Application of Creatine Kinase Isoenzymes for Detecting Pathophysiological Changes in Yellowtail Infected with *Lactococcus garvieae*

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ABSTRACT — Creatine kinase (CK) isoenzymes were examined as diagnostic indicators for pathophysiological changes in yellowtail infected with *Lactococcus garvieae*. Three isoenzymes of CK similar to those found in human were detected by agarose electrophoresis. CK isoenzymes in the brain, heart and intestine consisted of CK-MM, CK-MB and CK-BB. CK-BB was not detected in other tissues or plasma. Normal yellowtail plasma contained CK-MM and CK-MB, while CK-BB and CK-SP appeared in the plasma of infected fish. This phenomenon was found in both naturally and artificially infected fish. These results suggest that appearance of CK-BB and CK-SP in the plasma may reflect the pathophysiological changes due to the infection. Therefore, we consider that the examination of plasma CK isoenzymes is a pathophysiological diagnostic tool for the streptococcal infection in yellowtail.

Key words: creatine kinase, *Lactococcus garvieae*, isoenzyme, yellowtail, diagnosis, *Seriola quinqueradiata*, pathophysiology

Lactococcus garvieae infection has caused considerable economic losses in cultured yellowtail in Japan. Though bacteriological examination is essential for diagnosis of the disease, observation of pathophysiological changes in infected fish helps indirect diagnosis. We consider that pathophysiological diagnosis for bacterial infection in fish offer useful information concerning the severity of the disease, and culturists may be able to adopt suitable measures for preventing the disease based on such information. Blood examination is one of the means to know the changes. While it has been found that plasma alkaline phosphatase activity and glucose level increased in fish infected with *Lactococcus garvieae* or *Streptococcus iniae* (Ikeda and Minami, 1982; Kusuda and Ninomiya, 1992), it is still unknown which tissue injury relates to those changes. As typical symptoms of this disease are encephalitis and pericarditis, we consider that some injuries of brain and heart due

to the disease may give diagnostic indicators for *L. garvieae* infection.

Changes in creatine kinase (CK; E.C. 2.7.3.2) reflect pathological alterations in specific human organs. CK is a dimer of two subunits of, M and B, generating three different isoenzymes in mammalian cells: MM (CK-MM, muscle type), BB (CK-BB, brain type) and MB (CK-MB, myocardial type). In human, distribution of CK isoenzymes is tissue specific, e.g. skeletal muscle contains CK-MM, while brain and heart contains CK-BB and CK-MB, respectively. Thus, CK isoenzymes are frequently used as serum diagnostic markers for acute myocardial infarction, skeletal muscle disease and neurological injury (Sirag *et al.*, 1986; Hamburg *et al.*, 1991). If CK isoenzymes are also tissue specific in yellowtail like in human, examination of plasma CK isoenzymes in yellowtail may detect brain and heart injuries due to the disease.

Enzymological properties of CK in red sea bream, pacific mackerel and carp were studied by Nakagawa and Nagayama (1991). Bucher (1990) investigated the distribution of CK activity in organs of rainbow trout.

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Variation of CK isoenzymes has been studied in skeletal muscle, heart, brain and stomach of salmonid fish (Perriard *et al.*, 1972; Ferris and Whitt, 1978; White *et al.*, 1992). Williams *et al.* (1996) reported plasma CK isoenzyme patterns of healthy rainbow trout. It is known that the severe myopathy in "Sudden death syndrome" of Atlantic salmon was accompanied by a significant elevation of CK activity levels (Rodger *et al.*, 1991). The present paper describes distribution of CK isoenzymes in organs of yellowtail and verify whether plasma CK isoenzymes can be used for the diagnosis of the *L. garvieae* infection in yellowtail.

Material and Methods

Sample preparation, measurement of CK activity and analysis of CK isoenzymes for tissue distribution

Six healthy yellowtail *Seriola quinqueradiata* weighing 80–120 g were obtained from Oita Institute of Marine and Fisheries Science, Oita Prefecture, Japan. Blood samples were taken by cardiac puncture with heparinized syringes. The plasma was separated by centrifuging at $1,000 \times g$, 10 min at 4°C and stored at –80°C until used. Pieces of white muscle, brain, heart, intestine, kidney, spleen and liver taken from each fish were washed three times in Ringer's solution and stored at –80°C. The tissue materials were homogenized with 0.25 M sucrose solution on ice. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C and the supernatants were used for the analysis of CK activity and the isoenzymes.

CK activity was assayed using commercial kit (Oliver's method, Wako). Protein concentration of the analytical samples, except the plasma, was measured according to Lowry's method (1951). CK activities of each tissue were expressed as mU/mg protein.

Agarose gel electrophoresis was carried out by the clinical laboratory procedure using precast "special agarose electrophoresis film" with commercial electrophoresis system (CIBA Corning Co.). Samples were applied to the gel when the CK activity reached 1,000 mU/mL. Standard human serum was used for checking the separation of CK isoenzymes.

*Examination of CK isoenzymes in yellowtail infected with *L. garvieae**

Plasma samples were collected from fish naturally infected with *L. garvieae*, occurring in a farm in Kagoshima Prefecture. Bacteria isolated from the brain and/or pericardium of twenty one samples were examined for *L. garvieae*.

Artificial infection was done to confirm the results obtained from naturally infected fish. Ninety non infected fish weighing 110 g on the average were placed in 2 m³ tanks for challenge test and adjusted for 1 week. *Lactococcus garvieae* strain OT62262 isolated from diseased yellowtail in Oita Prefecture in 1996 was

employed in the experiment. The bacterium was cultured in Tood Hewitt Broth (Nissui) at 25°C for 24 hr and used. Fish were challenged by immersion in seawater containing *L. garvieae* OT62262 cells at a concentration of 4.1×10^8 CFU/mL for 15 min. Following the challenge, fish were held at water temperature of 23.0–25.0°C. Five fish each were caught by spoon net at 0, 1, 2, 3, 4, 5, 8 and 16 days post infection. Blood samples were obtained by the cardiac puncture using heparinized syringes fitted with 23 G needles. Samples of the brain, heart, kidney and liver were dissected aseptically, washed with sterilized Ringer's solution several times, and homogenized. Viable numbers of *L. garvieae* were estimated from spread plates of serial dilutions on brain heart infusion agar (BHIA; Difco). The blood samples were centrifuged ($1,000 \times g$, 10 min) to separate the plasma, which was kept at –80°C and later used for analysis. Plasma alkaline phosphatase activity was determined as reference using a CL-7100 automated biochemical analyzer (Shimadzu Co. Ltd.).

Results

Tissue distribution of CK activity and CK isoenzymes in yellowtail

Creatine kinase activity was detected in plasma and

Fig. 1. Creatine kinase isoenzymes in normal yellowtail. Lane numbers are shown as follows; 1: Human standard, 2: Brain, 3: White muscle, 4: Liver, 5: Heart, 6: Spleen, 7: Kidney, 8: Plasma. CK-MM, CK-MB and CK-BB were referenced by the electrophoretic mobility of human standard.

Fig. 2. Distribution of CK isoenzymes in tissues of normal yellowtail.

Ratios (%) between CK isoenzymes in the tissues of were almost same in 6 fish examined. Therefore, a representative result is shown in this figure.

all tissues examined. The activity was $4,761 \pm 2,253$ in white muscle, 740 ± 166 in brain, 297 ± 280 in heart, 443 ± 139 in intestine, 148 ± 45 in kidney, 191 ± 58 in spleen, 87 ± 116 mU/mg protein in liver and 41 ± 26 mU/mL in plasma. CK activity of white muscle was approximately 6-fold higher than that of the brain and more than 10-fold higher than those of other tissues. These results were similar to those obtained from rainbow trout (Bucher, 1990).

Three bands of CK isoenzymes appeared on agarose electrophoresis films, showing a similar pattern to human control (Fig. 1). CK-MM, CK-MB and CK-BB were tentatively identified by their electrophoretic mobility compared with the human standard serum. CK-MM could only be found in the white muscle, while CK-MM and CK-MB were observed in the kidney, spleen and liver. CK isoenzymes of the brain, heart and intestine consisted of three isoenzymes (CK-MM, CK-MB and CK-BB). CK-MM and CK-MB were detected in the healthy yellowtail plasma, while CK-BB could not be detected (Fig. 2).

Plasma CK isoenzymes in fish naturally infected with L. garvieae

Electrophoretic pattern of plasma CK isoenzymes in fish naturally infected with *L. garvieae* is shown in Fig. 3. While uninfected fish showed CK-MM and/or both CK-MM and CK-MB, diseased fish exhibited CK-BB and a novel band (CK-SP) in addition to CK-MM and CK-MB. CK-BB was detected in 12 among 21 diseased fish (57%). On the other hand, CK-SP were observed in 17 of infected fish (81%). Four diseased fish (19%) neither had CK-BB nor CK-SP like the healthy fish.

Fig. 3. Plasma CK isoenzymes in yellowtail naturally infected with *L. garvieae*.

Lane numbers are shown as follows; 1: Human standard, 2 and 3: Healthy fish, 4 and 5: Infected fish. A novel band (CK-SP) is found at the position between CK-MM and CK-MB.

Plasma CK isoenzymes in fish artificially infected with L. garvieae

Individual data of experimental fish are shown in Table 1. In artificial infection, *L. garvieae* was isolated from some organs (at least from one organ) in all examined fish from 1 to 8 days post infection. CK-BB was detected only in 1 among 5 fish and CK-SP was not

Table 1. Individual data of CK isoenzymes, plasma alkaline phosphatase activity and log numbers of *Lactococcus garvieae* in artificial infection

Specimen	No.	CK isoenzymes*		Alkaline phosphatase (IU/L)	Log number of <i>L. garvieae</i>			
		CK-BB	CK-SP		Brain	Heart	Kidney	Liver
Pre-infection	1	ND	ND	153	ND	ND	ND	ND
	2	ND	ND	198	ND	ND	ND	ND
	3	ND	ND	174	ND	ND	ND	ND
	4	ND	ND	200	ND	ND	ND	ND
	5	ND	ND	210	ND	ND	ND	ND
1 day post infection	1	ND	ND	138	ND	3.40	ND	ND
	2	D	ND	115	ND	ND	1.95	ND
	3	ND	ND	177	ND	ND	4.84	1.48
	4	ND	ND	135	2.46	3.95	3.68	3.23
	5	ND	ND	166	ND	ND	3.18	3.04
2 day post infection	1	ND	D	289	5.26	5.00	5.79	4.90
	2	ND	D	540	4.95	3.30	6.00	6.34
	3	ND	D	634	6.32	5.81	5.70	5.69
	4	D	ND	328	5.90	4.87	5.70	6.15
	5	ND	ND	688	7.93	4.91	5.88	6.34
3 day post infection	1	ND	D	1,032	6.45	3.30	4.15	3.34
	2	ND	D	1,338	6.94	5.80	5.78	4.67
	3	ND	D	802	8.11	6.51	5.52	5.30
	4	ND	D	76	5.69	4.79	1.60	4.79
	5	ND	D	2,480	5.70	7.40	4.60	3.59
4 day post infection	1	D	D	783	6.77	5.90	5.64	4.11
	2	ND	D	550	2.20	6.40	3.11	ND
	3	D	ND	73	ND	3.26	ND	ND
	4	D	D	760	2.78	5.90	3.77	3.92
	5	D	D	685	6.89	5.15	5.94	5.38
5 day post infection	1	ND	D	1,716	8.28	6.88	6.30	5.00
	2	D	D	1,632	6.69	7.60	6.00	5.08
	3	ND	D	507	4.52	7.49	4.81	5.69
	4	ND	D	984	7.72	5.72	5.41	4.11
	5	D	D	1,255	7.80	8.00	5.68	5.66
8 day post infection	1	ND	ND	78	6.86	6.49	6.18	4.70
	2	D	D	1,325	6.83	7.08	5.70	4.85
	3	D	ND	188	6.90	6.91	5.20	5.46
	4	D	D	937	7.94	6.08	4.57	5.28
	5	ND	D	623	7.48	7.20	3.90	3.95
16 day post infection	1	D	ND	183	ND	ND	ND	ND
	2	D	ND	172	ND	ND	ND	ND
	3	D	ND	142	ND	ND	ND	ND
	4	D	ND	126	ND	ND	ND	ND
	5	D	ND	118	ND	ND	ND	ND

* Results of creatine kinase (CK) isoenzymes present as follows; ND: not detected, D: detected.

detected in any fish at 1 day of post infection. Incidence of fish with CK-SP (80%) was higher than those of fish with CK-BB (40%) during the period from 2 to 8 days post infection. CK-SP was detected in almost all fish in which alkaline phosphatase activity was higher than the initial value. Such positive correlation was not observed between CK-BB and alkaline phosphatase. Incidence of fish in which neither CK-BB nor CK-SP could be detected was 8% of all the fish infected with the bacteria. Although the bacteria were not reisolated, CK-BB was observed in fish examined at 16 days post infection.

Discussion

Examination of plasma CK isoenzymes is useful to know the pathophysiological changes in yellowtail infected with *Lactococcus garvieae*. Our results indicate that CK isoenzymes of yellowtail are tissue specific and the plasma normally contains two CK isoenzymes, CK-MM and CK-MB. CK-BB and CK-SP, which are unusual in the plasma of healthy fish, were detected in that of fish infected with *L. garvieae* and the phenomenon was confirmed in both naturally and artificially infected fish.

It is important to know which tissue produces CK-BB and/or CK-SP appeared in the plasma. Though not confirmed, CK-BB in the plasma of infected fish may reflect leakage from some of the affected brain, heart and intestine because CK-BB was found only in those tissues. On the other hand, CK-SP was not contained in any tissue of the healthy fish. It is not evident that CK-SP relates to the injury of tissues. However, we consider that the presence of CK-SP in the plasma of infected fish reflect some pathophysiological change due to the disease. Further investigations of CK-SP is needed to understand better the pathophysiological condition of the diseased fish.

Examination of CK isoenzymes has an advantage over measurement of plasma alkaline phosphatase activity and glucose level in pathophysiological diagnosis for *L. garvieae* infection. The occurrence of CK-SP was found to be related to high plasma alkaline phosphatase activity. It is well known that the alkaline phosphatase levels increase in fish infected with *L. garvieae*. The normal reference values of plasma components in yellowtail have not been established and variations of plasma components levels in individuals are wide. However, normal standard is not necessary for reference of CK isoenzymes because estimation of CK isoenzymes is carried out by detecting substances which healthy fish do not have. Hence it is considered that detection of CK enzymes is a more useful diagnostic tool to study pathophysiological changes in fish infected with *L. garvieae*. Plasma CK activity of rainbow trout is influenced by the effects of starvation, spawning and seasons (Bucher, 1990), but there is no report about physiological changes in the CK isoenzyme patterns in yellowtail. Thus, the influence of these factors on plasma CK isoenzymes has to be investigated in order to use them for diagnosis of *L. garvieae* infection in yellowtail.

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