Effect of *Ulva* Meal Supplementation on Disease Resistance of Red Sea Bream

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The effect of *Ulva pertusa* meal supplementation on disease resistance was monitored by means of some pathological indices in red sea bream *Pagrus major* fed on a 5% *Ulva* meal supplemented purified diet.

The *Ulva* meal enhanced phagocytosis of the granulocytes against *Pasteurella piscicida*. In the fish immunized with formalin-killed *P. piscicida*, the blood lymphocyte number increased by feeding the *Ulva* meal, but the granulocyte number and agglutinin titer were not influenced.

Complement activities such as spontaneous hemolytic activity (SH₅₀) and bactericidal activity against *Escherichia coli* and *P. piscicida* tended to increase by the supplement.

Although the effect on prevention from artificial infection by *P. piscicida* was erratic, the *Ulva* meal supplementation was suggested to enhance disease resistance of the fish without impairment of growth. The importance of feeding algae was discussed on the basis of disease resistance.

Nutritional value of fish diet has mainly been evaluated by weight gain and survival rate. Under the present condition of intensive fish culture, economic loss caused by diseases and stressors is inevitable to some extent. As nutritional deficiency can impair defense mechanism to various diseases, nutrition/disease interaction cannot be ignored. Elimination of potential pathogen and enhancement of defense function are required for improvement of productivity. Therefore, the study of nutrient requirement should include the enhancement of resistance to infectious disease in intensive fish culture.

In this respect, availability of algae supplement to fish diet was established on enhancement of physiological functions.¹⁻⁷⁾ Whereas the mechanism of the effect is not known, there were some evidence that dietary algae closely connected with fish health. In addition to the increase of stress resistance, *Chlorella* extract supplementation increased disease resistance of ayu *Plecoglossus altivelis*.⁸⁾

In this study, we investigated the effect of *Ulva* meal supplementation on susceptibility of infectious disease in order to confirm the importance of algae fortification in feed formulation.

Materials and Methods

Fish Culture System

Two year old red sea bream *Pagrus major* of 41 g body weight were reared in 1984 and of 37 g in 1985 at the Fisheries Laboratory of Hiroshima University. They were divided into two dietary groups in indoor tanks. The tanks were continuously supplied with fresh sea water of which temperature ranged from 17 to 30°C (average 26°C) in 1984 and from 14 to 26°C (average 19°C) in 1985. Salinity was between 20-33‰ (average 29‰) in 1984, and 16-23‰ (average 27‰) in 1985. The fish reared for 136 days in 1984 and for 91 days in 1985 were subjected to a series of the experiment.

Diets

Fish reared on a commercial diet was used for the experiment. Control group was fed on the purified diet prepared by the method of Furuichi and Yone,⁹⁾ as given in Table 1. Experimental diet was prepared by supplementation of dried *Ulva pertusa* meal to the above diet. The fish were fed approximately 2% body weight per day.

Challenge Test

Resistance against infectious disease was com-

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Table 1. Composition of diet fed on red sea bream

Ingredients	Dietary group	
	Control	Experimental
Vitamin free casein	54	54
Gelatin	12	12
Dextrin	10	10
Pollack liver oil	9	9
Cellulose	3	3
Elicitor (L-Asp-Na)	1	1
Amino acids* ¹	3	3
Vitamins* ²	3	3
Minerals* ³	5	5
<i>Ulva</i> -meal	—	5
Total	100	105

*¹ L-Val 0.7, L-Arg·HCl 1.5, L-Tyr 0.2, L-Phe 0.6.*² Halver's vitamin mixture.*³ Salt Mixture No. 2 (ICN Nutritional Biochemical).

pared between two dietary groups by intraperitoneal (IP) injection and immersion inoculations. *Pasteurella piscicida* (strain HT-8416) obtained from a naturally infected yellowtail *Seriola quinqueradiata* in Tokushima prefecture was used for the challenge tests. The fish were intraperitoneally inoculated with 5×10^6 and 5×10^7 CFU per 100 g of fish. The immersion challenge was carried out to immerse in bacterial suspensions of 4×10^5 and 4×10^6 CFU/ml for 15 min. The presence of *P. piscicida* was checked in the dead fish.

Phagocytosis

The blood withdrawn from the Cuvierian duct with a plastic syringe. The blood (0.5 ml) and 0.05 ml of *P. piscicida* cell suspension (10 ml/ml) were mixed in a heparinized tube and kept in a shaking incubator at 20°C. After 30 min incubation, the blood was smeared on a slide glass and stained with May-Giemsa.¹⁰⁾ The fish starved for an additional 21 days in water temperature at 20.0–25.6°C was also submitted to the measurements. Phagocytosis was calculated as follows.

Phagocytic rate (%)

$$= \frac{\text{Number of phagocyte positive granulocytes}}{\text{Number of granulocyte}} \times 100$$

Phagocytic index

$$= \frac{\text{Total bacteria cell number in granulocyte}}{\text{Number of phagocyte positive granulocyte}}$$

Hematological Measurements

Hematocrit and hemoglobin were measured by microhematocrit method and cyanmethohemo-

globin method, respectively. Erythrocyte number was measured by Compur M1000 (Compur Electronic GMBH) based on turbidity method. The leucocyte number was represented as ratio to 10,000 erythrocytes on a slide glass.

Immune Response

As antigen, formalin-killed *P. piscicida* was injected into IP (1 mg/100 g body weight) with Freund's complete adjuvant (DIFCO). The antigen was reinjected at 14 days after first injection. The serum agglutinin titer was determined by microtiter method.

Complement Activity

Spontaneous hemolytic activity (SH_{50}) and bactericidal activity were measured as indicators of serum complement activity. SH_{50} was measured by the method described by Sakai.¹¹⁾ The rabbit erythrocytes (Nippon Bio-Test Lab.) of 10^9 cells/7.5 ml were reacted with serial dilution of serum at 30°C for 60 min. The ratio of collapsed erythrocytes were spectrophotometrically measured at 541 nm. One SH_{50} unit represents hemolysis of 50% of erythrocytes. Serum bactericidal activity to *P. piscicida* and *Escherichia coli* (strain WP2) was determined by the method of Iida and Wakabayashi.¹²⁾ The serum immunized by *P. piscicida* was also used for the measurement. A series of diluted serum was reacted with 10^8 /ml of bacterial cell suspension at 25°C for 3 h. The serially diluted mixture was cultured on Brain Heart Infusion Agar (Eiken) plates. The number of colonies was counted and viable cells were calculated. A definition of classical and alternative pathways in the complement was based on a difference in inorganic divalent cation requirements for activation of the complement by the procedure of Sakai.¹¹⁾

Results

Body weight gain and feed conversion efficiency were not influenced by the *Ulva* meal supplementation, as shown in Table 2. The mortality was caused by accidents of culture system.

Hematological measurements of the fish at 1984 are shown in Table 3. They were not different between the two groups. Plasmological properties were significantly different in nonesterified fatty acids and amino nitrogen levels.⁹⁾

Resistance against *P. piscicida* infection was compared by challenge tests (Table 4). Mortality curves related to the doses of inoculated bacterial

Table 2. Effects of *Ulva* meal feeding on growth

Year	Group	Mean body weight (g)		Growth rate	Feed efficiency (%)	Mortality (%)
		Initial	Final			
1984	Control	41.0	101	2.47	60.6	8.5
	Experimental	41.2	105	2.54	62.9	17.5
1985	Control	36.8	64.0	1.74	52.5	24.3
	Experimental	36.8	65.7	1.78	49.5	17.4

Table 3. Result of artificial infection with *P. piscicida*

Challenge method (year)	Dose (CFU/100 g or 1 ml)	Mortality (died/tested)	
		Control	Experimental
Injection* ¹ (1984)	5×10^7	75% (9/12)	50% (6/12)
	5×10^8	8% (1/12)	8% (1/12)
	0	0% (0/4)	0% (0/4)
Immersion* ² (1985)	4×10^8	92% (12/13)	92% (12/13)
	4×10^9	64% (9/14)	79% (11/14)
	0	0% (0/5)	0% (0/5)

*¹ Organism was intraperitoneally injected per 100 g of fish body weight. Mortality shows at 3 weeks after challenge. Water temperature 20.5°C (17.6–22.9°C).

*² Fish were immersed in viable cell suspension (CFU/ml) for 15 min. Mortality shows at 2 weeks after the challenge. Water temperature 27.5°C (26.1–28.5°C).

cell. *P. piscicida* could be isolated from the dead fish. In the IP injection challenge, the *Ulva* meal seemingly increased the resistance. However, the result of immersion challenge was contrary to that of IP injection challenge. The differences found in both challenge tests were not significant between the two groups.

Fig. 1 shows phagocytosis of granulocytes of the fish after 30 min incubation. The differences were observed in the reactant at 15 min incubation, as well. The phagocytosis was clearly lower in the starved fish. The experimental group was significantly higher than the control group in phagocytic rate ($p < 0.01$) and phagocytic index ($p < 0.05$). The *Ulva* meal supplementation

Table 4. Blood properties of *Ulva* meal fed red sea bream

Group	Hematocrit (%)	Hemoglobin (g/100 ml)	Erythrocyte ($10^8/\text{mm}^3$)	Leucocyte*	
				Lymphocyte	Granulocyte
Control	32.4 ± 4.0 (n=16)	6.36 ± 0.83 (n=13)	4.09 ± 0.45 (n=14)	119 ± 58 (n=8)	23 ± 15 (n=8)
Experimental	34.1 ± 3.8 (n=16)	6.34 ± 0.89 (n=14)	4.04 ± 0.42 (n=14)	135 ± 41 (n=8)	16 ± 13 (n=8)

* Leucocyte number per 10^4 erythrocyte.

Fig. 1. Effect of *Ulva* meal supplementation on phagocytosis of red sea bream. Results show mean $\pm$ SE.

Fig. 2. Effect of *Ulva* meal supplementation on leucocyte number and agglutinin titer in immunized red sea bream. Results show mean $\pm$ SE.

* $p < 0.001$ to control group.

enhanced the phagocytosis.

The agglutinating antibody titer and the leucocyte number were determined in the fish immunized with formalin-killed *P. piscicida* antigen (Fig. 2). The leucocyte number solely trended to increase by feeding the *Ulva* meal. But the granulocyte number and the agglutinin titer were not influenced by the *Ulva* meal supplementation. Booster dose effect was not observed in both groups.

Fig. 3 shows the serum complement activity. The activity of SH_{50} of the fish was sensitive in rabbit red blood cell rather than that of sheep. SH_{50} activity of the serum was stable even by cryopreservation in liquid nitrogen at -198°C for 11 days. The *Ulva* meal seemed to increase SH_{50} . Serum bactericidal activity against *E. coli* and *P. piscicida* was higher in the experimental

group, and the activity significantly discriminated in the serum at 14 days after immunization with *P. piscicida*.

To confirm activation system of pathway of complement was checked by inorganic divalent cation requirement. SH_{50} and bactericidal activity against *E. coli* and *P. piscicida* lost the activity by incubation at 48°C for 30 min. EDTA inhibited the bactericidal activity. The bactericidal activity of the immunized serum was labile in incubation at 48°C as well. While the serum exhibited the bactericidal activity under the co-existence of Mg^{++} and Ca^{++} , the absence of Ca^{++} entirely inhibited the activity. The bactericidal activity of the normal serum was owing mainly to the alternative pathway of the complement and the immunized serum was probably by classical pathway.

Discussion

With the development of intensive fish culture, infectious disease arises one of the important problems to solve. Susceptibility to bacterial infection is considerably responsive to nutritional state. In this respect, nutrient requirement has been considered in improvement of disease resistance. The necessity of ascorbic acid,¹³⁻¹⁵ pyridoxine,¹⁶ α -tocopherol,¹⁷⁻¹⁸ niacin,²⁰ and minerals^{14,18} as nutrients was confirmed on the basis of preventive effect for artificial infection and other indices for explanation of defense mechanism.

Dietary algae are important nutrient for maintaining the high physiological function. Efficacy of *Ulva* meal as feed supplement was assessed on improvement of physiological condition in the red sea bream by Nakagawa *et al.*⁶ Similar effects were observed in *Ulva* meal fed black sea bream *Acanthopagrus schlegelii*,^{4,7} *Chlorella*-extract fed ayu *Plecoglossus altivelis*,^{1,3,8} and kelp meal *Laminaria digitata* fed yellowtail *Seriola quinqueradiata*.⁵ In addition to these physiological merits, disease resistance to vibriosis and "chochin disease" of ayu,⁸ and nutritional disease of yellowtail were effectively prevented by algae supplementation.¹¹

Although the effect of *Ulva* meal on prevention from the artificial infection was erratic, the phagocytosis which may be an important factor for infectious disease resistance was found to increase. The increase of phagocytosis accompanied with lymphocytes and complement activity would mean to activate somewhat of defense mechanism by

Fig. 3. Effect of *Ulva* meal supplementation on complement activities in red sea bream. Results show mean $\pm$ SE. * $p < 0.01$ to control group.

feeding the *Ulva* meal. SH₅₀ activity was found to decrease under the influence of starvation, according to Sakai.^{11,21} The starvation also decreased the phagocytosis in this study. However, it was doubtful whether these low values of starved fish severely reflected decrease of resistance to infectious disease.

The lymphocyte are known to form the basis of cellular and humoral immunity.²³ Blazer and Wolke¹⁹ reported that cellular and humoral immunity of rainbow trout were enhanced by α -tocopherol. In channel catfish *Ictalurus punctatus*, Li and Lovell¹⁵ reported that humoral immunity was enhanced rather than cellular immunity by dose of a large quantity of ascorbic acid. The *Ulva* meal affected cellular immunity, judging from increase of phagocytosis and lymphocyte number. While no evidence was found that the *Ulva* meal enhanced antibody response, the complement was somewhat activated. The complement in the fish serum has two pathways in activation, as alternative and classical pathways.²⁴ In this study, SH₅₀ and bactericidal activity were found to be mainly attributed to alternative pathway. The bactericidal activity of

the immunized serum was due to classical pathway. The *Ulva* meal seemed to enhance both alternative and classical pathways.

The relationship between dietary algae and physiological condition was described in our previous papers in several fishes.^{1-3,22} The results obtained in this study would indicate the possibility that disease resistance intimately connected with dietary strategy. The improvement of metabolism might concern to stimulate the defense mechanism, because algae supplementation was largely associated with activation of lipid mobilization to energy.^{3,5-7} In addition, energy metabolism related to stress response and glucocorticoid level in the blood, as indicated by Li and Lovell.¹⁵

Algae were sometimes found in the digestive organ of wild red sea bream. The elucidation of defense mechanism needs to be considered ecology/nourishment interaction in fish. The possible explanation for thus effect of dietary algae might indicate the necessity of algae ingestion to the fish. Dietary regimen should be included for maintenance of high physiological activity as well as good growth.

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