Purification of pyruvate kinase from pig dental pulp

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Introduction

The purification of pyruvate kinase has been studied in various tissues¹⁻³⁾ and the enzyme is known to have a regulatory function within the glycolytic pathway. It is thought that pyruvate kinase is one of the key enzymes in glycolysis and that the interactions of the enzyme with various modifiers are most important in controlling the glycolytic rate. Enzymes involved in glycolysis in the dental pulp have been previously studied, such as lactate dehydrogenase⁴⁾, phosphofructokinase⁵⁾ and pyruvate kinase^{6,7)}. However, these enzymes were not completely purified.

In the present study pyruvate kinase has been purified from pig dental pulps.

Materials and Methods

Materials: Materials were purchased from the following sources: ADP, PEP and NADH, Oriental Yeast; lactate dehydrogenase, Beringer; phosphocellulose, Brown; and ampholine, LKB.

Dental pulps were separated from the lower jaws of freshly killed pigs obtained from the slaughter house. The pulps were stored frozen at -80°C until use.

Enzyme assay. Pyruvate kinase activity was measured at 25°C in a coupled assay system similar to that described by Bücher and Pfeidere⁸⁾. The assay mixture contained as final concentrations, 50 mM Tris-HCl buffer, pH 7.2, 100 mM KCl, 10 mM MgCl_2 , 0.5 mM phosphoenolpyruvate, 1 mM ADP, 0.15 mM NADH, and $10\text{ }\mu\text{g/ml}$ of

lactate dehydrogenase. The reaction was initiated by the addition of pyruvate kinase.

Protein concentration was determined by the methods of Lowry *et al.*⁹⁾ and Bensadoun and Weinstein¹⁰⁾. Polyacrylamide gel electrophoresis was carried out according to Davis¹¹⁾. The gel concentration was 5% an 8-cm length. Protein on the gel was stained with Coomassie brilliant blue.

Results and Discussion

Purification of pyruvate kinase.

Extract. Dental pulps were thawed, homogenized with 3 vols (v/w) of 10 mM potassium phosphate buffer, pH 6.0, containing 5 mM MgCl_2 , 5 mM 2-mercaptoethanol, 0.2 mM fructose-1,6-diphosphate (FDP), and 1 mM EDTA (buffer A) in a Polytron-homogenizer at 10,000 r.p.m. for 30 sec. The supernatant fraction (crude extract) was collected by centrifugation of the homogenate at $18,000\times g$ for 30 min.

Ammonium sulfate fractionation. Solid ammonium sulfate was added to the extract to make a concentration of 50% saturation. After being stirred for 30 min, the supernatant fraction was collected by centrifugation at $18,000\times g$ for 30 min. Solid ammonium sulfate was added to the supernatant to 70% saturation. After being stirred for 30 min, the precipitate was collected by centrifugation ($18,000\times g$, 30 min) and dissolved in buffer A.

Ammonium sulfate was removed from the protein fraction by gel filtration through a Sephadex G-25 column ($1.5\times 12\text{ cm}$) equilibrated with buffer A.

Phosphocellulose column chromatography.

Fig. 1 Elution pattern of pig pulp pyruvate kinase on phosphocellulose column chromatography. Protein fraction (5.0 ml) obtained by Sephadex G-25 gel filtration from 3 g of pig dental pulp was applied to phosphocellulose column (1.5×12 cm) equilibrated with buffer A. After the column had been adequately washed the same buffer, the enzyme was eluted with a linear gradient (0-500 mM KCl) in the same buffer. 1-ml fractions were collected.

Fig. 2 Isoelectric fractionation of pyruvate kinase from pig dental pulp. Electrophoresis was performed at 700 V for 20 h at 0°C .

The enzyme solution was applied to a phosphocellulose column (1.5×12 cm) equilibrated with buffer A¹. After the column was washed with buffer A (50 ml), the enzyme was eluted by a linear gradient of

KCl from 0 to 500 mM in buffer A. The flow rate was 30 ml/h and 2 ml fractions were collected. Almost all of the enzyme activity was detected in 6 fractions (No. 37-42) (Fig. 1). The active fractions were

Table 1 Purification step of pyruvate kinase from pig dental pulp

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	8.7	131	23.0	0.175	100
(NH ₄) ₂ SO ₄ fraction (50–70% sat.)	2.0	38.0	11.1	0.292	48.3
Phosphocellulose column	10.8	0.607	5.05	8.32	22.0
Isoelectric focusing	3.0	0.021	1.07	51.0	4.7

Fig. 3 Disk gel electrophoresis of pyruvate kinase on polyacrylamide gel. The purified enzyme (5 μ g) was applied, and gel concentration was 5%. Protein on the gel was stained with Coomassie brilliant blue. The enzyme activity was measured using 2 mm gel slices.

combined (11 ml) and concentrated to about 2 ml using a Collodion bag.

Isoelectric focusing. Isoelectric fractionation was carried out by the method of Verterberg and Svensson¹²⁾. Electrophoresis was performed at 700 V for 20 h at 0°C. The maximal activity was detected at pH 8.0 (Fig. 2). The active fractions (No. 49–51) were combined (3 ml) and concentrated to about 0.5 ml using a Collodion bag.

Electrophoresis. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis which coincided well with the position of enzyme activity revealed by assay of 2 mm gel slices (Fig. 3).

A summary of the purification procedure is presented in Table 1. Pyruvate kinase

from pig dental pulp was purified to a homogeneous preparation which had a specific activity of 51 units/mg of protein.

Pyruvate kinase has been purified from various tissues^{1–3)}. However, the purification of the enzyme from dental pulp is quite difficult because dental pulps are small in size and surrounded by hard tissues. By using a phosphocellulose column and isoelectric focusing, pyruvate kinase has been purified from 3 g of dental pulp from 8 pigs in this study. This purified enzyme preparation will now be used to study the regulation of glycolysis in dental pulp.

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