enzyme, 0.05 mg protein/mL, immobilized enzyme, 20 mg solid/mL. ■, 50°C; ▲, 55°C; △, 60°C; □, 65°C. For both soluble and immobilized enzyme, the starting activity was taken as 100%.

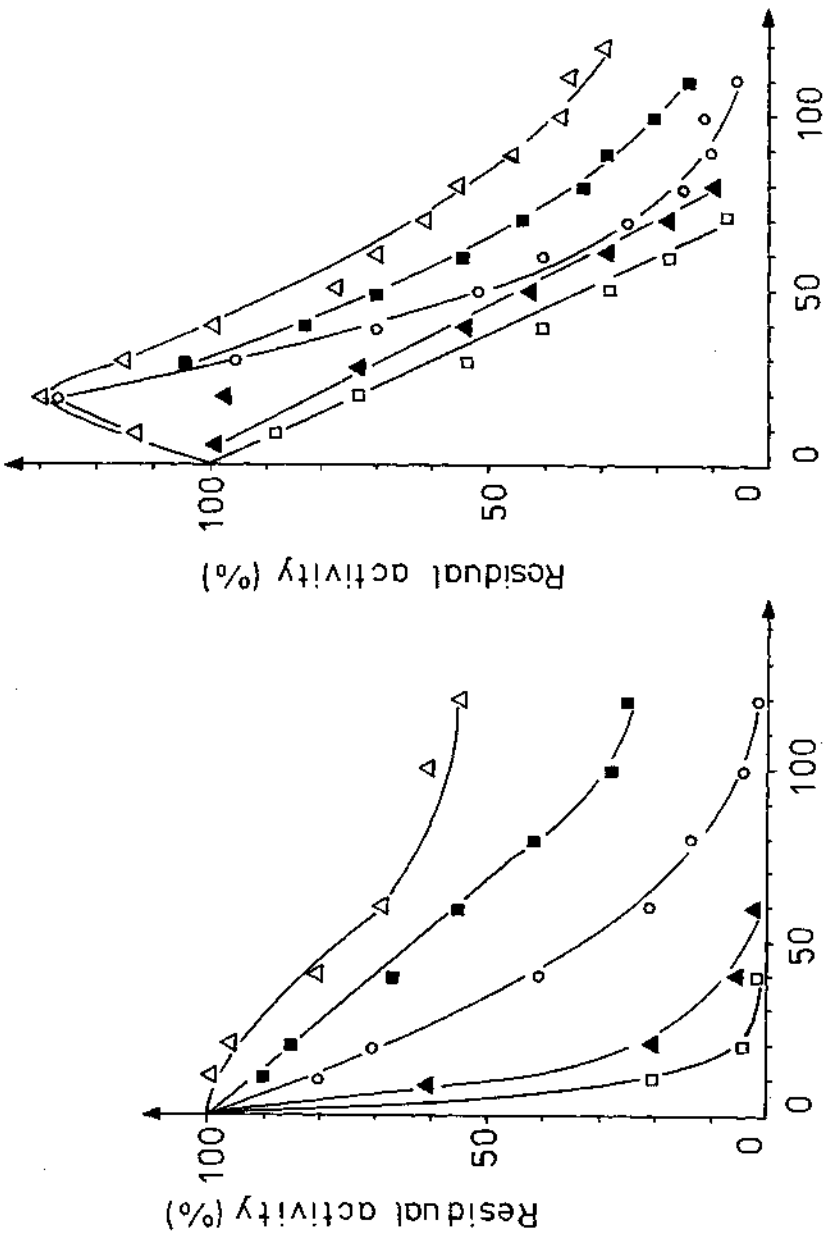

Fig. 4. pH-dependence of thermal stability ("pH stability") of soluble (A) and immobilized (B) aldolase at 55°C in absence of substrate. Experiments were carried out in 0.1 *M* phosphate buffer. Enzyme concentrations used: soluble enzyme, 0.05 mg protein/mL, immobilized enzyme, 20 mg solid/mL. ○, pH 6.0; ■, pH 6.5; □, pH 7.0; △, pH 7.5; ▲, pH 8.0. For both soluble and immobilized enzyme, the starting activity was taken as 100%.

transient activation was experienced. The immobilized enzyme did not show such an activation. On the basis of the progress curves, it was found that pH 6.5–8.5 the stability of the immobilized enzyme exceeded that of the soluble enzyme.

Resistance Against Urea

The denaturing effect of urea was investigated in the concentration range 1–5 *M* in 0.1 *M* phosphate buffer (pH 7.5) at 25°C. Treatments were performed in the absence of substrate. At 1–3 *M* urea concentration, the inactivation of the immobilized aldolase was preceded by a transient activation (Fig. 5B). At higher urea concentrations, the activation could not be detected. In the case of the soluble enzyme, no activation could be detected (Fig. 5A). At urea concentrations higher than 2 *M*, the immobilized aldolase was more resistant than the soluble enzyme against urea.

Storage Stability

The storage stabilities of the soluble and the immobilized aldolase were compared in water (pH 8.0) at 10°C. Under such conditions, the immobilized aldolase proved to be more stable than the soluble one. During storage for 10 d, the immobilized enzyme lost only 33% of its original activity, whereas the soluble enzyme was almost totally inactivated (the residual activity was about 5%).

Continuous Splitting of D-Fructose 1,6-Diphosphate

The practical use of Silochrome-aldolase was tested as concerns D-fructose 1,6-diphosphate splitting. A column reactor (0.8×2.6 cm) containing 5.4 U of aldolase activity and a 3.7 m*M* solution of D-fructose 1,6-diphosphate in 50 m*M* phosphate buffer (pH 7.5) as substrate were used. The effect of the flowrate on the substrate conversion is presented in Fig. 6. The reactor could be operated with good stability during 30 d (Table 1).

DISCUSSION

One of the most important groups of inorganic supports is the silica derivatives that are suitable for the preparation of immobilized enzymes of practical significance. In our experiments, a silica support possessing aldehyde functional groups was used to produce immobilized aldolase. The aldehyde groups of the support can produce Schiff base intermediates with the primary amino groups localized on the enzyme surface. The conjugate was stabilized by reduction with sodium borohydride. The activity loss revealed that a considerable proportion of the enzyme molecules

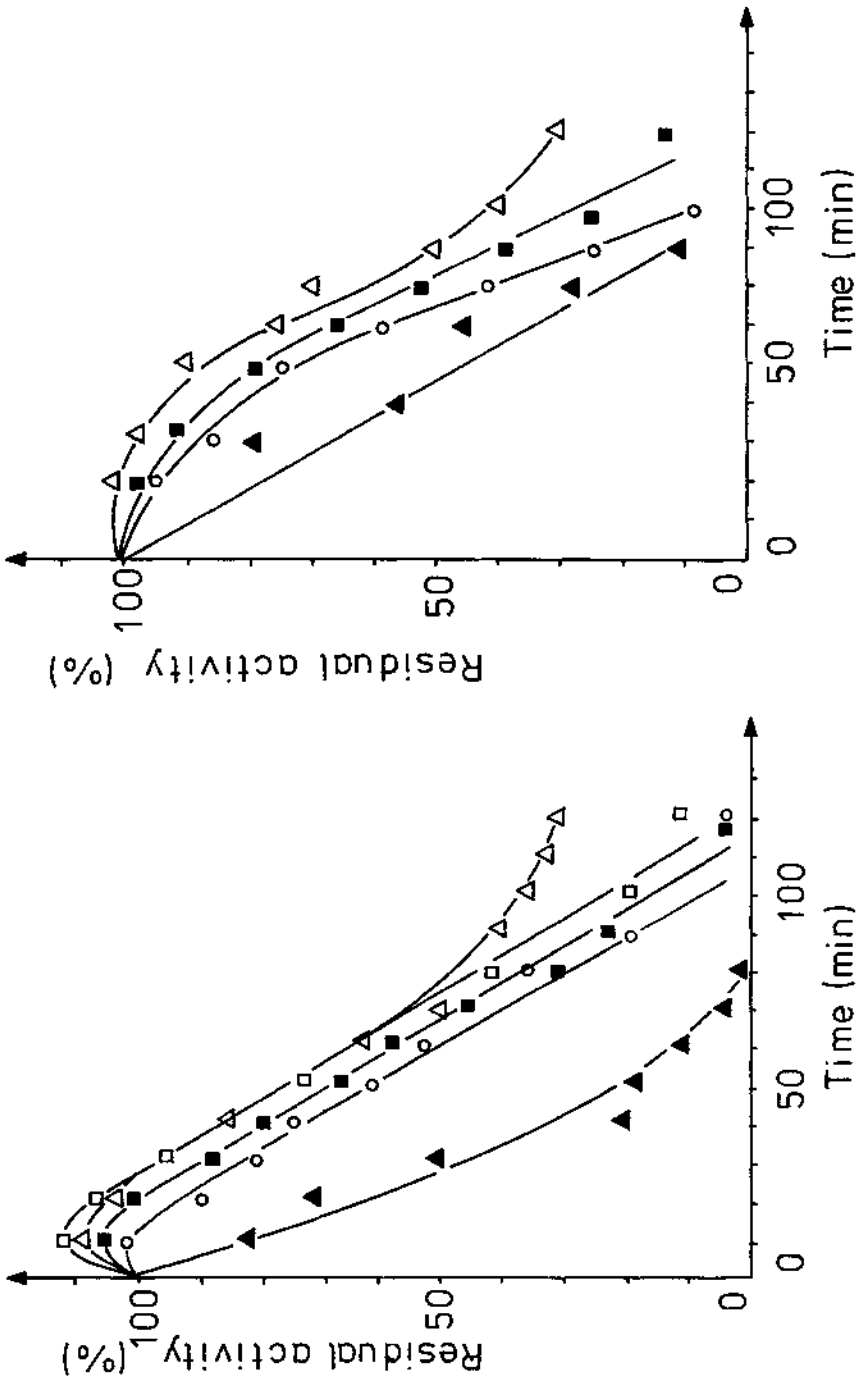

Fig. 5. Effects of urea on activities of soluble (A) and immobilized (B) aldolase. Incubation was carried out in 0.1 *M* phosphate buffer (pH 7.5) at 25°C in absence of substrate. Enzyme concentrations used: soluble enzyme, 0.05 mg protein/mL, immobilized enzyme, 20 mg solid/mL. Urea concentrations: △, 1 *M*; ■, 2 *M*; ○, 3 *M*; ▲, 4 *M*; □, 5 *M*. For both soluble and immobilized enzyme, the starting activity was taken as 100%.

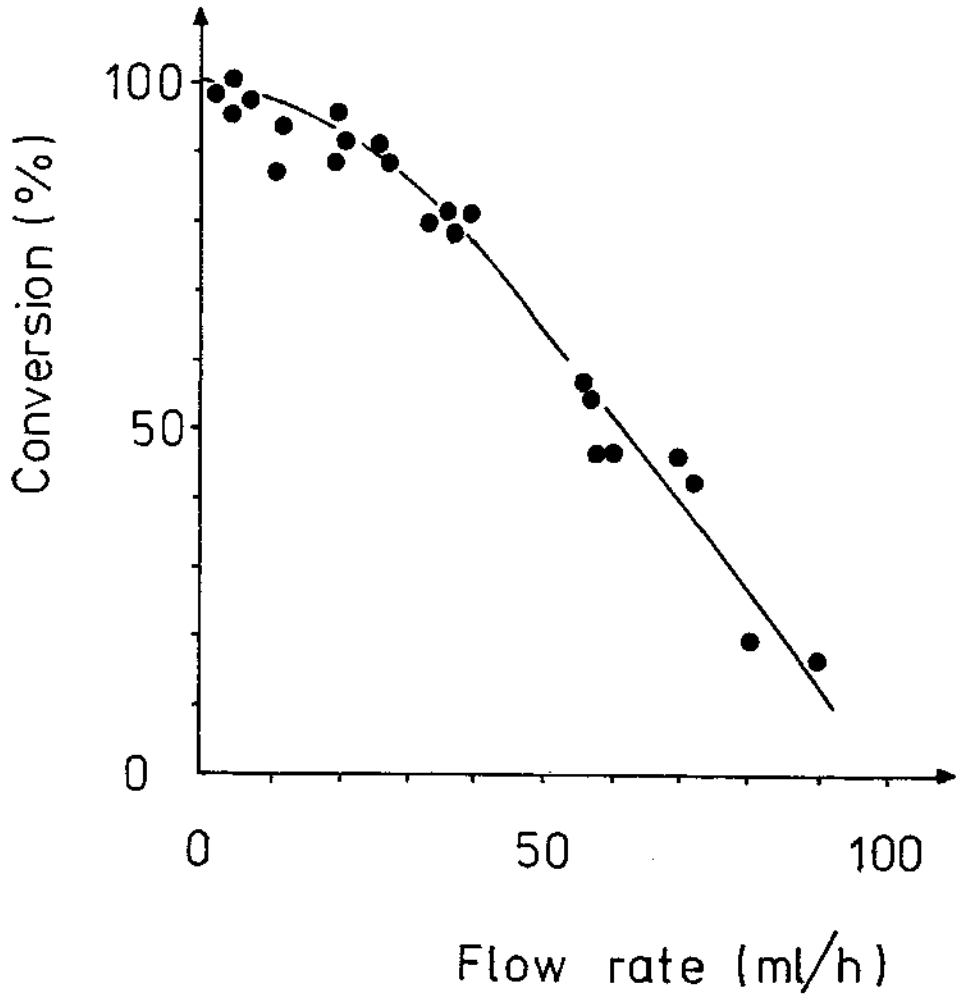

Fig. 6. Effect of flowrate on continuous splitting of D-fructose 1,6-diphosphate by immobilized aldolase at 25°C. The column reactor (0.8×2.6 cm) contained 5.4 U of aldolase activity. A 3.7 m*M* solution of D-fructose 1,6-diphosphate in 50 m*M* phosphate buffer (pH 7.5) was used as substrate. Conversion (%) = FDP_{split} / $FDP_{starting}$.

were inactivated during the coupling process. The inactivation was probably a result of the reaction circumstances and disadvantageous steric interactions, but the possible reaction of the lysyl sidechain on the active center with an aldehyde group of the support cannot be excluded. The silica supports have an acidic character (*13*), and the polyanionic microenvironment abolishes the tertiary structure of the enzyme. In this way, the lysyl sidechain becomes accessible for the aldehyde group. In spite of the inactivation, which is disadvantageous from an economic point of view, the activity loss was partly compensated by the enhanced conformational and operational stability of the active molecules.

Table 1
Operational Stability of Immobilized Aldolase[a]

Duration of operation, days	Activity of column, percent
0	100.0
3	91.9
4	100.0
5	86.5
8	89.2
17	94.6
24	91.9
29	91.9

[a]The column reactor (0.8×2.6 cm) contained 5.4 U of aldolase activity. A 3.7 mM solution of D-fructose 1,6-diphosphate in 50 mM phosphate buffer (pH 7.5) was used as substrate. The flow rate was 1.8 mL/h at 25°C.

The immobilization induces characteristic changes in the catalytic properties and stability of the enzyme. The charged supports form a special microenvironment for the bound enzyme molecules, in which their catalytic properties are altered (*14*). In the case of covalently attached enzymes, some of the charged amino acid sidechains take part in the bonding, and the altered charge pattern on the molecular surface also influences the catalytic properties (*15*).

In the covalent attachment of the aldolase to the silica support, the lysyl ε-amino groups probably took part, and by this means the charge pattern of the molecular surface was shifted in the negative direction. The charge shift might be reflected in the pH-activity profile of the immobilized aldolase. However, the acidic character of the support also appears to be important. A possible explanation of the higher $K_{m\,app}$ value of the immobilized enzyme is the operation of repulsive forces between the negatively charged matrix and the negatively charged substrate.

A hydrophilic microenvironment generally improves the conformational stability of enzymes. The Silochrome support is hydrophilic, and it therefore enhanced the resistance of the aldolase molecules against denaturation.

The immobilized aldolase could be used for the continuous splitting of D-fructose 1,6-diphosphate. The elaboration of both analytical and preparative applications is in progress.

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