39. Purification and Characterization of Arginine Kinase from Scallop Smooth Muscle

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Introduction. Arginine kinase, first discovered by Lohmann,¹⁾ catalyzes the reversible formation of ATP and arginine from phosphoarginine and ADP:

The reaction proceeding from left to right is arbitrarily designated as the forward reaction. The enzyme is found in a wide variety of invertebrate species, being especially abundant in muscle. In view of the function of phosphocreatine and creatine kinases in vertebrate muscles, it appears that arginine kinase also plays an important role in the contractile processes of invertebrate muscles. Little is known, however, about its precise physiological function. In this study, arginine kinase was prepared from scallop smooth muscle in a homogeneous state and some of its properties were described.

Materials and method. Unless otherwise stated, the following techniques were used to determine the activity of the enzyme. The activity of the forward reaction was determined according to the method of Morrison *et al.*²⁾ Reaction mixture contained 0.45 ml of 5 mM phosphoarginine, 1 mM ADP, 1 mM MgCl₂ and 5 mM MOPS buffer (pH 7.0). The tubes were incubated in a water bath at 25°C and the reaction was started by the addition of 0.05 ml of arginine kinase solution. At the end of the incubation period the reaction was stopped by the addition of 0.5 ml of 0.1 M EDTA-5M NaOH. The amount of arginine released during the reaction was determined in the same tube by the method utilizing Sakaguchi reaction³⁾ as described by Rosenberg *et al.*⁴⁾ To the incubated mixture 3 ml of water and 1 ml of developing reagent were added and the red colored arginine complex was measured at 535 nm after standing for 30 min at room temperature. Developing solution contained α -naphthol and diacetyl solubilized in ethanol; their final concentrations were 1% and 0.05%, respectively. The velocity of the reverse reaction was estimated by measuring inorganic phosphate (Pi) released from phosphoarginine by acid hydrolysis. The reaction mixture, 0.4 ml, containing 10 mM arginine, 5 mM ATP, 10 mM MgCl₂ and 5 mM MOPS buffer (pH 8.5), was incubated in a water bath at 25°C. The reaction was started by adding 0.1 ml of enzyme solution and stopped by the addition of 0.5 ml of 0.2 N TCA. The tubes were then placed in boiling water for 1 min. Under these conditions phosphoarginine is quantitatively hydrolyzed to arginine and inorganic phosphate. They were then rapidly cooled in an ice bath and liberated Pi was estimated by the method of Fiske and SubbaRow with a slight modification.⁵⁾

Amino acid analysis was carried out essentially by the method of Moore and Stein⁶⁾ with a Hitachi amino acid analyzer (Model KLA-3B). Samples of the enzyme were hydrolyzed in constant boiling HCl (6N) in evacuated, sealed tubes for 24 h. After evaporation of HCl, hydrolyzates were dissolved in pH 2.2 solution and analyzed. Total half-cystine was determined by measuring cysteic

Fig. 1. Chromatography of the fraction precipitated between 0.72 and 0.95 saturated ammonium sulfate of 1 mM EDTA extract from scallop smooth muscle. Crude arginine kinase fractionated with $(\text{NH}_4)_2\text{SO}_4$ (56 mg) was collected and dialyzed against 10 mM Tris-HCl (pH 8.0) and applied to a DEAE-Toyopearl column (2.1×8 cm) equilibrated with the same buffer solution and eluted by using a linear gradient formed for 10 mM Tris-HCl buffer (pH 8.0) and 0.1 M KCl containing the same buffer. KCl concentration is shown as a dotted line. Each fraction is 4 ml. $\times$, kinase activity.

Fig. 2. SDS-PAGE patterns of fractions obtained during purification of arginine kinase. Electrophoresis was carried out using 10% polyacrylamide gel containing 0.1% SDS. Lane 1, 1 mM EDTA extract from scallop smooth muscle. Lane 2, the fraction precipitated by 72% saturation of 1 mM EDTA extract. Lane 3, the fraction precipitated by 95% saturation of 1 mM EDTA extract. Lane 4, arginine kinase (fraction number 15) purified by DEAE-Toyopearl column chromatography.

acid produced by performic acid oxidation.⁷⁾ Tryptophan content was determined from the absorption spectrum of the enzyme dissolved in 6 M guanidine-HCl, pH 6.5, as described by Edelhoch.⁸⁾

SDS-PAGE (polyacrylamide gel electrophoresis) was performed according to the method of Porzio and Pearson.⁹⁾ The protein concentration was determined by biuret reaction¹⁰⁾ and also by the absorbance at 280 nm.

Results and discussion. Adductor smooth muscle (45 g), obtained from living scallop, *Patinopecten yessoensis*, in the Sea of Okhotsk, was blended in 5 volumes of a solution containing 1 mM EDTA and 10 mM Tris-HCl buffer (pH 8.0), and extracted for 30 min at 4°C. The mixture was centrifuged for

Table I. Yields and specific activities of fractions obtained during purification of arginine kinase from scallop smooth muscle (started with 45 g muscle)

Fraction	Volume	Protein	Specific activity (μ mol Arg/mg·min)	Total activity
	ml	mg		
Extract	95	694	7.3	5,059
Ammonium sulfate precipitate (0.72 saturation)	22	400	3.0	1,200
Ammonium sulfate precipitate (0.72 to 0.95 saturation)	16	56	69.0	3,864
Eluates	25	17	210.0	3,570

Fig. 3. Effects of all pH on the activities of the forward reaction ($\circ$) and the reverse reaction ($\triangle$) of arginine kinase. The forward and reverse reaction were carried out under the same assay conditions as described in "Materials and methods".

10 min at 8,000 rpm and to the resulting supernatant was added 0.1 volume of 0.5 M Tris-HCl buffer (pH 8.0). The solution was then brought to 0.72 saturation by the addition of solid ammonium sulfate. The precipitate was removed by centrifuging at 13,000 rpm for 10 min and the resulting supernatant was then brought to 0.95 saturation by further addition of solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 mM Tris-HCl buffer (pH 8.0). The resulting solution was dialyzed for 18 hrs against 2 changes of the same buffer. It was then applied to DEAE-Toyopearl column and eluted by using KCl linear gradient. As shown in Fig. 1, arginine kinase was eluted in the first peak. Fig. 2 shows SDS-PAGE patterns of fractions obtained in the course of purification. One of the fractions was shown to be homogeneous, its molecular weight being estimated to be 43 K. The yields and specific activities of fractions during the purification are summarized in Table I.

Effects of pH on the kinase activity are shown in Fig. 3. The pH-activity curve shows a broad optimum between 8 and 10 in the reverse reaction, resembling that of rabbit muscle type creatine kinase. The forward reaction involving proton shows a lower optimum between 6 and 6.5 as expected.

For rabbit muscle creatine kinase, Mg^{2+} , Mn^{2+} , Ca^{2+} and Co^{2+} have been found to act as activators. As shown in Fig. 4, the activity of arginine kinase in the forward reaction is markedly increased by Mg^{2+} , but not by Ca^{2+} quite unexpectedly. Mn^{2+} and Co^{2+} also activated the enzyme. No activation was seen with Ba^{2+} , Sr^{2+} , Ni^{2+} , Fe^{2+} , Cu^{2+} and Zn^{2+} (data not shown). In the reverse reaction, Mg^{2+} , Mn^{2+} , and Co^{2+} activated the enzyme, but there was no activation by Ca^{2+} (data not shown). The enzymatic activity somewhat decreased with increase in KCl concentrations (about 60% of the maximum at 0.6 M).

Fig. 4. Effects of metal ions on the arginine kinase activity in the forward reaction. Reaction mixtures and conditions were the same as described in "Materials and methods" except the presence of various metal ions of 1 mM or 1 mM EDTA.

Table II. Amino acid composition of arginine kinase*¹

	Scallop	Lobster
Asx	44	38
Thr	17	17
Ser	20	16
Glx	33	39
Gly	35	28
Ala	28	25
Val	21	22
Met	10	8
Ile	16	17
Leu	37	28
Tyr	9	9
Phe	17	19
Lys	40	27
His	11	8
Arg	16	16
Pro	12	12
Cys* ²	9	5
Trp* ³	3	3
Total	378	337

*¹ Values are mol of amino acid/mol of arginine kinase. The molecular weight of scallop arginine kinase was estimated to be 43,000 according to the pattern of SDS-PAGE and the values for lobster arginine kinase were derived Blethen *et al.*¹¹⁾ *² Determined as cysteic acid after performic acid oxidation and hydrolysis in 6 N HCl for 18 hr.⁷⁾ *³ Determined by the spectrophotometric method of Edelhoch.⁸⁾

The amino acid composition of scallop arginine kinase was similar to that of lobster arginine kinase as shown in Table II. However, Lys, Leu and Cys contents of scallop arginine kinase were higher than those of lobster kinase. Smaller differences were observed among some other residues.

The phylogenetic significance of remarkable difference in distribution in the animal kingdom between arginine kinase and creatine kinase has long been a matter of discussion. To inquire into this problem, isolation and characterization of both enzymes from different kinds of species must be carried out before anything else. In this respect, the studies on arginine kinase have so far been very much behind those on creatine kinase. The development of a simple method of purifying arginine kinase in this article may be an important forward step in pursuing this interesting problem.

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