無脊椎動物におけるアルギニンキナーゼの出現と分布

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Occurrence and Distribution of Arginine Kinase in Crustacea with Special Reference to Kurumaebi *Penaeus japonicus* Collected from Lake Hamana and Nearby Area

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Arginine kinase activities were determined in the whole body or in the muscle of fifteen crustacean species. It could be confirmed that the enzyme activities were high in *Penaeus japonicus*, *Portunus sanguinolentus* and *Portunus trituberculatus*. The highest enzyme activity in *Penaeus japonicus* was found in the swimmeret muscle and this was followed by the dorsal and ventral muscles. In the case of *Portunus trituberculatus*, the highest activity was obtained for the coxa muscle followed by the palm muscle. The highest enzyme activity in *Procambarus clarki* was exhibited by the dorsal muscle.

The fluctuation of enzyme activity due to growth was observed in *Penaeus japonicus* whose body weight ranged from 0.31 mg to 4 g. The enzyme activity per 1 g of whole body increased with the growth of body weight of *Penaeus japonicus*. Specific activity, which is the activity per 1 mg of protein also increased but reached to almost a constant level at 0.6 g of body weight in *Penaeus japonicus*. The enzyme activity in the dorsal muscle of *Penaeus japonicus* which weighed from 1 to 25 g exhibited an increasing tendency with increase of body weight. Starch gel electrophoresis did not show the presence of isoenzymes of arginine kinase during the growth period of *Penaeus japonicus*.

In order to determine the effects of environmental factors on enzyme activity, the rearing experiment of *Penaeus japonicus* was carried out at 15, 20 and 25°C. The enzyme activity was almost the same at these different rearing temperatures. The rearing of *Penaeus japonicus* at different salinity levels, such as 25, 34 and 42.5‰ was carried out but the enzyme activity did not change due to these different salinities.

All the naturally occurring phosphorylated guanidino compounds function as stores of high energy phosphate-bonds. Phosphoryl groups of the phosphagens are transferred to ADP and form ATP as a result of enzymatic reactions catalyzed by phosphotransferases or kinases. The presence of arginine kinase (EC 2.7.3.3) in crude extracts of crab muscle, which catalyzes the following reaction,

$$\text{ATP}+\text{Argine} \rightleftharpoons \text{AP}+\text{ADP}$$

was demonstrated by Lohmann.[1] The widespread occurrence of arginine kinase among invertebrates and the presence of only creatine kinase in vertebrates have led to the suggestion that arginine kinase is one of the most primitive enzymes in the line of evolution. Detailed studies of arginine kinase have been carried out for lobster *Homarus vulgaris* and American lobster *Homarus americanus* (Virden *et al.*[2]; Blethen and Kaplan[3,4]) but only a little attention has been paid to kurumaebi, the prawn *Penaeus japonicus* and gazami, the crab *Portunus trituberculatus*. On the other hand, in Japan, kurumaebi is commercially cultured and it is being envisaged to culture gazami in the near future. This study was mainly carried out to observe the role of arginine kinase, which plays an important role in muscle contraction of these organisms, at various environmental conditions. Such a study can contribute to achieve a progress in planning out the ecological requirements of the culture systems.

Materials and Methods

Sampling

A number of marine invertebrate samples such

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* Abbreviations: AP: Arginine phosphate; DTT: Dithiothreitol; PEP: Phosphoenolpyruvate.

as numaebi *Paratya compressa*, ushiebi *Penaeus monodon*, kumaebi *Penaeus semisulcatus*, Kurumaebi *Penaeus japonicus*, hiraisogani *Gaetice depressus*, taiwangazami *Portunus pelagicus*, futababenitsukegani *Thalamita sima*, gazami *Portunus trituberculatus*, janomegazami *Portunus sanguinolentus*, ishigani *Charybdis japonica*, tatejimafujitsubo *Balanus amphitrite* and short-necked clam (asari) *Tapes philippinarum* were collected from Lake Hamana. Amerikazarigani *Procambarus clarki*, brine shrimp *Artemia salina* and krill *Euphausia* sp. were purchased from a commercial source. Wamushi *Brachionus plicatilis* and copepod *Tigriopus* sp. which were cultured at Fisheries Laboratory, The University of Tokyo were used.

Extraction

Immediately after being caught, the samples were dissected out and were minced with scissors. The minced samples were homogenized with 5 volumes of ice-cold water. The homogenate was centrifuged at 14,000 rpm for 30 min and the supernatant was used to determine the occurrence and distribution of arginine kinase.

Determination of Enzyme Activity

The general equation for the enzyme activity can mainly be expressed as follows.

$$\text{Arginine}+\text{ATP} \rightleftharpoons \text{AP}+\text{ADP}$$

1. Forward Reaction Enzyme activity in the forward direction was measured at 30°C in Hitachi model 200–20 spectrophotometer by monitoring the oxidation of $NADH_2$ at 340 nm as described by Hird and McLean[5] with some modifications. The reaction mixture of 3 m*l* contained in the final concentration 50 mM Tris-HCl buffer (pH 8.2), 1 mM PEP, 1 mM $MgSO_4$, 1 mM ATP, 0.3 mM $NADH_2$, 15 mM arginine, 0.33 mM DTT, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase and an appropriate amount of arginine kinase.

In addition to the above coupled enzyme reaction, the activity of the forward reaction was determined by estimating inorganic phosphate released from AP due to acid hydrolysis. The method used was similar to that of Morrison *et al.*[6] with some modifications. A final reaction volume of 0.4 m*l*, contained 50 mM Tris-HCl buffer (pH 8.0), 4 mM ATP, 25 mM arginine, 4 mM $MgSO_4$ and an appropriate amount of arginine kinase. The reaction was carried out at 30°C for 10 min and stopped by adding 0.4 m*l* of 0.2 N TCA. The tubes were placed in a boiling water bath for 1 min. They were then rapidly cooled by agitation in an ice bath and inorganic phosphate released from AP was estimated by the method of Chen *et al.*[7]

2. Reverse Reaction The activity in the reverse direction was determined by observing the reduction of NADP at 340 nm and 30°C in a coupled assay according to the method of Volmer *et al.*[8] with some modifications. The reaction mixture of 3 m*l* contained in the final concentration 50 mM Tris-HCl buffer (pH 7.4), 0.4 mM AP, 10 mM $MgSO_4$, 5 mM ADP, 13.3 mM glucose, 3 mM NADP, 16 units of hexokinase, 5 units of glucose 6-phosphate dehydrogenase and an appropriate amount of arginine kinase.

Rearing of Kurumaebi

The rearing experiments of kurumaebi were carried out twice at the Fish Culture Farm at Seto Inland Sea in Ehime Prefecture. The first rearing experiment (Experiment 1) was carried out from April 18 to May 23, 1987 and the average body weight of specimens increased from 0.31 to 10.5 mg within this period. Sampling dates and the respective average body weights are presented in Table 5.

The second rearing experiment (Experiment 2) was started on June, 5 1986 at the same place and was continued up to August 9 in the same year. The average body weight of specimens at the end of the experiment was around 5 g. Whole body krill meal was used as the feed for these prawns during the first two weeks, and then gradually it was mixed with a commercial diet, until they were one month old. After 2 months, prawns were fed with a commercially prepared diet. Sampling dates are indicated in Table 6 and samplings were done from June 5 to August 9 at an interval of three days.

Enzyme Assay

About 20–30 specimens were selected randomly and homogenized with 10 volumes of 100 mM Tris-HCl buffer (pH 8.0) containing 1 mM mercaptoethanol. Arginine kinase activity was determined by means of the coupled enzyme reaction in the forward direction as explained.

Protein Assay

Protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as standard.

Localization of Enzyme on Starch Gel Electrophoresis

The electrophoresis was done by using 87 mM Tris-boric acid buffer (pH 9.0) containing 1 mM EDTA as described by Ayala *et al.*.[10] A small amount of the extract was absorbed on a 5×5 mm piece of Toyo filter paper No. 2, and then was placed in the electrophoretical starch gel. Electrophoresis was carried out with a constant current of 40 mA per slab for 7 h. The staining solution used to locate arginine kinase contained 100 mM Tris-HCl buffer (pH 7.6), 1.2 mM ADP, 5.5 mM AMP, 5 mM $MgCl_2$, 5 mM AP, 7.4 mM glucose, 0.9 mM NADP, 2 units of glucose 6-phosphate dehydrogenase and 5 units of hexokinase. The concentrations of nitro blue tetrazolium and phenazine methosulfate were adjusted to 0.16 and 0.04 mg/m*l*, respectively. The total volume of the reaction mixture was 10 m*l*. The incubation was carried out in the dark at 37°C for 90 min. Gels were stained with coomassie brilliant blue R-250 for proteins.

Rearing of Kurumaebi at Different Temperatures

Kurumaebi specimens weighing 13.0 g in average were divided randomly into three groups of 20 each and were reared in 750 *l* capacity polypropylene tanks. A sand bed of 7 cm thickness was spread on the bottom of the tanks throughout the experiment. Each of the tanks was filled with 150 *l* of sea water. The sea water was filtered through a bed of chemical fiber to remove detritus and particulate matters before being supplied into the tanks. Aeration was provided by pumping air through 10 cm long aquarian air stones. The initial 7°C water temperature was raised up gradually and kurumaebi were subjected to constant 15, 20 and 25°C acclimation temperatures within 48 h. A constant 34‰ salinity level was maintained throughout the experiment. Two kurumaebi specimens were sampled every two days. They were killed by cutting off their heads immediately and were frozen at −20°C until use. During the experiment, kurumaebi were fed with crushed shellfish daily.

Rearing of Kurumaebi at Different Salinities

Kurumaebi specimens weighing 17.0 g in average were divided randomly into three groups of 30 each. Three salinity levels, 25, 34 and 42.5‰ were prepared by using artificial sea water and the experiment was carried out in the same polypropylene tanks which had been used for the temperature experiment with a 6 cm thick sand bed. The salinities were maintained throughout the experiment and the water temperature was kept at 25°C. They were fed with crushed shellfish daily. Three kurumaebi specimens were sampled every 2 days. They were killed and frozen as explained above.

Enzyme Assay

Arginine kinase activity was measured by means of the coupled enzyme reaction in the forward direction as explained. The dorsal muscle of kurumaebi was used for the enzyme assay.

Results

Table 1 shows the occurrence and distribution of arginine kinase in several species of crustacean. In our preliminary investigation, several fish samples of yellowtail, tilapia, sillago (kisu), honmoroko, flatfish and red sea bream were subjected for the determination of arginine kinase activity in their red muscle, white muscle and liver. It was unable to detect any occurrence of arginine kinase in those samples (data not shown). The enzyme activity could be detected in wamushi, copepod and brine shrimp at low levels. A significant activity could not be detected in fujitsubo body muscle extract. Kurumaebi arginine kinase showed an activity which ranged from 1,020 to 3,940 μmol/min·g of dorsal muscle. An increasing tendency of the activity was observed with the increase of body weight. The enzyme activities of numaebi, ushiebi and kumaebi were found to be less than that of kurumaebi. Gazami revealed the highest enzyme activity among the crab species including hiraisogani, taiwangazami, futababenitsukegani, janomegazami and ishigani. As a consequence, kurumaebi and gazami arginine kinases were much more superior in activity to those of all the samples which were tested.

Table 2 shows the distribution of arginine kinase in different tissues of kurumaebi. The dorsal and ventral muscles exhibited activities of 1,450 and 1,160 μmol/min·g, respectively. The highest activity was obtained from swimmeret muscle and it revealed 2,060 μmol/min·g. The telson and antennal muscles exhibited 770 and 650 μmol/min·g, respectively. A significant activity could not be seen in the intestine.

Table 3 shows the distribution of arginine kinase activity in various tissues of gazami. The highest activity was obtained from the coxa muscle and it was 2,170 μmol/min·g. The palm muscle exhibited 1,290 μmol/min·g activity, while arginine kinase activities in the brain, nerve cord and heart were 340, 120 and 210 μmol/min·g, respectively.

Table 1. Distribution of arginine kinase

Class	Common name	Scientific name	Body weight (g)	Tissue	Activity (μmol/min·g)
Rotatoria	wamushi	*Brachionus plicatilis*		Whole body	10
Crustacea	brine shrimp	*Artemia salina*		Eggs	40
	〃	〃		Whole body	30
	krill	*Euphausia* sp.	0.8	〃	15
	numaebi	*Paratya compressa*	0.8	〃	65
	ushiebi	*Penaeus monodon*	35.2	Dorsal muscle	1,040
	kumaebi	*Penaeus semisulcatus*	7.6	〃	1,100
	kurumaebi	*Penaeus japonicus*	1.3	〃	1,020
	〃	〃	2.0	〃	1,340
	〃	〃	3.1	〃	1,160
	〃	〃	10.6	〃	1,670
	〃	〃	12.0	〃	1,770
	〃	〃	14.7	〃	2,230
	〃	〃	17.0	〃	3,180
	〃	〃	19.8	〃	3,380
	〃	〃	24.6	〃	3,940
	hiraisogani	*Gaetice depressus*	3.0	Body muscle	120
	taiwangazami	*Portunus pelagicus*	99.8	〃	600
	futababenitsukegani	*Thalamita sima*	18.2	〃	840
	gazami	*Portunus trituberculatus*	145.9	〃	1,350
	〃	〃	34.7	〃	220
	janomegazami	*Portunus sanguinolentus*	20.3	〃	1,760
	ishigani	*Charybdis japonica*	0.3	Whole body	330
	〃	〃	1.0	〃	420
	〃	〃	2.9	〃	410
	〃	〃	108.5	Palm muscle	720
	copepod	*Tigriopus* sp.		Whole body	80
	tatejimafujitsubo	*Balanus amphitrite*	0.2	Body muscle	*ND
Pelecypoda	short-necked clam	*Tapes philippinarum*	6.2	〃	40

* ND: Not detectable.

Table 2. Distribution of arginine kinase in different tissues of kurumaebi *Penaeus japonicus*

Tissue	Activity (μmol/min·g)
Dorsal muscle	1,450
Ventral muscle	1,160
Swimmeret muscle	2,060
Telson muscle	770
Uropod muscle	240
Antennal muscle	650
Brain+Nerve cord	110
Stomach	10
Intestine	*ND
Heart+Blood vessel	270
Eyes	20

Body weight: 34.7 g.
* ND: Not detectable.

Table 3. Distribution of arginine kinase in different tissues of gazami *Portunus trituberculatus*

Tissue	Activity (μmol/min·g)
Body muscle	220
Palm muscle	1,290
Coxa muscle	2,170
Brain	340
Nerve cord	120
Eggs	10
Stomach	230
Intestine	60
Heart	210

Body weight: 35.2 g.

The enzyme activities in the intestine and in the eggs were very low as compared to those of other organs.

Distribution of arginine kinase activity in different tissues of amerikazarigani is presented in Table 4. The higher activities could be seen in the dorsal muscle, palm muscle and uropod muscle, and the values obtained were 1,920, 1,820 and 1,370 μmol/min·g, respectively.

The enzyme activities during the very early growth period of kurumaebi in rearing Experiment

1 are shown in Table 5. The average body weight of the specimens was from 0.31 to 10.5 mg and the activity increased from 11 to 234 μmol/min·g, of whole body.

Table 4. Distribution of arginine kinase in different tissues of amerikazarigani *Procambarus clarki*

Tissue	Activity (μmol/min·g)
Dorsal muscle	1,920
Telson muscle	1,030
Palm muscle	1,820
Walking leg muscle	829
Uropod muscle	1,370
Heart	456
Gut	156
Gastric gland	82
Nerve cord	166
Liver	72
Gills	26
Eyes	90

Body weight: 25.6 g.

Table 6 shows the results of Experiment 2. Arginine kinase activity increased gradually from 176 to 699 μmol/min·g of whole body when the body weights were gained from 0.053 to 5.51 g. The specific activity increased from 5.5 to 15.6 μmol/min·mg protein when the body weight increased from 0.053 to 0.621 g. But the specific activity remained almost constant after kurumaebi gained 0.6 g of body weight, only one month later. The relationships among the enzyme activity, specific activity and average body weight gained are plotted in Fig. 1.

Composite pictures of the starch gel electrophoretic patterns of the above samples are presented in Fig. 2. The electrophoretic patterns were different from each other, whereas only a single band of arginine kinase appeared at the same location irrespective of sample.

Fig. 3 represents the enzyme activities and the specific activities of kurumaebi reared at 15, 20 and 25°C. The specific activity of kurumaebi reared at 15°C exhibited 39 μmol/min·mg pro-

Table 5. Changes in arginine kinase activity during the growth of kurumaebi (Experiment 1)

Sampling date		Av. body weight (mg)	Protein content (mg/g)	Activity (μmol/min·g)	Sp. activity (μmol/min·mg protein)
April	18	0.31	38.4	11	0.3
〃	27	2.52	34.5	142	4.1
May	2	3.19	37.0	96	2.6
〃	8	4.74	36.4	227	6.2
〃	13	10.30	32.2	225	7.0
〃	18	8.64	34.9	234	6.7
〃	23	10.50	34.0	234	6.9

Table 6. Changes in arginine kinase activity during the growth of kurumaebi (Experiment 2)

Sampling date		Av. body weight (g)	Protein content (mg/g)	Activity (μmol/min·g)	Sp. activity (μmol/min·mg protein)
June	5	0.053	32.0	176	5.5
〃	8	0.058	25.3	212	8.4
〃	11	0.043	29.0	212	7.3
〃	14	0.059	24.8	262	10.6
〃	17	0.106	22.8	258	11.3
〃	20	0.121	36.0	292	8.1
〃	23	0.263	22.3	337	15.1
〃	29	0.284	26.8	364	13.6
July	2	0.462	27.0	376	13.9
〃	5	0.428	29.3	382	13.0
〃	8	0.621	29.3	456	15.6
〃	11	0.603	32.3	477	14.8
〃	28	2.59	37.0	635	17.2
August	8	5.51	42.5	699	16.4
〃	9	4.01	42.0	640	15.2

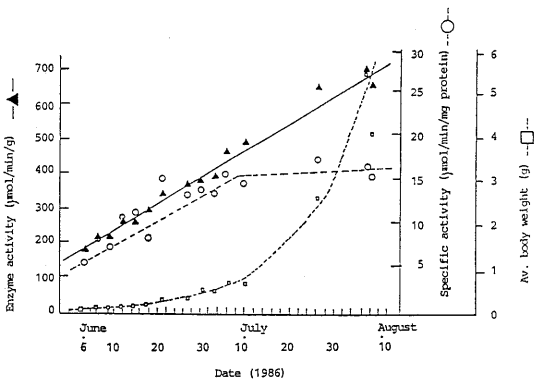

Fig. 1. Changes in arginine kinase activity during the growth of kurumaebi (Experiment 2).

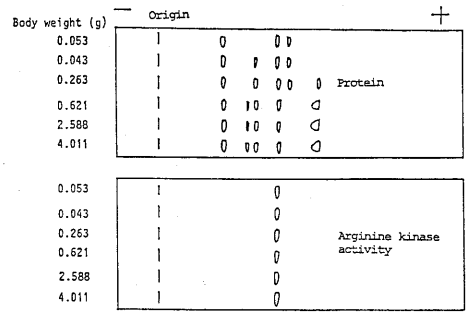

Fig. 2. Electrophoretic patterns of arginine kinase at different growth stages of kurumaebi.

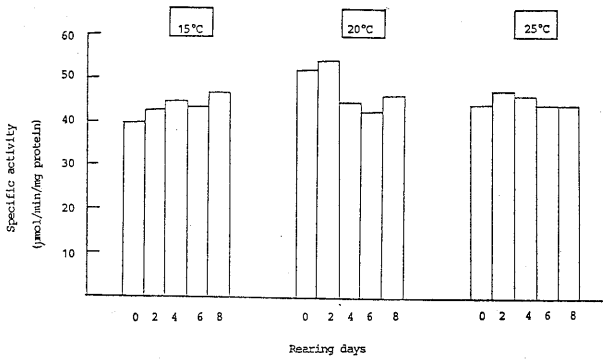

Fig. 3. Arginine kinase activity of the dorsal muscle of kurumaebi reared at different temperatures.

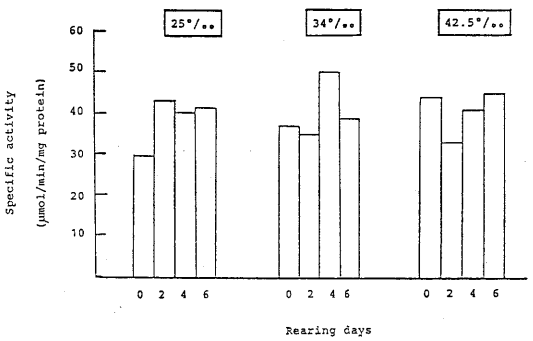

Fig. 4. Arginine kinase activity of the dorsal muscle of kurumaebi reared at different salinities.

tein on the initial day of the experiment. On the same day these values in kurumaebi reared at 20 and 25°C were quite high, being 52 and 44 μmol/min·mg protein, respectively. After 8 days at 15°C the specific activity of kurumaebi increased to 46 μmol/min·mg protein. At the end of the experiment the specific activity of kurumaebi which were reared at 15, 20 and 25°C was around 45 μmol/min·mg protein. The enzyme activities did not vary significantly due to these different temperature levels.

Fig. 4 shows the arginine kinase activities of kurumaebi at 25, 34 and 42.5‰ salinity levels. A significantly low activity could be seen in kurumaebi which were reared at 25‰ salinity level.

The specific activity gradually increased from 29 to 41 μmol/min·mg protein during the 6 day period of the experiment. The specific activity of kurumaebi at 34‰ showed a uniform trend throughout the experiment. The specific activity observed in kurumaebi on the initial day at 42.5‰ salinity level was 44 μmol/min·mg protein and it is slightly higher than those at 25 and 34‰ levels. At the end of the experiment the specific activities in the specimens from the three different salinity levels were around 40 μmol/min·mg protein.

Discussion

The presence of arginine kinase could be confirmed in almost all the invertebrate samples which were analyzed during the present investigation.

The enzymes of kurumaebi and gazami revealed the higher activities among the samples which were subjected to the investigation. A significant difference in activity could not be seen between wild kurumaebi caught from Lake Hamana and those purchased from kurumaebi culture farms. Also, imported kurumaebi from Taiwan exhibited the same activity. One of the most interesting observations of this study was the detection of an increasing tendency of the activity with the increase of body weight. The enzyme was abundant in the muscle, and highly contractile muscles revealed the highest activity. This phenomenon agrees with the major biological role of creatine kinase in vertebrates during the muscle contraction.

The arginine kinase activities in various tissues of kurumaebi, gazami and amerikazarigani varied from each other. The enzyme activity in the muscle was always higher than those of other organs. This may be due to the necessity of higher energy requirements by the highly contractile muscles. It has also been reported by Lang *et al.*[11] that the presence of arginine kinase in the myofibrillar structure of *Drosophila melanogaster* varied in different types of muscle.

Environmental factors affect the metabolic rates of crustaceans in a significant manner. Such factors may affect on the activity of arginine kinase of kurumaebi and gazami as far as cultural practices are concerned. But the enzymatic activities did not change significantly due to different temperatures at 15–25°C and salinities at 25–42.5‰ in kurumaebi. When kurumaebi were exposed to different environmental conditions, initial fluctuations of enzyme activities observed might be due to the effect of sudden change in the environmental condition. However the organisms recovered from the shock at the end of the experiment as revealed by stabilized enzyme activity.

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