Journal of Aquatic Animal Health
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uahh20

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Version of record first published: 09 Jan 2011.

To cite this article: Barbara S. Horney, Allan L. MacKenzie, Richard J. Cawthorn, Robert J. MacMillan & Claude Morris (2001): Measurement of Arginine Kinase Activity in Hemolymph of American Lobsters, Journal of Aquatic Animal Health, 13:4, 328-333

To link to this article: http://dx.doi.org/10.1577/1548-8667(2001)013<0328:MOAKAI>2.0.CO;2

Journal of Aquatic Animal Health 13:328–333, 2001

Measurement of Arginine Kinase Activity in Hemolymph of American Lobsters

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Abstract.—The concentration of arginine kinase (AK) in the hemolymph of lobsters may aid in the identification of muscle diseases in these animals in the same way that creatine kinase activity in blood plasma does in mammalian species. Both manual and automated analysis methods were developed for measuring AK levels in the hemolymph of the American lobster *Homarus americanus*. A commercial reagent that is used to measure creatine kinase in human blood serum was modified by substituting phospho-L-arginine for creatine phosphate as the substrate to allow measurement of the AK enzyme. Stability of the enzyme in lobster hemolymph plasma was less than 3 h at room temperature (22°C), 6 h when refrigerated (2–5°C), and 24 h when frozen (−20°C). Comparisons were made between the manual and automated protocols using three lobster hemolymph plasma samples. The average recovery in the latter relative to the former was 103%. The automated assay was linear up to 1,940 U/L. Precision studies were conducted using the automated procedure with low and high AK plasma samples. Coefficients of variation were less than 2% for the within-run ($N = 15$) and less than 3.5% for the between-run assays ($N = 7$). A nonparametric description (range, 9–3,322 U/L; median, 111 U/L; 25th−75th percentiles, 71–304 U/L) was applied to the data from the automated method for AK concentrations in lobster plasma using 95 healthy lobsters obtained during the 1995 spring season in Lobster Fishing Area 26A, Prince Edward Island. No significant differences in AK levels were detected between male and female lobsters. Identifiable increases in hemolymph AK activity were detected in 5 out of 10 lobsters 44 d after they were experimentally infected with the ciliate parasite *Anophryoides haemophila*. Our applications will facilitate consistent analysis of AK levels in the hemolymph of the American lobster.

The lobster industry of North America relies extensively on short- and long-term maintenance of lobsters in holding units prior to marketing. Determination of the health of these animals to ascertain which populations are suitable for long-term storage and to maintain good health during impoundment should result in decreased losses during storage and subsequent shipment to market. Previous studies examined changes in lobster serum protein levels in relation to feed availability, temperature (Stewart et al. 1967, 1972), and alterations during the molt and reproductive cycles (Barlow and Ridgway 1969). Mercaldo-Allen (1991) has identified changes in serum levels for calcium, chloride, inorganic phosphorus, magnesium, potassium, sodium, osmolality, and total protein over the molt cycle. These parameters are nonspecific indicators of decreased condition or disease and have a wide range of biological variation that makes interpretation of the concentrations difficult (Glynn 1968; Barlow and Ridgway 1969; Castell and Budson 1974; Mercaldo-Allen 1991). Additional information on health status could be provided if increases in arginine kinase (AK) activity, which indicate enzyme leakage across muscle cellular membranes, prove to be a specific marker of muscle cellular damage like increases in creatine kinase (CK) activity in the serum or plasma of mammals (Wolf 1991; Nockels et al. 1996).

Alterations in the blood levels of many compounds can indicate physiologic stress or disease in mammals and fish. Increased leakage of intracellular enzymes from organs into the blood follows cellular damage (Kramer 1989). The failure of normal regulation of extracellular electrolytes and minerals or the decreased excretion of waste compounds is also used to indicate diseased states in several species. An early indicator of disease in lobsters would be highly advantageous to processors for management and marketing decisions. Arginine kinase belongs to the same family of enzymes as CK and is assumed to be involved in intracellular energy transport in cardiac and skeletal muscle (Dumas and Camonis 1993). Increased plasma activity of CK is used as an early indicator of skeletal muscle disease in mammals (Wolf 1991). Increased plasma CK activity has also been associated with stress in calves (Nockels et al. 1996). Arginine kinase occurs in arthropods (Mor-

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Received October 13, 2000; accepted May 27, 2001.

rison 1973) and is especially abundant in the abdominal flexor muscle tissue of European lobsters *Homarus vulgaris* (Newsholme et al. 1978). Observations in our laboratory suggest that the level of AK in lobster hemolymph can be an indicator of muscle damage caused by the scuticociliate *Anophryoides haemophila* (Cawthorn 1997). Given the usefulness of plasma CK activity alterations in identifying disease and stress in mammals, an AK assay under various biological or environmental conditions may prove to be a useful indicator of stress or disease in lobsters. In this study, we investigated the feasibility of developing an application for measuring AK levels in the serum and plasma of American lobsters *Homarus americanus*.

Methods

Lobster source and housing.—Canner lobsters (carapace length, 65–81 mm; weight, 190–400 g) were purchased from a local supplier during the fall 1994 and spring 1995 seasons in Lobster Fishing Area 26A, Prince Edward Island, and transferred to the Aquatic Animal Facility, Atlantic Veterinary College. They were housed in compartmentalized units in a saltwater recirculation system (2 ± 1°C) containing both particle and biological filters with a 12 h light:12 h dark photoperiod. The temperature and dissolved oxygen content (range, 7.5–10.5 mg/L) of the system were monitored on a continual basis. Additional water quality parameters, namely, pH (range, 7.9–8.4), salinity (range, 28–32‰), un-ionized ammonia (<0.1 mg/L), nitrates (<20 mg/L), and nitrites (<0.1 mg/L), were monitored weekly. Lobsters were fed one mussel (*Mytilus edulis*) with a cracked shell weekly. Lobsters were maintained in accordance with the guidelines of the Canadian Council on Animal Care and under the supervision of the Animal Care Committee, University of Prince Edward Island.

Sampling.—Samples (approximately 1.5 mL) were collected from the ventral abdominal sinus under aseptic conditions by swabbing the surface with a 70% solution of alcohol prior to removing the hemolymph with a 3-cm^3 syringe fitted with a 20-gauge needle. Samples were kept on ice in the syringe (<20 min) until they were transferred to polypropylene microcentrifuge tubes and centrifuged at 1,000 × gravity for 15 min at 4°C to remove hemocytes and delay coagulation (Martin and Hose 1995). The hemolymph plasma was removed and kept on ice until AK levels were measured (always within 4 h of sampling).

Assay development.—Arginine kinase levels were determined using both benchtop techniques and an automated biochemical analyzer (Hitachi 911, Boehringer Mannheim Canada, Laval, Quebec) with a modified commercial reagent (Diagnostic Chemicals, Ltd., Charlottetown, Prince Edward Island). The commercial reagent measures creatine kinase activity using the creatine-phosphate-dependent production of adenosine triphosphate from adenosine diphosphate (Oliver 1955) through the hexokinase and glucose 6-phosphate dehydrogenase coupled reaction (German Society for Clinical Chemistry 1977; Gerhart et al. 1979). The modification of the reagent involved using 5 mM of phospho-L-arginine (P-5139, Sigma Chemical Company, St. Louis, Missouri) as substrate instead of the creatine phosphate (Tombes and Shapiro 1989). Manual analysis was carried out in duplicate at 37°C and 340 nm with a spectrophotometer, as described in the reagent insert, using arginine kinase from lobster tail muscle (A-3389, Sigma Chemical) as a control sample. Automated analysis was carried out on the biochemical analyzer using an application for creatine kinase analysis available through the Diagnostic Chemicals technical service. The assay was performed using a K factor,[1] which should be determined for the specific instrument being used.

Enzyme stability and assay validation.—Stability of the AK enzyme was evaluated by monitoring its level in two hemolymph plasma samples over time. These samples were aliquoted and stored at room temperature (22°C) as well as under refrigerated (2–5°C) and frozen (–20°C) conditions. Enzyme levels were measured initially and after 3, 6, 24, 48, and 168 h in the room temperature and refrigerated samples. The frozen samples were analyzed at 24, 48, and 168 h. All stability samples were analyzed using manual techniques. Agreement between the manual assay and automated analyzer results was verified by comparing three lobster plasma samples covering the range of the assay.

Linearity of the assay was determined using the automated analyzer and saline dilutions of a high-level AK hemolymph plasma. The observed results were compared with theoretical recoveries based on the dilutions. Samples with AK values above linearity were routinely diluted with saline and reassayed.

Precision studies for the automated procedure

[1] To determine the K factor, consult the Hitachi 911 instrument manual, calibration method section, or contact Diagnostic Chemicals, Ltd., technical service.

TABLE 1.—Stability of the arginine kinase enzyme activity (U/L) in American lobster hemolymph at three temperatures, as obtained by the manual assay; percent recoveries are given in parentheses.

Time (h)	Room temperature (22°C)	Refrigerated (2–5°C)	Frozen (−20°C)
	Sample 1		
0	122		
3	116 (95)	100 (82)	
6	108 (89)	102 (84)	
24	12 (10)	107 (88)	108 (89)
48	0 (0)	102 (84)	111 (91)
168	0 (0)	74 (61)	79 (65)
	Sample 2		
0	713		
3	444 (62)	692 (97)	
6	275 (39)	683 (96)	
24	6 (0)	550 (77)	669 (94)
48	0 (0)	446 (63)	520 (73)
168	0 (0)	171 (24)	259 (36)

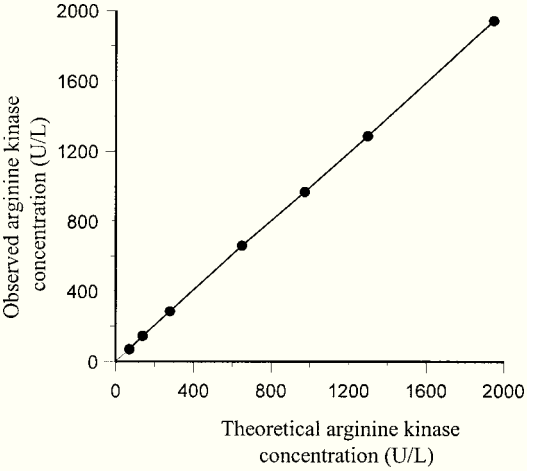

FIGURE 1.—Linearity assessment of an automated assay for arginine kinase (AK) using a high-level (1,946 U/L) American lobster hemolymph plasma diluted in 0.85% saline. The observed AK concentrations correlate closely (within 5%) with the expected concentrations.

were conducted with plasma hemolymph samples, and coefficient of variation (100·SD/mean) calculations were performed. Two hemolymph samples were analyzed in 15 replicates in the same trial to obtain a within-run precision check. To obtain the between-run precision, two hemolymph samples were analyzed in seven trials. Each trial consisted of an individual analysis on the instrument preceded by fresh calibration with an AK control for quality assurance. Hemolymph samples were kept on ice, and a fresh aliquot used for each run to minimize sample degradation. Total analysis time was restricted to 2 h. The analytical sensitivity of the procedure using the automated analyzer was determined using saline dilutions of one of the samples used in the within-run precision determination.

Statistical treatment.—A computer software program (Minitab 1992) was used for statistical calculations. AK activity in lobster plasma was determined using the automated method for 95 healthy lobsters (45 males and 50 females) maintained for 2 weeks in the Aquatic Animal Facility, Atlantic Veterinary College, under approximate pound conditions. A nonparametric description (the median and 25th−75th percentiles) was obtained for the AK concentrations in lobster plasma. A Mann–Whitney rank-sum test was employed to check for a significant difference ($\alpha < 0.05$) in AK levels between sexes.

Infection trial.—A preliminary study was conducted to evaluate the effect of *A. haemophila* infection on hemolymph AK levels. Baseline hemolymph AK activity was measured in 20 lobsters. Then 10 lobsters were injected with *A. haemophila* (5,000 organisms, injected into the ventral tail sinus) using filtered seawater as the transport vehicle, while another 5 were injected with filtered seawater alone (to determine the effect of the transport medium) and the remaining 5 were noninjected controls. Hemolymph AK activity was measured at 15, 30, 44, and 61 d postinfection.

Results

The stability of the AK enzyme in hemolymph plasma under different temperature regimes is presented in Table 1. Average (samples 1 and 2) recoveries based upon initial measurement were 79% after 3 h at room temperature, 90% after 6 h of being refrigerated, and 91% after 24 h of being frozen. Three plasma samples were analyzed for AK levels using both manual techniques (57, 121, and 1,810 U/L) and the automated analyzer (53, 127, and 2,018 U/L). Average recovery for the automated procedure compared with the manual assay was 103%. The automated procedure was considered to be linear to at least 1,940 U/L because dilutions of hemolymph plasma with a high AK level gave observed results within 5% of those that were expected (Figure 1). The results of the precision study are summarized in Table 2. Coefficients of variation for the within-run and between-run analyses were less than 3.5% for all calculations. An approximation of the lower limit of sensitivity for the automated assay was obtained

TABLE 2.—Precision of the data obtained by automated analyses for two concentrations of arginine kinase (AK) in the plasma of American lobster.

Statistic	Concentration	
	Low	High
Within run (N = 15)		
Mean AK concentration (U/L)	83	142
Standard deviation	1.30	1.94
Coefficient of variation[a]	1.57	1.37
Between run (N = 7)		
Mean AK concentration (U/L)	92	251
Standard deviation	3.13	3.21
Coefficient of variation[a]	3.40	1.28

[a] 100 · SD/mean.

with a 14-fold saline dilution of the low-level (83 U/L) hemolymph plasma sample used in the within-run precision study. Recovery (6 U/L) was 100%.

The median arginine kinase concentration in 95 healthy lobsters maintained for 2 weeks at the Atlantic Veterinary College under simulated pound conditions was 111 U/L (25th−75th percentiles, 71–304 U/L; range, 9–3,322 U/L). No significant difference was detected between males and females.

The baseline hemolymph AK activity of the 20 lobsters in the infection trial ranged from 59 to 1,241 U/L (Table 3). Arginine kinase activity was above the range obtained from the 95 healthy lobsters in 1 of 10 ciliate-infected lobsters at day 30 and in 5 of 10 ciliate-infected lobsters at day 44. Five of the infected lobsters had died, and one additional lobster had markedly increased AK activity at day 61. Neither the seawater-inoculated or noninoculated lobsters exhibited an increase in AK activity at any of the sampling times.

Discussion

It is our opinion that a demonstrated decrease in enzyme activity of 20% or more in one or both of the hemolymph samples evaluated for enzyme stability indicates an unsatisfactory stability at that time and temperature. Because AK in lobster hemolymph plasma is not stable at room temperature (recovery < 80% after 3 h) and has limited stability under refrigerated (6 h at 2–5°C) and frozen (24 h at −20°C) conditions, we conclude that analysis would best be performed under automated conditions, which reduce analysis time and potential sample degradation while increasing accuracy. Further, we recommend that all samples be kept on ice until analyzed and that AK measurement be made on the day of sampling.

TABLE 3.—Arginine kinase concentrations (U/L) in the hemolymph plasma of American lobsters initially (baseline) and 15, 30, 44, and 61 d after inoculation with the blood ciliate *Anophryoides haemophila,* inoculation with filtered seawater, or no inoculation. Lobsters were housed in a recirculating system with water temperature of 2 ± 1°C. The letter C means that the sample was clotted and unusable, the letter D that the lobster died before sampling day 61.

Treatment	Time (d)				
	Baseline	15	30	44	61
Ciliate					
1	1,241	489	246	394	969
2	514	C	C	541	1,929
3	571	601	C	172	1,894
4	596	113	2,886	155,300	D
5	586	80	300	2,008	151,550
6	226	9	1,042	6,750	D
7	123	74	1,837	8,100	D
9	225	254	6,516	7,450	D
9	306	280	C	146	1,464
10	59	293	963	29,450	D
Seawater					
11	375	270	43	189	8
12	403	375	101	C	366
13	102	10	22	48	19
14	354	129	73	106	35
15	231	53	36	61	75
No inoculation					
16	182	66	37	179	102
17	185	36	53	54	90
18	167	113	36	78	392
19	304	73	50	58	448
20	118	0	29	19	51

Excellent agreement was achieved between the manual and automated assay methods. The average recovery variation (automated compared with manual for three samples) was 103%, with no individual sample recovery variation greater than 11.5%. Linearity for the automated procedure was determined to prevail until at least 1,940 U/L (Figure 1). This value does not reflect the effects of reagent degradation (which may develop during storage) because all the reagents used in our study were freshly prepared and used within 24 h. The saline dilutions used to determine our linear range resulted in values within 5% of the theoretical calculations. In the linearity assessment performed herein, the lowest evaluated AK level was approximately 70 U/L. This sample performed well in the linearity assessment (the theoretical yield was 70 U/L), but lower levels were not assessed. Acceptable recovery was obtained during the analytical sensitivity check, indicating linearity and sensitivity to less than 10 U/L for the assay. The precision of the automated assay is acceptable,

with coefficients of variation of less than 2% for the within-run evaluation and less than 4% for the between-run evaluation.

Arginine kinase concentrations were assessed in lobsters held under approximate pound conditions to provide a working reference range. A nonparametric description was chosen to best describe this range when data resisted transformation to normality. Some of the sample variability may reflect the inadvertent aspiration of muscle fluid, which would contain high concentrations of this enzyme, in the collection process. Employing great care in the sampling procedure, so that hemolymph is collected from the ventral tail segment closest to the thorax just off the midline—avoiding the ventral nerve cord and ventral abdominal artery (without deep penetration)—would help assure collection of hemolymph and not muscle fluid in future investigations.

The purpose of this communication is to describe a method of measuring AK activity in lobster hemolymph. Using this method, we demonstrated increased hemolymph AK concentrations in lobsters experimentally infected with *A. haemophila*. These preliminary results suggest that measuring the activity of this enzyme in hemolymph from lobsters affected by systemic disease could be useful in disease identification or in investigations of disease pathogenesis in much the same way that plasma CK activity is for diseased or stressed mammals (Wolf 1991; Nockels et al. 1996).

In summary, we have developed both manual and automated applications to measure AK levels in the hemolymph of the American lobster. Further work is required to assess the importance of this enzyme as an early indicator of disease states in this lobster.

Acknowledgments

Project funding was from Diagnostic Chemicals Limited (DCL), Charlottetown, Prince Edward Island; DCL was supported in part by the Industrial Research Assistance Program of the National Research Council of Canada. The authors thank the Max Bell Foundation for program funding to the AVC Lobster Science Center, University of Prince Edward Island.

References

Barlow, J., and G. J. Ridgway. 1969. Changes in serum protein during the molt and reproductive cycles of the American lobster (*Homarus americanus*). Journal of the Fisheries Research Board of Canada 26: 2101–2109.

Castell, J. D., and S. D. Budson. 1974. Lobster nutrition: the effect on *Homarus americanus* of dietary protein levels. Journal of the Fisheries Research Board of Canada 31:1363–1370.

Cawthorn, R. J. 1997. Overview of ''bumper car'' disease-impact on the North American lobster fishery. International Journal for Parasitology 27:167–172.

Dumas, C., and J. Camonis. 1993. Cloning and sequence analysis of the DNA for arginine kinase of lobster muscle. Journal of Biological Chemistry 268: 21599–21605.

Gerhart, W., J. Waldenstrom, and W. Gruber. 1979. EDTA effect on creatine kinase (CK) and on the SCE reagent. Scandinavian Journal of Clinical and Laboratory Investigations 39:737–742.

German Society for Clinical Chemistry. 1977. Standardization of methods for the estimation of enzyme activities in biological fluids. Standard method for the determination of creatine kinase activity. Journal of Clinical Chemistry and Clinical Biochemistry 15:255–260.

Glynn, J. P. 1968. Studies on the ionic, protein, and phosphate changes associated with the moult cycle of *Homarus vulgaris*. Comparative Biochemistry and Physiology 26:937–946.

Kramer, J. W. 1989. Clinical enzymology. Pages 338–360 *in* J. J. Kaneko, editor. Clinical biochemistry of domestic animals, 4th edition. Academic Press, San Diego, California.

Martin, G. G., and J. E. Hose. 1995. Circulation, the blood, and disease. Pages 465–495 *in* J. R. Factor, editor. Biology of the lobster *Homarus americanus*. Academic Press, San Diego, California.

Mercaldo-Allen, R. 1991. Changes in the blood chemistry of the American lobster, *Homarus americanus*, H. Milne Edwards, 1837, over the molt cycle. Journal of Shellfish Research 10:147–156.

Minitab. 1992. Minitab statistical software, standard version, release 9.1 for VAX/VMS. Minitab Computer Software, State College, Pennsylvania.

Morrison, J. F. 1973. Arginine kinase and other invertebrate guanidino kinases. Pages 457–486 *in* P. D. Boyer, editor. The enzymes, volume 8. Academic Press, New York.

Newsholme, E. A., I. Beis, A. R. Leech, and V. A. Zammit. 1978. The role of creatine kinase and arginine kinase in muscle. Biochemical Journal 172:533–537.

Nockels, C. F., K. G. Odde, and A. M. Craig. 1996. Vitamin E supplementation and stress affect tissue α-tocopherol content of beef heifers. Journal of Animal Science 74:672–677.

Oliver, I. T. 1955. A spectrophotometric method for the determination of creatine phosphokinase and myokinase. Biochemical Journal 61:116–122.

Stewart, J. E., J. W. Cornick, D. M. Foley, M. F. Li, and C. M. Bishop. 1967. Muscle weight relationship to serum proteins, hemocytes, and hepatopancreas in the lobster, *Homarus americanus*. Journal of the Fisheries Research Board of Canada 24:2339–2354.

Stewart, J. E., G. W. Horner, and B. Arie. 1972. Effects of temperature, food, and starvation on several physiological parameters of the lobster *Homarus americanus*. Journal of the Fisheries Research Board of Canada 29:439–442.

Tombes, R. M., and B. M. Shapiro. 1989. Energy transport and cell polarity: relationship of phosphagen kinase activity to sperm function. Journal of Experimental Zoology 251:82–90.

Wolf, P. L. 1991. Abnormalities in serum enzymes in skeletal muscle diseases. American Journal of Clinical Pathology 95:293–296.