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Studies on Myokinase in the Muscle of Fishes-IV

Myokinase from Skipjack Muscle

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Abstract

Myokinase from skipjack muscle was purified by gel filtration using Sephadex G-75 and phosphocellulose chromatography using adenosine triphosphate (ATP) and adenosine monophosphate (AMP) as substrates for the elution. Molecular weight of the enzyme was 25,000 from the result of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Optimum pH of the enzyme was 7.5. EDTA inhibited the enzyme and re-activation of the enzyme treated with EDTA was achieved by dialysis against Tris-Cl buffer (pH 7.6) containing 5 mM of $MgCl_2$, $MnCl_2$ and $CoCl_2$. The purified enzyme catalyzes transphosphorylation reaction only between ATP and AMP.

Myokinase (ATP: AMP phosphotransferase EC 2.7.4.3) is plentiful in tissues where the turnover of energy from adenine nucleotide is great, such as muscle and mitochondria. The importance of myokinase in biological system is in its involvement in the maintenance of equilibrium among the adenine nucleotides. On the other hand, in our laboratory we isolated some enzymes from fishes and studied on catalytic properties and molecular properties of enzymes to know denaturation of enzymes or proteins during frozen storage. Because denaturation of proteins during frozen storage is one of the most important problem.

Myokinase from rabbit muscle, porcine muscle, rat liver and yeast were isolated, and physical properties and kinetics were studied[1]. As described previously we isolated myokinase I and II from carp muscle using DEAE-cellulose and CM-cellulose[2)3)]. In this report we isolated myokinase from the muscle of skipjack (*Katsuwonus pelamis*) using phosphocellulose and investigated catalytic properties and molecular properties of the enzyme.

Experimental procedure

Enzyme assay During the purification procedure the activity of myokinase was measured by the method using pyruvate kinase with excess phosphoenolpyruvate (PEP) together lactate dehydrogenase and excess NADH[4]. The assay mix-

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ture was 3.0 mM ATP, 1.5 mM AMP, 0.6 mM PEP, 0.3 mM NADH, 1.2 mM $MgSO_4$, 116 mM KCl, 100 mM Tris-Cl buffer (pH 7.6), 8 units of pyruvate kinase and 11 units of lactate dehydrogenase. The oxidation of NADH at 30°C was followed by chages in absorbance at 340 nm with a Hitachi EPU-2A spectrometer. For the investigation of catalytic properties the activity of the enzyme was measured by the method described previously[2]. The assay mixture was 25 mM Tris-Cl buffer (pH 7.6) and 2.5 mM ADP. ATP produced by the enzyme was separated by Dowex 1 × 4 (Cl type, 100-200 mesh), and the amount of ATP was calculated from the molar absorption coefficient at 260 nm. Protein was determined by the method of LOWRY *et al.*[5]. One enzyme unit of myokinase is defined as the consumption or the production of 1 μmole of ATP per min at 30°C.

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis in the absence or presence of sodium dodecyl sulfate was performed according to the method of DAVIS[6] or WEBER *et al.*[7]. The determination of molecular weight of the enzyme was done using bovine serum albumin (M. W. 68,000), pyruvate kinase (M. W. 57,000), RNA polymerase-subunit (M. W. 39,000) and trypsin inhibitor (M. W. 21,000) as markers.

Materials ATP, ADP and AMP were obtained from Yamasa Soybean Souce Co. PEP, CTP, CMP, GTP, ITP, IMP, UTP, UMP, pyruvate kinase, lactate dehydrogenase, RNA-polymerase and trypsin inhibitor were purchased from Boehringer Mannheim Co. Bovine serum albumin (Fraction V) was obtained from Armour Pharmaceutical Co. Phosphocellulose was obtained from Brown Co. and Dowex 1 × 4 (100-200 mesh) was purchased from the Dow Chemical Co.

Result

Purification of myokinase from skipjack muscle. Myokinase from skipjack muscle was purified by a method slightly modified from that of NODA *et al.*[8]. Frozen muscle was minced and homogenized with 4 volumes of 0.01 M phosphate buffer (pH 7.0) by Tarrax for 10 min. Homogenate was centrifuzed at 8,000 × g for 20 min.

Phosphocellulose was equilibrated with 0.01 M phosphate buffer (pH 7.0) and evenly packed in a 12 cm diameter glass filter. Filter paper (Toyo No. 2) was laid upon phosphocellulose. Myokinase in the dialyzed extract prepared as above was applied to phosphocellulose column (26G2 glass filter) and the column was washed with 0.01 M phosphate buffer (pH 7.0) adequately. Phosphocellulose which adsorbed myokinase was removed from glass filter and repacked in a column (4 cm diameter, 50 cm high). The column was washed with the same buffer. Myokinase was eluted by 0.01 M phosphate buffer (pH 7.0) containing 0.5 mM of AMP and ATP.

Fractions of myokinase was pooled, concentrated by ultrafiltration membrane (Amicon UM-10) and applied to Sephadex G-75 column (2.6 cm diameter, 90 cm high) equilibrated with 0.01 M phosphate buffer (pH 7.0). Eluting profile is

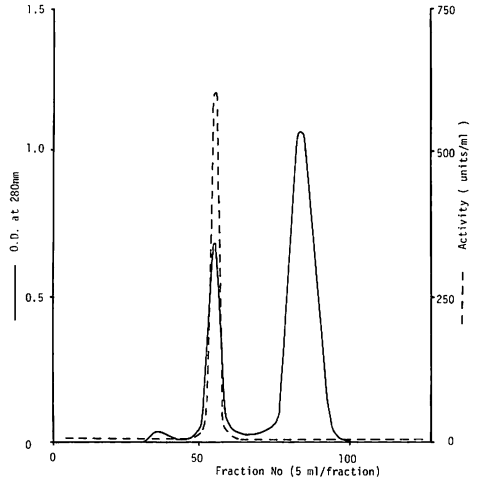

Fig. 1. Gel filtration chromatography of myokinase fractionated by phosphocellulose column chromatography.
Column size; 2.6 cm (diameter) × 90 cm (high)
Flow rate; 12 ml/h

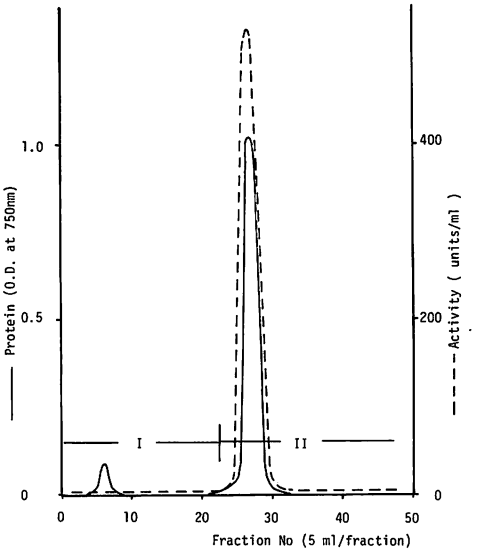

Fig. 2. Elution profile of phosphocellulose column chromatography.
I 0.01 M phosphate buffer (pH 7.0)
II 0.01 M phosphate buffer (pH 7.0) containing 0.5 mM of ATP and AMP.
Column sizw; 1.2 cm (diameter) × 15 cm (high)
Flow rate; 24 ml/h

shown in Fig. 1.

Myokinase separated by Sephadex G-75 was pooled, concentrated by ultrafiltration membrane and applied to phosphocellulose column (1.2 cm diameter, 15 cm high). Myokinase was eluted with 0.01 M phosphate buffer containing 0.5 mM of AMP and ATP as shown in Fig. 2.

The purification procedure is summarized in Table 1.

Table 1. Purification of myokinase from skipjack muscle

Fraction	Volume (ml)	Protein (mg)	Units	Specific Activity (units/mg)	Purification
Homogenate	2,000	16,600	65,560	4	
Phosphocellulose	10	51	17,748	348	87
Sephadex G-75	9	10	10,800	1,080	270
Phosphocellulose	30	8	8,806	1,074	268

Homogeneity and molecular weight of the enzyme prepared From rechromatography by phosphocellulose the single symmetrical activity peak showed uniform specific activity. In polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate a single band was detected as shown in Fig. 3.

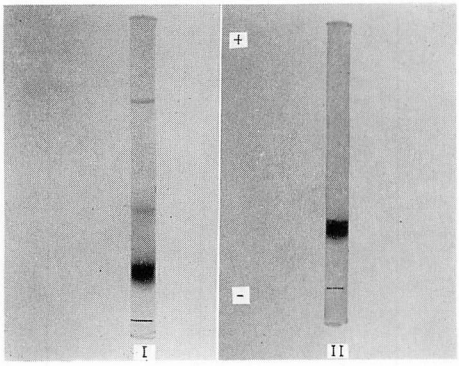

Fig. 3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

I Enzyme fractionated by chromatography on phosphocellulose.
II Enzyme fractionated by rechromatography on phosphocellulose.
Concentration of acrylamide was 7.5 %.

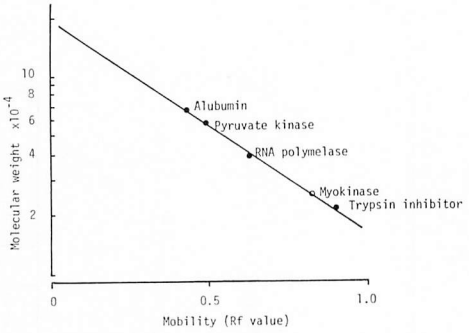

Fig. 4. Molecular weight determination by polyacrylamide gel electrophoresis in the ptesence of sodium dodecyl sulfate.

Concentration of acrylamide and N, N′-methylene-bis (acrylamide) was 7.5 % and 0.12 % respectively. Marker proteins were bovine serum albumin (68,000), pyruvate kinase from rabbit muscle (57,000), RNA-polymerase from *E. coli* (α-subunit, 39,000) and trypsin inhibitor (21,500).

Molecular weight of myokinase was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. From Fig. 4. molecular weight of myokinase was 25,000.

Optimum pH Activity of myokinase was assayed by incubating a mixture of 2.5 mM ADP and 37.5 mM phosphate buffer or Tris-Cl buffer at 37°C for 10 min. As shown in Fig. 5., optimum pH of myokinase was 7.5.

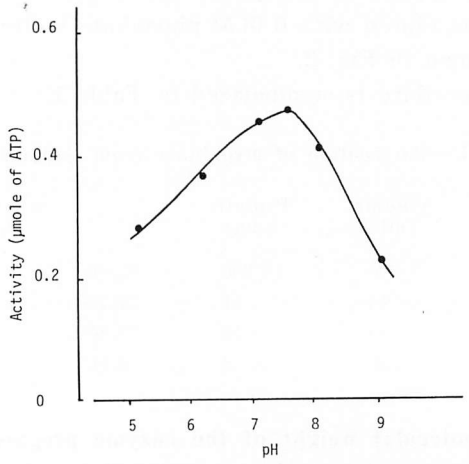

Fig. 5. Effect of pH on the activity of myokinase from skipjack muscle.

pH 5-8; 0.038 M phosphate buffer
pH 9; 0.038 M Tris-Cl buffer

Effect of EDTA Effect of EDTA on myokinase activity was investigated. The enzyme and 50 mM EDTA were incubated at 0°C for 3 days. After the appropriate periods of incubation the enzyme was dialyzed and the residual activity was assayed.

As shown in Fig. 6-a, EDTA inactivated myokinase almost completely. However re-activation of the enzyme treated with EDTA was achieved by dialysis with 0.05 M Tris-Cl buffer (pH 7.6) containing 5 mM of $MgCl_2$, $MnCl_2$ or $CaCl_2$ (Fig. 6-b).

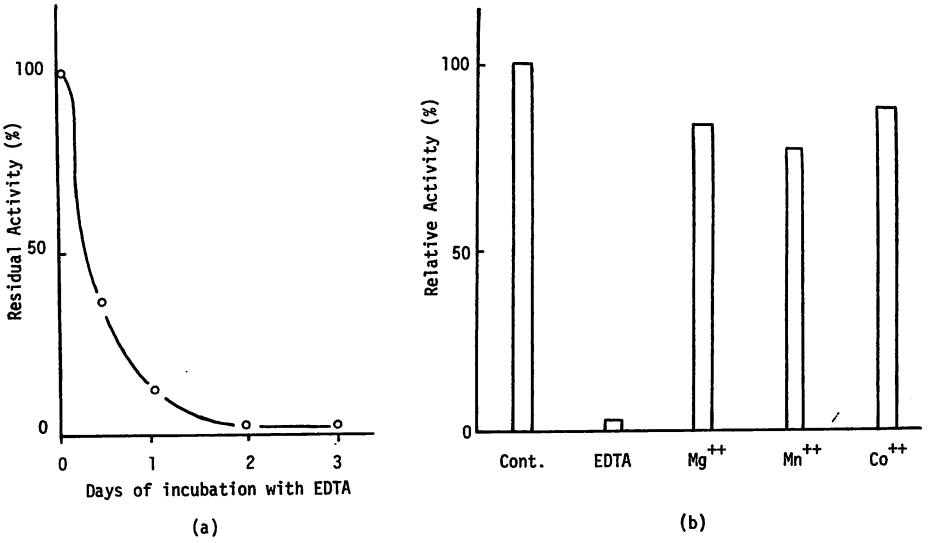

Fig. 6. a) Effect of EDTA on the activity of myokinase. Enzyme was incubated in 0.05 M Tris-Cl buffer (pH 7.6) containing 0.05 M EDTA at 0°C.
b) Re-activation of the enzyme treated with EDTA by 5 mM of bivalent metal ions.

Effect of bivalent metals such as Mg^{++}, Ca^{++}, Mn^{++}, Co^{++} and Zn^{++} on myokinase was also investigated. Mg^{++}, Ca^{++} and Co^{++} increased the activity of the enzyme from 1.2 to 1.4 times.

Table 2. Nucleotide specificity

Substrates	Relative reaction rates
ATP+AMP	100
ATP+c-AMP	0
ATP+IMP	0
ATP+CMP	0
ATP+GMP	0
ATP+UMP	0
GTP+AMP	0
UTP+AMP	0

Nucleotide specificity Nucleotide specificity was investigated using the assay mixture containing ATP+XMP or XTP+AMP. ATP produced by the enzyme was determined by the enzymatic method described in "Experimental procedure". Table 2 shows the result of investigation. The enzyme catalyzed the only ATP +AMP.

Discussion

Using the buffer containing ATP and AMP, which are substrates for myokinase, for eluting the enzyme from phosphocellulose column was very useful to purify and shortened the periods of purification procedure. Overrall periods for the purification was about 10 days.

Homogeneity of the purified enzyme was recognized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The specific activity of the purified enzyme was 1,080 units per mg of protein and 270 times higher than that of the enzyme in extract. Specific activity of myokinase, which were tested for homogeneity, from rabbit muscle, porcine muscle and bovine liver were 2,200, 1,810 and 1,062 units per mg of protein respectively.

We reported previously myokinase I and II from carp muscle[2)3)]. However there was no isozyme from skipjack muscle. Molecular weight of the enzyme from skipjack muscle was 25,000 and that of myokinase I and II were 27,000 and 22,000 respectively.

EDTA inhibited the activity of the enzyme from skipjack muscle remarkably as well as myokinase II from carp muscle[9)]. Re-activation of the enzyme treated with EDTA was obserbed by dialyzing against 0.05 M Tris-Cl buffer (pH 7.6) containing 5 mM $MgCl_2$, $CaCl_2$ and $CoCl_2$. Re-activation of myokinase II from carp muscle treated with EDTA was also obserbed by dialyzing against 0.05 M Tris-Cl buffer (pH 7.6) containing 5 mM $MgCl_2$ and $MnCl_2$, but not obserbed by the buffer containing $CoCl_2$[9)].

Myokinase from skipjack muscle was not so activated by any of bivalent metal ions, Mg^{++}, Ca^{++}, Mn^{++}, Co^{++} and Zn^{++}. On the other hand myokinase II from carp muscle was activated by Mn^{++} and Co^{++} by 120 and 160% respectively[9)]. For myokinase from rabbit muscle and yeast, the order of reactivity of the ions has been found to be $Mg^{++}>Ca^{++}>Mn^{++}>Ba^{++}$[1)].

It was found that either of substitution of AMP by another purine mononucleotides and ATP by another purine trinucleotides led to no activity. This specificity of two substrates in myokinase from skipjack muscle is very high as well as that of carp muscle. However the nucleotide specificity of myokinase from rabbit muscle, bovine liver and yeast is not so high as that of skipjack or carp muscle[1)10)]. In these enzymes, some activity was obserbed when ATP was substituted for 2′-dATP, CTP, GTP, UTP and ITP, but was not obserbed when AMP was substituted for another nucleoside monophosphate.

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