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An aqueous extract from *Sargassum* sp. enhances the immune response and resistance against *Streptococcus iniae* in the Asian sea bass (*Lates calcarifer* Bloch)

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Abstract The effects of the hot water extract from *Sargassum* sp. on the growth performance, immune responses, oxidative stress, and resistance of Asian sea bass (*Lates calcarifer*) to *Streptococcus iniae* were investigated. Four groups of fish were fed on the basal diet containing the extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} diet for 30 days. Thereafter, the fish from each group were divided into equal halves. The first half was injected intraperitoneally with 0.85 % NaCl, and the second half was injected with *S. iniae* (2×10^3 cfu $fish^{-1}$). At the end of the 30 days feeding trial, there were no significant differences in final body weight, weight gain, feed conversion ratio, and hepatosomatic index among four groups. Plasma protein, total immunoglobulin (Ig), and lysozyme messenger RNA (mRNA) levels in fish fed on diets containing 1.0 and 2.0 g kg^{-1} of *Sargassum* sp. extract, however, became increased in a non-dose-dependent manner. When fish were exposed to the bacteria, at 24 h, there were significantly ($p < 0.05$) higher levels of hematocrit, red blood cell and white blood cell, Ig, and serum lysozyme in fish fed on diet containing 2.0 g kg^{-1} of *Sargassum* sp. extract than those of the fish fed the control diet, and the highest survival rate was also observed in this group. In addition, fish receiving the seaweed extract were able to suppress lipid peroxidation especially at 24-h post *S. iniae* challenge. These findings thus suggested that *Sargassum* sp. extract can be used as an immunostimulant in Asian sea bass.

Keywords *Sargassum* sp. · Brown algae · Sea bass · *Lates calcarifer* · Antibacterial activity · Immune responses

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Introduction

The Asian sea bass is an economically important fish species in Thailand and other Asian countries. However, diseases caused by *Streptococcus iniae* have been reported to occur frequently in the culture of many fish species globally including in Asian sea bass farmed in Thailand (Agnew and Barnes 2007; Suanyuk et al. 2010). The use of antibiotics and chemotherapeutics as prophylactic measures has been widely criticized for its negative impacts like immunosuppression and the accumulation of residues in the tissues of aquatic animals and the environment (Samuelsen et al. 1992). Therefore, the use of immunostimulants which are natural compounds has been widely advocated to control aquatic diseases. Seaweeds are a source of immunostimulant compounds which offer potential benefits on the immune response of fish and its resistance against pathogens (Cheng et al. 2007, 2008).

Sargassum is a largely unexploited brown seaweed genus in the Phaeophyceae. In Thailand, *Sargassum* spp. are found on both the Gulf of Thailand and the Andaman Sea coasts (Noiraksar and Ajisaka 2008). The dominant polysaccharides derived from *Sargassum* sp. like fucoidan and alginate are

considered to be immunostimulants (Immanuel et al. 2012a, b). Differences in the extraction method used with *Sargassum* spp. produce a variety of biologically active components with different structures and different functional properties (Kantachumpoo and Chirapart 2010). Extraction by hot water is a popular method of extraction because it has been confirmed to be safe and to have no side effects on the environment in animal trials. While many studies have focused on hot water extracts from *Sargassum* spp. administered as immunostimulants to improve immune activity and disease resistance in shrimp, these effects in fish have rarely been investigated. The only study traced was that of Fujiki et al. (1992) reporting that hot water extracts from several species of brown algae including *Sargassum autumnale* injected intraperitoneally in the common carp (*Cyprinus carpio*) increased resistance against *Edwardsiella tarda* and increased the resistance of yellowtail (*Seriola quinqueradiata*) against streptococcal infection. This study was based only on the effect on mortality rate, with no information about the effect on the immune system of the fish. From the reasons mentioned above, therefore it is interesting to investigate the effects of the hot water extract from *Sargassum* sp. collected from the coast of Songkhla Province in Thailand, administered as a dietary supplement to the Asian sea bass on modulation of immunity, oxidative stress, and resistance to bacterial pathogen *S. iniae*.

Materials and methods

Preparation of the seaweed extract

About 100 kg of *Sargassum* sp. (SG-0044) was collected from the coastal area of Songkhla Province, south of Thailand. Its morphology has been described by Noiraksar and Ajisaka (2008). Once obtained, the seaweed was washed thoroughly with fresh water and air-dried. The dried seaweed (10 kg) was then milled to particle size less than 2 mm and stored in air-tight plastic bottles at room temperature. To prepare the extract, the ground seaweed was autoclaved in deionized water (seaweed: water = 1:10 *w*/*v*) at 120 °C for 3 h. The resulting extract was then filtered through a nylon mesh (300 μm pore size), spray-dried, and kept in a desiccator. The percentage yield of the extract was 1.14 % based on the dried weight of seaweed.

The proximate composition of the *Sargassum* sp. extract was determined according to official methods of analysis (AOAC 1995). The monosaccharide component analysis was conducted based on a high performance liquid chromatography (HPLC) system equipped with a column Zorba x NH_2 (4.6 × 250 mm, 5 μm); the mobile phase was 75 % acetonitrile:25 % H_2O and a flow rate of 0.5 mL min^{-1}. The sugar component of the crude extract was analyzed using fucose, mannose, fructose, glucose, galactose, and sucrose as standards.

Experimental diets

Four experimental diets containing different levels of *Sargassum* sp. extract were used in this study. The basal diet contained 0.5 % carboxymethylcellulose (CMC) and *Sargassum* sp. extract was added to the test diets at levels of 0.5, 1.0, and 2.0 g kg^{-1} with a corresponding decrease in the amount of CMC. A further unsupplemented control diet was also prepared. Each diet was stored in polyethylene bags at −20 °C in the dark until used. The proximate composition of the ingredients and the experimental diets was determined according to official methods of analysis (AOAC 1995) as shown in Table 1.

Bacteria culture

The strain of bacteria pathogenic to Asian sea bass, *Streptococcus iniae*, was kindly provided by the Coastal Aquatic Animal Health Research Institute, Department of

Table 1 Composition of the experimental diets (g kg^{-1}) in this study

Ingredients	Experimental diets			
	Sargassum sp. extract in diet (g kg^{-1})			
	0	0.5	1.0	2.0
Fish meal	500	500	500	500
Squid liver	40	40	40	40
Soybean meal	155	155	155	155
Broken-milled rice	110	110	110	110
Wheat flour	100	100	100	100
Sargassum sp. extract	–	0.5	1	2
CMC	5	4.5	4	3
Fish oil	50	50	50	50
Soybean oil	30	30	30	30
Butylated hydroxytoluene (BHT)	1	1	1	1
Mineral mixture[a]	5	5	5	5
Vitamin mixture[b]	3	3	3	3
Vitamin C	1	1	1	1

The mineral and vitamin mixtures and the vitamin C were produced by KCS Pharmatic Co., Ltd., Thailand

[a] Per kg of mineral mixture: iron 12,000 mg; copper 12,000 mg; zinc 15,000 mg; manganese 6000 mg; iodine 200 mg; selenium 25 mg; magnesium 50,000 mg; calcium 100,000 mg; phosphorus 80,000 mg

[b] Per kg of vitamin mixture: vitamin A 600,000 IU; vitamin D3 200,000 IU; vitamin E 6000 IU; vitamin K 1200 mg; vitamin B1 5000 mg; vitamin B2 6000 IU; vitamin B6 5000 mg; vitamin B12 6 mg; niacin 20,000 mg; pantothenic acid 16,000 mg; folic acid 1000 mg; biotin 200 mg; Endox Dry 20,000 mg

Fisheries, Songkhla, Thailand. Bacteria stocks kept frozen in glycerol at −80 °C were restreaked on tryptic soy agar plate and incubated for 24 h at 35 °C after that the bacteria were grown in tryptic soy broth supplemented with 1.5 % NaCl for 24 h at 35 °C. The bacteria cells were collected by centrifugation and the supernatant was discarded then the pellets washed in sterile phosphate buffered saline (PBS, pH 7.4) three times and resuspended in normal saline (0.85 % NaCl). The optical density of the bacterial suspension was measured using a spectrophotometer then adjusted the *S. iniae* cell number to 6.5×10^9 cfu mL^{-1}.

Experimental design

Asian sea bass fingerlings purchased from a private farm in Songkhla, Thailand, were shipped to the laboratory and cultured in 10 t concrete pond for 2 weeks. During the acclimation period, fish were fed twice daily on the control diet (unsupplemented). After acclimation period of 2 weeks, fish of 80.59 ± 13.95 g mean weight were placed into a 1000 L round tank containing 800 L of filtered seawater for 12 tanks with 80 fish each. Triplicate tanks of fish were randomized fed on one of experimental diet to satiation at 8.00 a.m. and 5.00 p.m. for 30 days. At the end of feeding period, blood samples, liver, and head kidney were collected from 15 fish of each treatment to study the immune responses and oxidative stress. After the first sample collection, the fish from each treatment were divided into two groups and one group was injected intraperitoneally (*i.p.*) with 100 μL of 0.85 % NaCl, the other group being injected *i.p.* with *S. iniae* (2×10^3 cfu $fish^{-1}$). The samples of blood, liver, and kidney were again collected from 15 fish of each treatment at 0, 3, 24, 48, and 96 h post injection.

Blood samples were collected from the caudal veins of fish in each group and their livers were rapidly removed. The blood sample was divided into two portions; the first portion was mixed with heparin at a dose of 150 unit mL^{-1} for hematocrit determination, blood cell count, blood smear, and plasma collection. The plasma was separated from the heparinized blood by centrifuging the blood at 1500×*g* for 5 min at 4 °C, and the supernatant was transferred to 1.5 mL microtube then kept at −80 °C for total protein and total immunoglobulin assays. Another portion of blood was allowed to clot for 1 h at room temperature and for more than 5 h at 4 °C before centrifugal separation at 1500×*g* for 5 min at 4 °C. The serum was collected and kept at −80 °C for alternative complement activity and for lysozyme activity assays. The livers were excised, rinsed in cold normal saline, weighed, and stored at −80 °C for determination of the hepatosomatic index and lipid peroxidation assay. The head kidneys of three fish from each group were fixed in TRIzol reagent (Invitrogen) and kept at −80 °C for lysozyme gene expression analysis.

During the experimental period, the water temperature ranged from 27.0 to 28.9 °C, the salinity was between 19 and 25 ppt and maintained dissolved oxygen (DO) more than 4.5 mg L^{-1}. The ammonia-N concentration was monitored and adjusted to 0.21–0.48 mg L^{-1} by water exchange one third every 2 days.

Total immunoglobulin assay

The plasma total immunoglobulin (Ig) was determined following the method of Siwicki and Anderson (1993). The assay was based on the measurement of total protein contents in plasma using a Lowry's micro protein determination method prior to and after precipitating the immunoglobulin molecules by employing a 12 % solution of polyethylene glycol (Sigma). The difference in the protein contents was taken as the Ig content for Asian sea bass.

Alternative complement activity assay

This assay was conducted by a method modified from Yano (1992) and Ortuno et al. (1998) by using rabbit red blood cells (RaRBC, National Laboratory Animal Center, Mahidol University, Thailand). Briefly, RaRBC were washed and adjusted to 2×10^8 cells mL^{-1} in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01 M). A quantity of 100 μL of the RaRBC suspension was lysed with 3.4 mL of distilled water, and the absorbance of the hemolysate was measured at 414 nm against distilled water to obtain the 100 % lysis value. The test serum was appropriately diluted and different volumes ranging from 40 to 100 μL were made up to 100 μL total volume before being allowed to react with 40 μL of RaRBC in a test tube. After incubation at room temperature for 90 min with occasional shaking, 1.25 mL of saline solution was added to each test tube and the mixture was centrifuged at 1600×*g* for 5 min at 4 °C. The optical density of the supernatant was measured using a microplate reader at 405 nm. A lysis curve was obtained by plotting the percentage of hemolysis against the volume of serum added. The dilution corresponding to 50 % hemolysis mL^{-1} was expressed as ACH50 unit mL^{-1}.

Lysozyme activity assay

Serum lysozyme activity was measured according to the methods of Obach et al. (1993) and Demers and Bayne (1997) based on the lysis of the lysozyme sensitive Gram-positive bacterium *Micrococcus lysodeikticus*. Briefly, dilutions of standard hen egg white lysozyme (Sigma) ranging from 0 to 14 mg mL^{-1} in 0.1 M phosphate citrate buffer, pH 5.8, and undiluted serum samples (25 μL) were placed into wells of a 96-well plate in triplicate. A quantity of 175 μL of a 75 mg mL^{-1} suspension of the above bacteria (Sigma)

prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm at room temperature using a microplate reader. The lysozyme concentrations were calculated from the known standard curve of lysozyme from hen egg white and reported as μg lysozyme mL^{-1} serum.

Lipid peroxidation assay

Lipid peroxidation was measured by employing thiobarbiturate reactive substances (TBARs) assay as described by Jaczynski and Park (2003). Briefly, 0.1 g liver samples were homogenized in 1 mL of ice-cold 50 mM potassium phosphate buffer, pH 7.8, and centrifuged at 7,000×*g* for 20 min at 4 °C. Then, 0.25 mL of homogenate was mixed with 1.25 mL of reagent assay mixture (1 % BHT, 8 % sodium dodecyl sulfate, and 0.8 % thiobarbituric acid in 20 % acetic acid) and put in a boiling water-bath for 30 min. The samples were cooled to room temperature and centrifuged at 2,000×*g* for 5 min. The supernatants were measured using a spectrophotometer at 532 nm using malondialdehyde (MDA) as a standard.

Lysozyme gene expression

RNA extraction The head kidney was homogenized in 1 mL TRIzol reagent to disrupt the cells and release the RNA. It was then placed on ice and 0.2 mL of chloroform was added. After vigorous shaking and incubation at room temperature for 2 to 3 min, the samples were centrifuged at 12,000×*g* at 4 °C for 15 min. The lower phase and white protein inter-phase were discarded, while the clear upper phase containing the RNA was aspirated and placed in new microtubes. An equal volume of cold isopropanol was added and the solution was allowed to stand at room temperature for 10 min before being centrifuged at 12,000×*g* at 4 °C for 15 min. The supernatant was discarded and the pellets washed in 1 mL cold 75 % ethanol followed by centrifugation at 12,000×*g* at 4 °C for 5 min. After the final wash, the ethanol was removed and the pellets were air-dried for 5 min and redissolved in DEPC-treated water (Sigma). The concentration and the purity of the extracted RNA were measured using a spectrophotometer. The RNA was stored at −80 °C.

cDNA synthesis A 1 μg μL^{-1} total RNA was used to produce a complementary DNA (cDNA) using an iScript cDNA Synthesis Kit (Bio-Rad). Briefly, RNA was incubated with 4 μL of 5XiScript reaction mix, 1 μL of iScript reverse transcriptase, and 10 μL of nuclease-free water. The total volume was 20 μL. The reaction mixtures were incubated for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C with the reaction ended at 4 °C and the cDNA stored at −20 °C until being used for the next step.

PCR of lysozyme gene PCR amplifications were performed in a 25-μL final reagent mixture containing 2.5 μL of 10× high fidelity PCR buffer, 0.5 μL of 10 mMdNTP mixture, 1 μL of 50 mM $MgSO_4$, 1 μL of primer, 5 μL of the cDNA obtained (200 ng μL^{-1}), and 0.1 μL of PlatinumTaq DNA Polymerase high fidelity (Invitrogen). A thermocycler was used with the following program: 2 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C (25 cycles) followed by an over-extension step for 5 min at 72 °C. The primers specific to lysozymes (GenBank: EU136178.1) and β-actin (GenBank: GU188683) of *Lates calcarifer* were designed as the sequences listed in Table 2. A PCR with primers for β-actin was performed with all samples as a positive control for RT-PCR, since β-actin is constitutively expressed in the head kidney. These PCR products also allowed verification that equivalent amounts of cDNA were present in the different samples and amplifications of the different lysozymes genes were compared among the samples.

The PCR products (5 μL) were run on stained 2 % agarose gel (SYBR Safe stain; Invitrogen). Electrophoresis was run at 120 V for 25 min, and the products were subjected to UV visualization and densitometric analysis with a Gel Doc print (Vilber Lourmat). A 50-bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Photo-Capt software (Vilber Lourmat).

Statistical analysis

All data were reported as mean ± SD and the variance of data was analyzed using one-way ANOVA and differences of mean values were compared using the Duncan test. Significance was judged at $p < 0.05$.

Results

Proximate compositions of the extract and experimental diets

The proximate composition of the *Sargassum* sp. extract used in this study was found to be 4.68 ± 0.16 % moisture, 12.37 ± 0.21 % protein, 58.23 ± 0.19 % carbohydrate, and 24.72 ± 0.13 % ash. The HPLC analysis of its sugar component

Table 2 Primer sequences of lysozyme and β-actin gene for Asian sea bass (*L. calcarifer*)

Genes	Primer sequences	GenBanks
β-actin	F: 5′ TGG AGA AGA TCT GGC ATC AC 3′ R: 5′ AGG TCA TAG CTC TTC TCC AG 3′	GU188683
Lysozyme	F: 5′ TGC ATC ACA CAC CAT GGC AA 3′ R: 5′ CAT CCA CGT TGT CAT AGG AG 3′	EU136178.1

Table 3 Proximate composition of experimental diets

Parameters	Experimental diets			
	Sargassum sp. extract in diet (g kg^{-1})			
	0	0.5	1.0	2.0
Protein	42.95 ± 1.06	43.26 ± 0.17	42.80 ± 0.38	44.18 ± 0.83
Crude fat	14.73 ± 0.35	13.92 ± 0.97	12.73 ± 0.92	12.78 ± 1.28
Ash	16.77 ± 0.13	16.33 ± 0.09	17.07 ± 0.50	16.44 ± 1.13
Fiber	0.72 ± 0.12	0.75 ± 0.21	0.95 ± 0.06	0.85 ± 0.03
Carbohydrates	24.82 ± 1.22	25.72 ± 1.00	26.44 ± 1.46	25.74 ± 1.08

Means in a row are not significantly different ($p > 0.05$, $n = 3$)

showed that the extract contained mostly fucose (1120 mg L^{-1}) whereas mannose, fructose, glucose, galactose, and sucrose were less abundant (<200 mg L^{-1} each). The proximate composition of the experimental diets presents in Table 3. There were no significant differences among the compositions of the four experimental diets.

Growth performances and hepatosomatic index

During 30 days feeding period, although the average feed intakes of fish receiving the extract at 0 and 0.5 g kg^{-1} diet were significantly higher ($p < 0.05$) than those of fish which received the extract at 1.0 and 2.0 g kg^{-1} diet, there were no differences of weight gain and feed conversion ratio and hepatosomatic index among four treatments as the results shown in Table 4.

Hematological values

There were no significant differences ($p > 0.05$) of the hematological values (Fig. 1a–c) among four groups of fish that received different levels of the seaweed extract at 30 days post feeding of experimental diets (initial) and after injection with NaCl. In *S. iniae* injection groups, however, some differences occurred. Three hours after bacterial exposure, the %hematocrit of 1.0 g kg^{-1} group became significantly lower ($p < 0.05$) than that of 2.0 g kg^{-1} group (Fig. 1a). At 24 h after *S. iniae* injection, however, the hematocrit values of fish receiving the seaweed extract at 0.5 and 2.0 g kg^{-1} diets were similar and significantly increased as compared to the control value (0 g kg^{-1}) ($p < 0.05$) (Fig. 1a). Also the red blood cell (RBC) number of fish fed on diet containing the extract at 2.0 g kg^{-1} was significantly higher ($p < 0.05$) than that of the control fish at 3, 24, and 48 h post *S. iniae* injection (Fig. 1b), and no significant difference in RBC count was observed between fish receiving the seaweed extract at 1.0 and 2.0 g kg^{-1} diets after 48 h (Fig. 1b). At 3 h after bacterial injection, there was a significant decrease in the number of white blood cell (WBC) of 1.0 g kg^{-1} group as compared to those of the control and 0.5 g kg^{-1} groups ($p < 0.05$) (Fig. 1c). However, the WBC of 1.0 and 2.0 g kg^{-1} groups significantly increased at 24 h post *S. iniae* challenge as compared to that of the control group, but only that of 2.0 g kg^{-1} group still maintained significantly higher level ($p < 0.05$) after 48 h (Fig. 1c).

Table 4 Growth performances and hepatosomatic index of Asian sea bass fed the experimental diets containing different levels of *Sargassum* sp. extract for 30 days

Parameters	Experimental diets			
	Sargassum sp. extract in diet (g kg^{-1})			
	0	0.5	1.0	2.0
Initial weight (g $fish^{-1}$)	81.64 ± 14.31	79.96 ± 13.67	79.96 ± 13.57	80.82 ± 14.87
Final weight (g $fish^{-1}$)	105.49 ± 18.52	102.93 ± 19.74	99.44 ± 18.93	101.42 ± 17.13
Weight gain[a] (%)	29.22 ± 2.87	28.74 ± 6.66	24.36 ± 1.19	25.51 ± 1.96
Feed intake[b] (g $fish^{-1}$)	32.89 ± 1.13[b]	32.17 ± 0.60[b]	26.23 ± 2.40[a]	26.45 ± 1.88[a]
FCR[c]	1.39 ± 0.16	1.45 ± 0.35	1.35 ± 0.09	1.29 ± 0.10
HSI[d] (%)	1.33 ± 0.31	1.41 ± 0.70	1.46 ± 0.38	1.41 ± 0.35

Means in a row with the same letters are not statistically different ($p > 0.05$, $n = 80$)

[a] $\text{Weight gain} = \frac{100 \times [\text{final mean body weight (g)} - \text{initial mean body weight (g)}]}{\text{initial mean body weight (g)}}$

[b] Feed intake = dry feed consumed (g)/body weight (g)

[c] Feed conversion ratio = feed intake (g)/weight gain (g)

[d] Hepatosomatic index = [liver weight (g) × 100]/body weight (g)

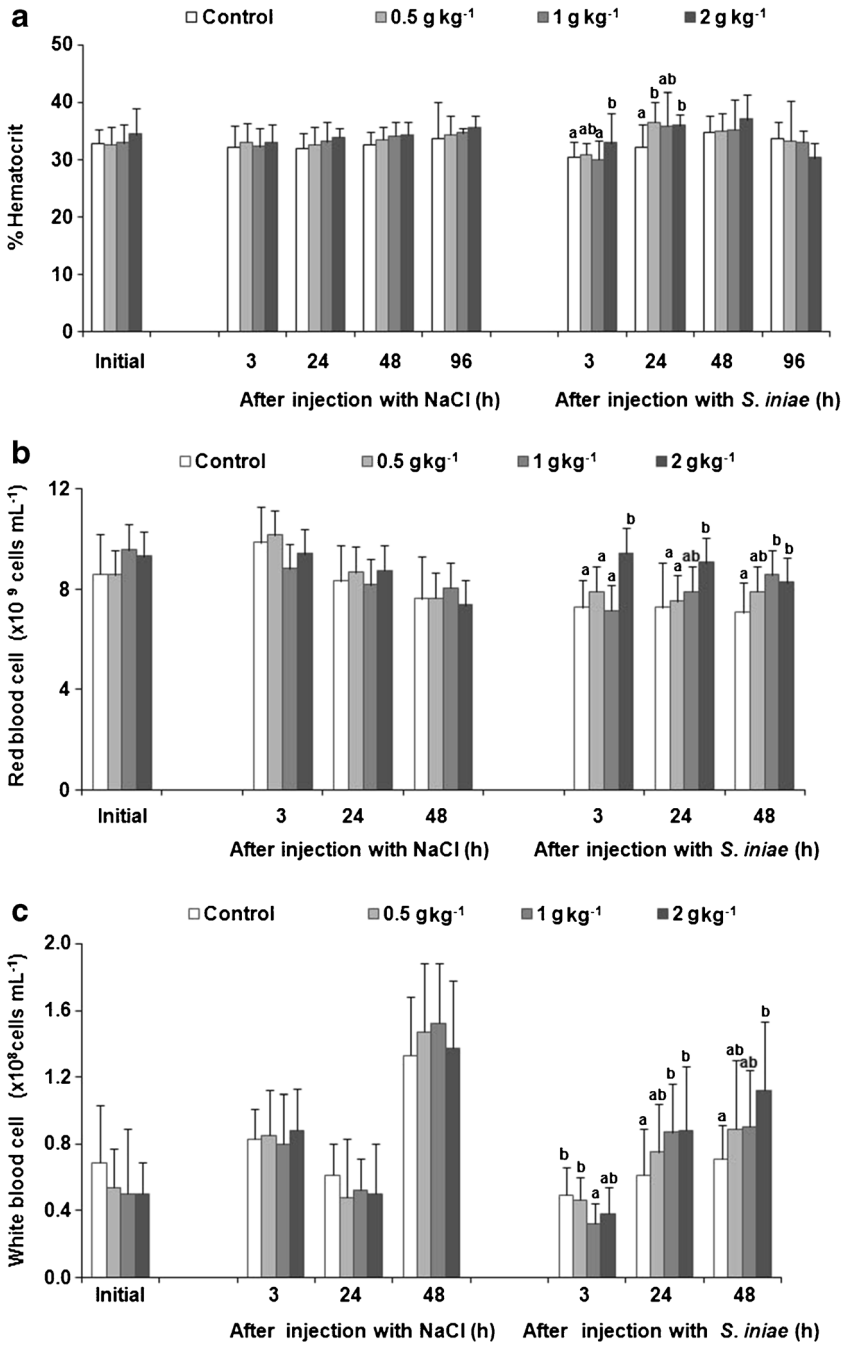

Fig. 1 **a** Hematocrit. **b** Red blood cell count. **c** White blood cell count of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 30 days (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± SD. Statistical differences ($p < 0.05$, $n = 15$) between groups are indicated by *different letters* over the bar graphs

Plasma protein levels

After being fed the experimental diets for 30 days (initial), total plasma protein and total immunoglobulin (Ig) levels of the groups of fish fed diet containing the extract at 1.0 and 2.0 g kg^{-1} were significantly higher ($p < 0.05$) than the other groups (Fig. 2a, b). Such elevated protein and Ig levels of both groups, however, were found decreased after NaCl and bacterial injections. Although there were no significant differences ($p > 0.05$) among NaCl injection groups, total protein and Ig concentrations in the plasma of fish after the injection with bacteria had significant differences (Fig. 2a, b). The plasma protein level of the control fish significantly decreased ($p < 0.05$) at 24 h post *S. iniae* injection as compared to the level at 3 h, and then gradually increased from 48 to 96 h (Fig. 2a). The protein levels of fish receiving the extract at 0.5 g kg^{-1}, however, were significantly higher than their respective control values ($p < 0.05$) at 24 and 48 h (Fig. 2a). Similar results were obtained for Ig. The Ig concentrations of fish treated with the seaweed extract at all levels were

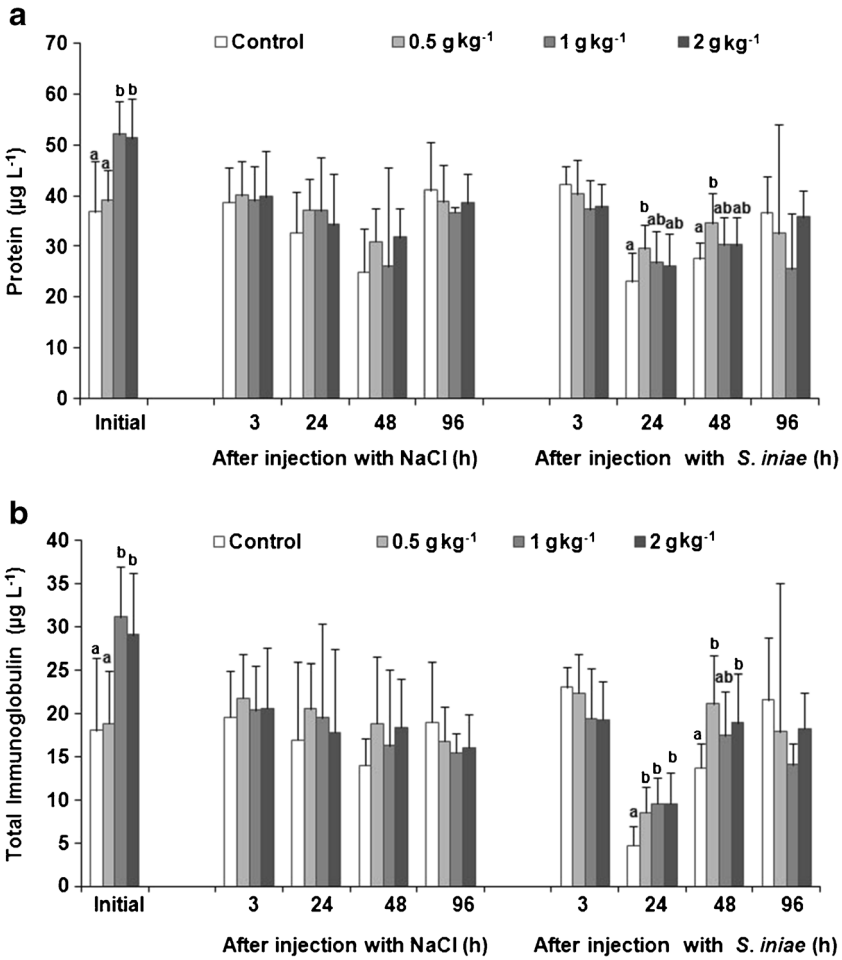

Fig. 2 **a** Protein and **b** total immunoglobulin in the plasma of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 30 days (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 15$) between groups are indicated by *different letters* over the bar graphs

significantly higher than that of the control fish ($p < 0.05$) at 24 h post *S. iniae* injection (Fig. 2b), but the difference between 1.0 g kg^{-1} and control groups became non-significant ($p > 0.05$) after 48 h (Fig. 2b).

Lysozyme activity

After 30 days feeding and after injection with NaCl, there were no significant differences of serum lysozyme activity among the treatments as shown in Fig. 3. The enzyme activity of the control fish gradually decreased from 3 to 48 h after bacterial injection, and significant differences ($p < 0.05$) among groups thus occurred at those time points. Three hours after injection with the bacteria, the lysozyme activity of fish that received diets containing the extract at 1.0 and 2.0 g kg^{-1} were lower than that of the control fish ($p < 0.05$). The lysozyme of fish receiving diets containing the extract at all levels, however, became significantly higher than that of the control

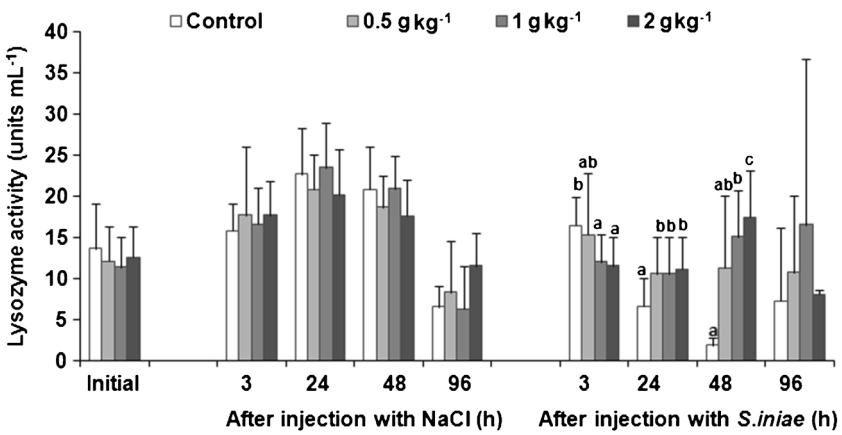

Fig. 3 Serum lysozyme activity of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 30 days (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 15$) between groups are indicated by *different letters* over the bar graphs

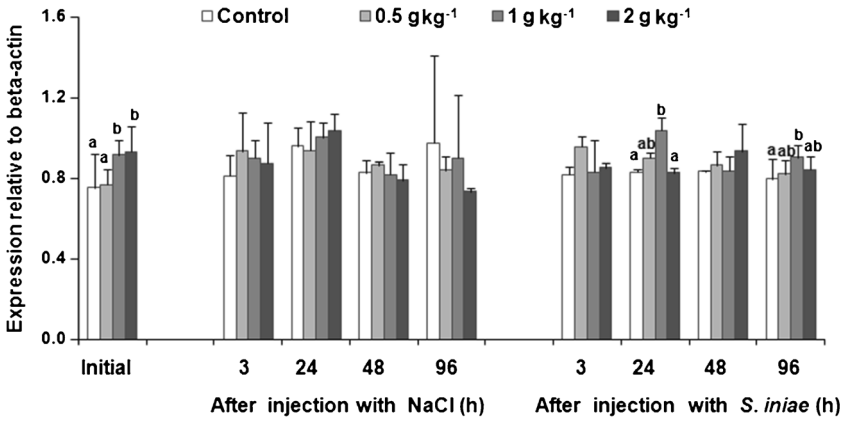

Fig. 4 Lysozyme gene expression from the head kidney of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 4 weeks (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 3$) between groups are indicated by *different letters* over the bar graphs

fish ($p < 0.05$) after 24 h of bacterial injection. At 48 h, their increases were greater and seemed to follow a dose-dependent pattern (Fig. 3).

Lysozyme gene expression

Figure 4 shows the lysozyme gene expression in head kidney of fish that received four different levels of the extract. After being fed diets supplemented with or without extract for 30 days (initial), there were significant differences ($p < 0.05$) in the expression of this gene. The relative messenger RNA (mRNA) abundance in the groups fed the extract at 1.0 and 2.0 g kg^{-1} diets were greater than those of the control and 0.5 g.kg^{-1} groups ($p < 0.05$) (Fig. 4). However, the expression of the gene between the groups was not significantly different ($p > 0.05$) at any time points after injection with NaCl. For *S. iniae* groups, there were some changes at 24 and 96 h after the injection. The mRNA levels of the lysozyme gene in fish receiving the extract at 1.0 g kg^{-1} diet were significantly higher ($p < 0.05$) than those of the control fish (Fig. 4).

Complement activity

There were no significant differences ($p > 0.05$) of the complement activity of fish that received four levels of the extract after 30 days (initial) and post NaCl injection as shown in Fig. 5. After 3 h of *S. iniae* injection, however, there was a decrease in the activity of 2 g kg^{-1} group as compared to those of 0.5 and 1.0 g kg^{-1} groups ($p < 0.05$) (Fig. 5). At 48 h after injection with the bacteria, the complement activity of fish receiving the extract at 1.0 and 2.0 g kg^{-1} were significantly higher than that of the control fish ($p < 0.05$) (Fig. 5).

TBARs levels

After being fed the control and the seaweed extract supplemented diets for 30 days (initial), there were no significant differences ($p > 0.05$) among the TBARs levels of four groups. Also their levels at each time point post NaCl injection were not significantly different from each other, as shown in Fig. 6. Post *S. iniae* injection at 3 h, there were still no significant differences of the TBARs levels among fish that received four different levels of the extract. The control values, however, gradually increased from 3 to 96 h after bacterial injection. The TBARs levels of fish receiving the seaweed extract at 0.5, 1.0, and 2.0 g kg^{-1} were similar and significantly decreased ($p < 0.05$) as compared to that of the control fish at 24 h after the injection. At 48 h, however, only the TBARs value of 2 g kg^{-1} group was significantly lower than that of the control group (Fig. 6). There were no significant differences among the TBARs levels of four groups at 96 h after injection with the bacteria.

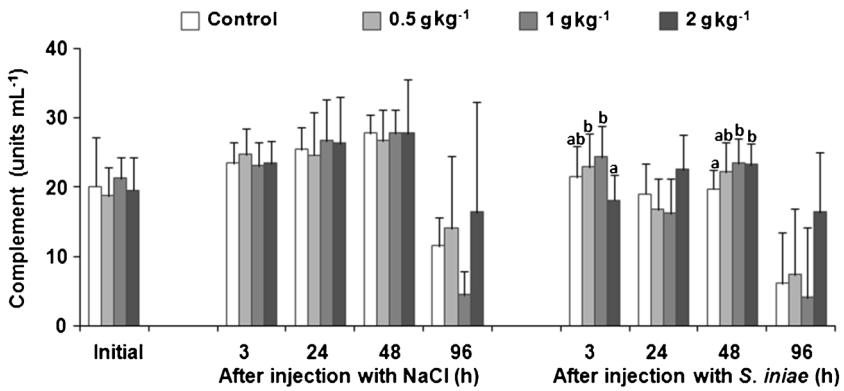

Fig. 5 Complement activity of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 4 weeks (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 15$) between groups are indicated by *different letters* over the bar graphs

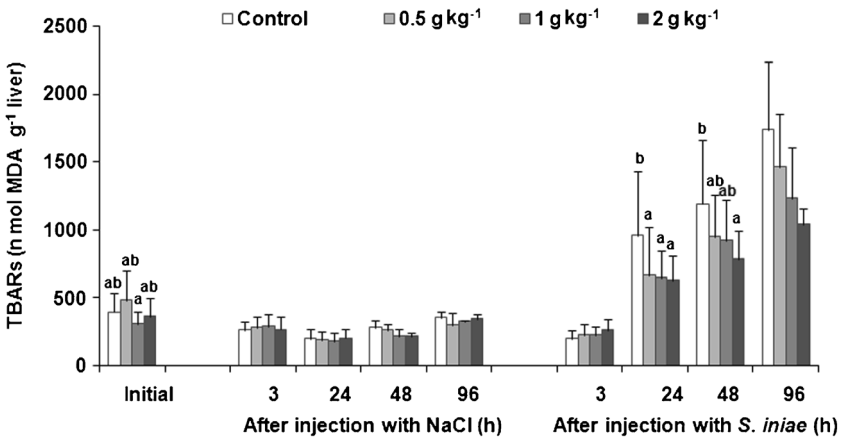

Fig. 6 TBARs of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 4 weeks (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 15$) between groups are indicated by *different letters* over the bar graphs

Survival rates

All the fish that received four different levels of the *Sagassum* sp. extract for 30 days (initial) and after NaCl injection were survived as shown in Fig. 7. After *S. iniae* injection, however, some fish died. The survival rate of fish that received 2.0 g $kg^{-1}$of the extract was 98.1 % which was significantly higher ($p < 0.05$) than that of the control fish (94.29 %), whereas the fish fed the extract at 0.5 and 1.0 g kg^{-1} gave the same survival rate of 96.19 % at 24 h post bacterial exposure. After 48 and 96 h, the survival rates of fish in all treatments became lower but were not significantly different ($p > 0.05$) from each other (Fig. 7).

Discussion

The contents of the hot water extract from *Sargassum* sp. prepared by spray-drying method in the present study were different from those of the freeze-dried hot water extract from *S. hemiphyllum* (10.1 % moisture, 38.5 % crude protein, 8.4 % crude fat, 12.2 % ash, and 30.8 % total carbohydrate) (Hwang et al. 2010). In our extract, the levels of moisture and protein were lower but that of ash was higher indicating more inorganic constituents. It also contained mostly carbohydrate (approximately 58 %) but no lipids. The main sugar component in the extract was fucose as also reported by Kantachumpoo and Chirapart (2010). Therefore, the chemical composition of *Sargassum* seaweed extracts varies depending on the species of seaweed, the environment, and the extraction method.

The proximate analysis of all the fish diets revealed that their crude protein levels at 42–44 % and lipid levels at 12–15 % met the requirements of Asian sea bass according to Ambasankar et al. (2009). Although 30 days of the *Sargassum* sp. extract administration did not change the growth performance of the Asian sea bass, it would still be interesting to use fish at a smaller initial size and to increase the duration of the feeding trial to investigate any influences of the same extract on the growth rate of Asian sea bass in the future studies.

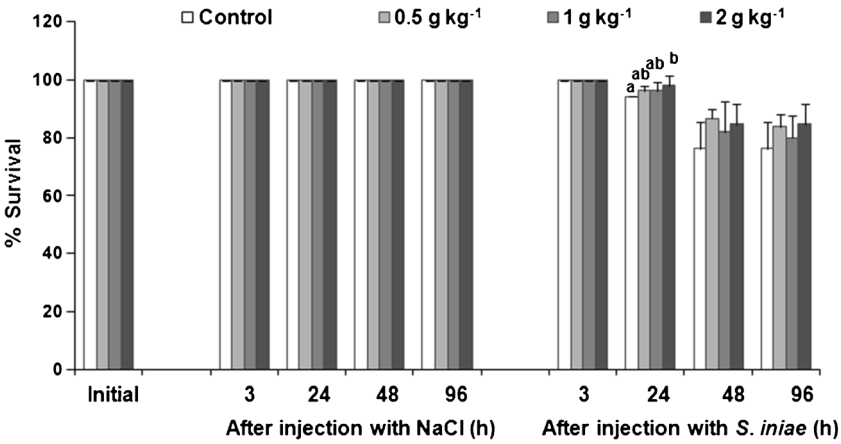

Fig. 7 Survival rate of Asian sea bass fed diets containing *Sargassum* sp. extract at 0, 0.5, 1.0, and 2.0 g kg^{-1} after 4 weeks (initial), after injection with NaCl and *S. iniae* at 3, 24, 48, and 96 h. Data represent the mean ± S.D. Statistical differences ($p < 0.05$, $n = 3$) between groups are indicated by *different letters* over the bar graphs

At the end of 30 days feeding trial, the extract at all levels did not produce any significant effects on the hematocrit, blood cells, lysozyme, complement, TBARS, and also the survival rate of the fish. However, a significant increase in plasma protein, immunoglobulin (Ig), and lysozyme gene expression levels was observed in the 1.0 and 2.0 g kg^{-1} groups. The reason for such findings is still not known. We speculate that the differences were caused by biological variation among the fish rather than the extract administration since the levels increased similarly in a non-dose-dependent manner. It seems likely also that the total plasma protein was elevated because of the increase of Ig.

After the fish receiving the extract were injected with NaCl, we could not detect any significant changes in all the parameters from the respective control over the whole study period. These results thus suggest that NaCl did not induce immunomodulation in the fish. In addition, all of them maintained their levels similar to the initial values, except WBC that became much higher after 48 h of NaCl injection by an unknown cause. As significant increase in total WBC count usually indicates an activation of immune system, we then suspect that there would probably be some kind of exogenous signal that triggered the immune response process in these fish either during or after blood sample collection at the previous time point (24 h).

In this study, there was a reduction in hematological parameters including hematocrit, RBC and WBC counts, plasma protein, and Ig in the Asian sea bass upon infected with *S. iniae*, as reported previously (Wanman et al. 2005). The extract especially at 2.0 g kg^{-1} diet, however, showed the positive effects on these parameters mostly at 24–48 h post bacterial challenge. Similar to our findings, the extract of the brown seaweed, *Ascophyllum nodosum*, has been shown to be capable of increasing RBC and WBC counts in stressed lambs (Saker et al. 2004). Among Ig levels of the four treatments at different time points after *S. iniae* injection, those measured at 24 h were found to be much lower than at 3 h, but gradually increased after 48 h, while the levels of total WBC were increased rather than decreased. In this regard, we then assume that the levels of circulating Ig fluctuated in concert with the change of B lymphocyte population in the *S. iniae* infected fish, based on the fact that they are the main source of Ig production. At 24 h after the injection, the number of bacterial cells probably dropped as a result of general body immune defenses after the initial exposure to the pathogens, leading to a subsequent decrease in B cell activation followed by the lowering of Ig levels. It is interesting, therefore, to determine both the B cell and Ig levels in the fish over time to prove the above assumption.

The alternative pathway of complement activity emerges as a powerful non-specific defense mechanism, protecting fish from a wide range of potentially invasive organisms (Müller-Eberhard 1988). The water-soluble polysaccharides extracted from brown seaweeds such as fucoidan from *Laminaria cichorioides* and sodium alginate from *Laminaria digitata* and *A. nodosum* have been found to promote the complement activity (Zvyagintseva et al. 2000; Bagni et al. 2005). In the present study, there was a significant increase in complement activity together with lysozyme activity in the Asian sea bass fed diet containing the *Sargassum* sp. extract at 1.0 and 2.0 g kg^{-1} after 48 h of bacterial injection. Both of the activities have also been found increased when brown algal alginate was fed to sea bass (*Dicentrarchus labrax*) for 15 days (Bagni et al. 2005).

Lysozymes are hydrolytic enzymes with bactericidal property. They cause damage mainly to Gram-positive bacteria like *Bacillus* spp. and *Streptococcus* spp. by cleaving β-1, 4 linkages between N-acetylmuramic acid and N-acetylglucosamine residues of peptidoglycan in the bacterial cell walls (Ibrahim et al. 2001). In the present study, serum lysozyme activity of the control fish declined gradually from 3 to 48 h after *S. iniae* challenge implying an increase in the utilization of the enzyme molecules as the bacterial cells multiplied, and then they elevated after 96 h for maintaining homeostasis. The alternative complement has been known to act against bacteria in conjunction with lysozyme (Wang et al. 2009). In comparison to those of the lysozyme activity, its levels in the control fish after the bacterial exposure seemed to decrease later. However, the increased lysozyme and complement levels seen in the Asian sea bass receiving the seaweed extract suggest that both of them were enhanced together in order to increase resistance of the fish against *S. iniae*.

The results of lysozyme gene expression measured in head kidneys were rather different from those of serum lysozyme activity. Feeding the *Sargassum* sp. extract at 1.0 and 2.0 g kg^{-1} for 30 days appeared to increase the expression to a similar extent. This difference was then considered not due to the stimulatory effect of the extract. The number of lysozyme transcripts of NaCl-injected fish also remained at the initial levels and did not change significantly throughout the study period. These findings thus indicate that NaCl did not modulate the expression of the lysozyme gene. In fish, lysozymes are present in macrophages residing in the organs prone to the risk of bacterial invasion, especially head kidney being recognized as the major site of lysozyme secretion (Paulsen et al. 2001; Yuan et al. 2008). In the present study, we could detect a slight but significant increase in the lysozyme transcript abundances only in the fish fed with of 1.0 g kg^{-1} and after 24 and 96 h post injection with the bacteria. Taken together, the results from the measurements on lysozyme activity and mRNA expression indicate that the extract did not strongly regulate the expression of lysozyme gene, and as suggested earlier, the

gradual decrease in serum lysozyme levels observed after the bacterial challenge was resulted from the increasing rate of lysozyme utilization upon the bacterial expansion rather than a decrease in the enzyme production.

Changes in the environment and the conditions of infection have been known to cause an increased level of lipid peroxidation (Benzie 1996). Previously, Lim et al. (2002) have reported that the extract from *S. siliquastrum* inhibited hemolysis and suppressed lipid peroxidation in vitro. In the present study, we also demonstrated that there was an increase in lipid peroxidation (expressed as TBARs level) in the liver of the Asian sea bass upon *S. iniae* injection whereas the *Sargassum* sp. extract suppressed it. The findings that fucoidan from *L. japonica* was able to prevent the increase of lipid peroxide in the serum, liver, and spleen of diabetic mice (Li et al. 2002) and that the serum MDA contents significantly decreased when juvenile yellow catfish were fed fucoidan from *S. horneri* (Yang et al. 2014) also suggest that fucoidan in our *Sargassum* sp. extract would act as the active material in this case.

Infection by *S. iniae* has been reported to cause mass mortality in the culture of Asian sea bass (Bromage and Owens 2009; Suanyuk et al. 2010). In the present study, by employing the same *S. iniae* challenge dose as that of Bromage and Owens (2002), the first mortality was observed at 24 h post injection as reported by Taniguchi (1983) and Bromage et al. (1999). At that time point, however, the survival rate of fish receiving 2.0 g kg^{-1} *Sargassum* sp. extract was significantly higher than those of the control fish and the fish fed diet containing 0.5 and 1.0 g kg^{-1} extract. These results thus indicate that a dietary supplementation of *Sargassum* sp. extract was able to increase the resistance against *S. iniae* from its ability to enhance immune system in the Asian sea bass.

The finding that our seaweed extract contained fucose in abundance implies that the extract consisted of a large amount of fucoidan (Wijesinghe and Jeon 2012). This polysaccharide acts as a ligand for selectins, carbohydrate-binding proteins that exhibit antibacterial activity via recognition and binding to complementary oligosaccharides on the cell wall or plasma membrane of the associated bacteria cells. As a result, the pathogens are immobilized and prevented from multiplication (Lasky 1995). Therefore, fucoidan in the *Sargassum* sp. extract could help alleviate *S. iniae* infection by attenuating the bacterial cell expansion in the fish.

In conclusion, we demonstrated for the first time that the hot water extract from *Sargassum* sp. was able to enhance the humoral immune response and resistance against pathogenic *S. iniae* in the Asian sea bass (*Lates calcarifer*) by improving the immune parameters as well as increasing the survival rate of the fish post bacterial infection. Therefore, this seaweed extract has a potential to be used as a dietary supplement in the Asian sea bass culture.

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