J. Fish. Sci. Tech. 3(1), 52~63, 2000

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY
© The Korean Fisheries Society

Characterization of a Substance from *Photobacterium damsela* subsp. *piscicida* that Non-specifically Binds to Streptavidin

Tae Sung Jung*, Kim D.Thompson[1], Aelexandra Adams[1] and Myung Joo Oh[2]

Department of Microbiology, College of Medicine, Gyeongsang National University, Chinju 660-751, Korea

[1]Aquatic Vaccine Unit, Institute of Aquaculture, University of Stirling, Stirling, Scotland, FK9 4LA

[2]Department of Fish Pathology, Yosu National University, Yosu 550-749, Korea

(Received January 2000, Accepted March 2000)

Non-specific reaction has been a problem in doing, especially, research and diagnosis for infectious agents. Avidin-biotin-peroxidase complex (ABC) techniques has widely been used to amplify a reaction. *Photobacterium damsela* subsp. *piscicdia* (formerly *Pasteurella piscicida*) exhibited a capacity to bind with streptavidin non-specifically. The band, estimated 26 K Da in Western blotted paper, was blocked with biotin but incompletely. In an attempt to explore an involvement of the non-specific substance in attaching piscine cells, cell attachment test performed using anti- *Ph. d.* subsp *piscicida* sera raised mouse and rabbit exhibited slightly blocking effects for Mediterranean (I736) and significantly for Japanese (Sp 92144) isolate. Biotin decreased the attachment ability significantly for Sp92144 but it was not effective to I736. Both isolates showed greatly enhanced attachment ability with poly-L-lysin. The non-specific binding substance was contained in bacterial extracellular products (ECPs). The substance was able to purified with 2-imminobiotin affinity column, the purified substance appeared to have 4 bands in silver staining, and had a carbohydrate branch. This purified substance showed cytotoxic effects selectively between 5 piscine cell lines. Moreover, it stimulated rainbow trout macrophage in terms of reduction of cytochrome c as well as yeast phagocytosis, significantly.

Key words: *Photobacterium damsela* subsp. *piscicida*, streptavidin, non-specific binding, extracellular products (ECPs), cell toxicity, non-specific immune response

Introduction

Pasteurellosis, caused by *Photobacterrium damsela* subs *piscicida* (*Ph. d.* subsp. *piscicida*), has caused huge economic damage to the sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) and yellowtail (*Seriola quinqueradiata*) culture industries in the Mediterranean and Japan (Rodgers and Furones, 1998; Kusuda and Kawa, 1998).

Although the virulence factors of the pathogen are not fully understood, the extracellular products (ECPs) of *Ph. d.* subsp *piscicida* are found to be toxic in gilthead sea bream, turbot (*Psetta maxima*) and rainbow trout (*Oncorhynchus mykiss*) (Magarinos et al. 1992). They have also shown to be haemolytic *in vivo* in experimentally infected gilthead sea bream, causing significant decrease in the number of circulating red blood cells. They are also capable of eliciting an inflammatory response (Noya et al. 1995). Fish injected with ECPs develop severe lesions in the liver and gills, suggesting the presence of toxin(s) which may be important in the pathogenesis of *Ph. d.* subsp *piscicida*. When the isolates of the bacterium from different geographical origins were cultivated under iron limited conditions, most of the ECPs exhibited elevated levels of caseinase activity (Bakopoulous et al. 1997 a). Recently, Jung et al. (2000) showed the presence of sialic acid on the surface of *Ph. d.* subsp.

*To whom correspondence should be addressed.
82-55-751-8747 (phone) / 82-55-759-1588 (fax)
E-mail address: tsj01@hotmail.com

piscicida using a Glycoprotein determination kit purchased from Bio-Rad. The kit can detect either carbohydrate structures in glycoprotein molecules or sialic acid depending on how the kit is used. The kit relies on the specific oxidation of the carbohydrate and the labeling of the carbohydrate with biotin which can subsequently be reacted with streptavidin-alkaline phosphatase. Streptavidin is a protein produced by *Streptomyces avidinii* which can bind up to four molecules of vitamin H, D-biotin. The interaction with biotin is noncovalent but extremely tight, the dissociation constant of about 10^{-15} M being about 10^3-10^6 times higher than that of typical antigen antibody interaction (Diamandis and Christopoulos, 1991). The molecular weights of the sialic acid varied between the different isolates of *Ph. d.* subsp. *piscicida,* with the Japanese isolates having a lower molecular weight sialic acid than the Mediterranean isolates. Different sensitivities to sialidase were also exhibited between these isolates.

Avidin-biotin-peroxidase complex (ABC) is frequently used to amplify antibody or molecular based reaction involved in both research and in diagnosis (Wilchek and Bayer, 1990). As well as 2-iminobitin, the analog of biotin cyclic guanidino, also interacts with avidin/strepavidin in a pH-dependent manner. This allows biologically active streptavidin reporter molecules to be purified free from damaged proteins and unconjugated molecules by affinity chromatography (Fudem-Goldin and Orr, 1990). However, sometimes a non-specific reaction results when the avidin binds to tissue section and serum (Rao and Chakraborti, 1989; Jones et al. 1987).

Non-specific binding, at the same molecular weight as sialic acid band, has been noted when ABC was used. It has also been observed with the ECP of the bacterium. Attempts are made here to reduce the level of non-specific binding of *Ph. d.* subsp *piscicida* when ABC is used. The substance at 26 kDa, perhaps sialic acid, was purified by affinity chromatography and the effects of this substance on fish cell lines and on head kidney macrophages *in vitro* were then examined.

Material and Methods

Bacteria preparation

Two isolates of *Ph. d.* subsp. *piscicida,* I736, and Sp92144, (Jung et al. 2000), were stored at -70℃ on PROTECT® beads (Technical Service Consultants LTD) until required. The beads were placed in tryptone soya broth (TSB) containing 2% (w/v) NaCl and the bacteria allowed to grow for 16 h at 22℃. The bacteria were harvested by centrifugation at 2900 g for 20 min at 4℃, washed twice with sterile phosphate buffered saline (PBS: 0.02 M $NaH_2PO_4 \cdot 2H_2O$, 0.02 M $Na_2HPO_4 \cdot 2H_2O$, 0.15 M NaCl, pH 7.2), and bacterial pellets finally resuspended in PBS. The absorbance of each bacterial suspension was adjusted to 1.0 at 610 nm using PBS. This was equivalent to approximately 2×10^8 c.f.u $m\ell^{-1}$ as determined from a standard curve of bacterial concentration *versus* absorbance at 610 nm.

Non-specific reaction of the 26 kDa in Western blot analysis

Ph. d. subsp. *piscicida,* I736, (2×10^8 c.f.u $m\ell^{-1}$) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 22℃. The separated bands were then transferred to a nitro-cellulose membrane (Jung et al. 2000). The membrane was cut into small strips which were placed into incubation trays (Bio Rad). Each strip was treated differently and the non-specific reaction at 26 kDa band then examined. The strips were washed for 10 min with PBS or Tris Buffered Saline (10 mM Tris, 5 M NaCl, pH 7.5) containing 0.1% Tween 20. The treatment of the strip are indicated in Table 1.

Cell attachment

Two isolates, I736 and Sp92144, were prepared and adjusted to 2×10^8 bacteria $m\ell^{-1}$. The bacteria were then incubated with either anti- *Ph. d.* subsp. *piscicida* rabbit serum (Bakopoulos et al. 1997c) or with anti- *Ph. d.* subsp. *piscicida* mouse serum diluted in 1/100 in PBS, 0. 5 M biotin, 0. 01% Poly-L-lysine for 30 min at 22℃. Coated bacteria were washed with PBS by centrifugation at 2900g for 20 min at 4℃. Untreated bacteria were used as a control. The ability of the treated bacteria to attach to CHSE-214 cells in a 96 well plate was assessed as previously described by Jung et al. (2000).

Table 1. Treatment of strips Western blot containing of whole cell, *Ph. d.* subsp. *piscicida.*

Treatment	Reagents	Treatment time (min)	Source
1	M. W marker		Bio-Rad
2	Carbohydrate detection (Oxidation, Biotin labeling and streptavidin-alkaline phosphatase)	20, 60 and 60	Jung et al. (2000) and GDK
5	Biotylated anti-mouse (1/100) and streptavidin-HRP(1/100)	60 and 60	SAPU
6	Biotylated anti-rabbit (1/100) and streptavidin-HRP(1/100)	60 and 60	SAPU
7	Streptavidin-HRP(1/100)	60	SAPU
10	Anti-rabbit-HRP(1/100)	60	SAPU
11	Anti-mouse-HRP(1/100)	60	SAPU
13	Biotin(0.5M) and streptavidin-HRP(1/100)	120 and 60	Sigma and SAPU

GDK: Glycoprotein Determination Kit (Bio-Rad, 2000 Alfred Nobel Drive, Hercules CA, USA)
SAPU: Scottish Antibody Production Unit, Lanark, Scotland
HRP: Horseradish peroxidase

Purification of the 26 kDa substance with streptavidin

The 26 kDa material was purified from the ECP of *Ph. d.* subsp. *piscicida* according to Hofmann et al. (1980) with slight modifications. Isolate I736 was cultured as described above, and the supernatant of the bacteria was collected by centrifugation at 2900 g, for 30 min at 4℃. The bacterial pellets were removed, and supernatants were concentrated by reverse dialysis (10 kDa M.W. cut-off) using polyethylene glycol (PEG; M.W. 8,000). Concentrated ECP were centrifuged to remove any remaining bacteria, then passed through a 0.22 μm microfilter. They were dialysised against distilled H_2O overnight, changing the water three times over the dialysis period. A sample of the supernatant was subjected to Western blot analysis to confirm the presence of the 26 kDa band. The pH of the supernatant was adjusted pH 11 with 1 M NaOH, before adding 1 M NaCl. Purification of the 26 kDa material was performed by affinity chromatography (Bio-Rad) at 22℃ using 2-imminobiotin affinity beads (Sigma). The column was equilibrated with 50 mM sodium carbonate (pH 11) containing 1 M NaCl buffer, at a speed of 20 mℓ/min. The column was washed with equilibrating buffer until the detector (280 nm) reached baseline before adding the concentrated supernatant. The substance was eluted from the column using 50 mM ammonium acetate (pH 4) containing 0.5 M NaCl, and collected as 1 mℓ aliquots. It was not possible to detect the 26 kDa substance at 280 nm, so an enzyme linked immunosorbent assay (ELISA) was used to determine the level of the 26 kDa material in each fraction. The eluted substance was placed in bicarbonate buffer (pH 9. 6) (Sigma) and added to the ELISA plate at 30 $\mu\ell$ of substance in 110 $\mu\ell$ of buffer well^{-1}. PBS and elution buffer were used as a controls. After 1.5 h at 22℃, glutaraldehyde (0.005% v/v) diluted in PBS was added to the wells (50 $\mu\ell$ well^{-1}) for 20 min. The plates were washed with high salt washing buffer (HSWB: 0.02 M Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.8) with a 5 min soak on the last wash. Streptoavidin-HRP (SAPU), diluted 1 in 1000 with PBS plus 0.01% tween 20 was addded to the wells (100 $\mu\ell$ well^{-1}) for 40 min, before again washing the plate with HSW. Chromogen/substrate [120 $\mu\ell$ of 43 mM tetraethyl benzidine dihydrochloride in 2 M acetic acid added to 12 mL of substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4 containing 0.33% v/v H_2O_2)] was added to each well (100 $\mu\ell$ well^{-1}). The color reaction was stopped after 10 mins by the addition of 50 $\mu\ell$ well^{-1} of 2 M H_2SO_4. The reaction was read spectrophotometrically at 450 nm (Dynatech ELISA reader). The fractions which were positive in the ELISA were pooled and its immunostimulatory properties examined.

Silver staining and carbohydrate determination

The concentration of ECPs were measured by Bradford (Bio Rad), and the concentration of purified substance total carbohydrate substance by Dubois et al. (1956). Briefly, the purified substance (200 $\mu\ell$) was mixed with 5% aqueous phenol, then 1 mℓ of sulphric acid (H_2SO_4) was quickly added

to the bottom of the tube and again mixed well. The tube was left 22℃ for 15 min and read at 490 nm. The concentration of the substance was determined using standard curve prepared by 1 mg $m\ell^{-1}$ glucose. The substance purified from the column (0.85 ng $m\ell^{-1}$), partially concentrated ECPs (10 μg $m\ell^{-1}$) and whole bacteria (2×10^8 c.f.u $m\ell^{-1}$) were subjected to 12% SDS-PAGE. The gels were stained with a silver staining kit® (Sigma). The same sample transferred to a nitrocellulose membrane and the total carbohydrate present was examined using a glycoprotein determination kit (Bio-Rad) following the method of Jung et al. (2000).

Cytotoxicity test

Cell toxicity test was assessed using a conventional trypan blue exclusion method (Braunbeck, 1993). Five cell lines of piscine origin: sea bass larvae (*Cetropristis striata*) (SBL) cell line; epithelioma papulosum cyprini (*Cyprinus carpio*) (EPC); bluegill sunfish (*Lepomis macrochirus*) (BF-2); chinook salmon embryo (*Oncorhynchus tshawytscha*) (CHSE-214) and rainbow trout gonad (*Oncorhynchus mykiss*) (RTG-2) cells, were used to examine the toxicity of the purified substance. All the cell lines were cultured with minimum essential medium (MEM) plus 10% Foetal Calf Sera (FCS) in a CO_2 atmosphere except SBL which was maintained with Leibovitz-15 (L-15) plus 10% FCS in a non-CO_2 incubator. The cells were subcultured into 96 tissue culture plates, and maintained in a CO_2 incubator (except SBL) at 20 ℃ until confluent. The cells were washed three times with PBS, before adding 100 $\mu\ell$ $well^{-1}$ medium containing 5% FCS and the purified substance at 85, 17, 3.4 and 0.68 ng $well^{-1}$. Control wells, without the substance, were also included. After 3 days, the plates were washed twice with PBS, and 20 $\mu\ell$ of trypsin (2.5 mg $m\ell^{-1}$) (Sigma) was added to the wells. Trysinization was terminated by the addition of 80 $\mu\ell$ of PBS, containing 5% FCS, and 100 $\mu\ell$ of 0.25% trypan blue. Released cells were counted using a hemocytometer under a lighter microscope.

Fish husbandry

Thirty rainbow trout, 200 g, were maintained in a flow-through dechlorinated water system at ambient temperature at the Institute of Aquaculture, University of Stirling. After acclimation of one week, the non-specific response of the head kidney macrophages of the fish to the purified substance was examined.

Head kidney macrophages isolation

Macrophages were isolated from the head kidney and culture as described by Secombes (1990). The isolated macrophages were added to the wells of a 96-well microtiter plate (Nunc) (100 $\mu\ell$ $well^{-1}$ of a 2×10^7 cells $m\ell^{-1}$), and also to the wells of a sterile 8-well glass slide (Lab-TeK®, Nunc) (0.4 $m\ell$ at 5×10^6 cells $m\ell^{-1}$). Cells were allowed to adhere for 2 to 3 h at 20℃, before washing six times with Hanks balanced salt solution (HBSS). Culture medium (L-15 containing 5% heat inactivated FCS and Penicillin/Streptomycin) was added to the monolayers at 100 $\mu\ell$ $well^{-1}$ which were then incubated overnight at 20℃.

The numbers of macrophages with monolayers were counted as described by Secombes (1990). Macrophage activation factor (MAF) was also obtained from mixed leukocyte culture also described by Secombes (1990).

Respiratory bust activity of rainbow trout macrophages

Extracellular superoxide anion production

Extracellular generation of superoxide anion by trout macrophages was quantified spectrophotometrically in 96 well microplates be the reduction of cytochrome c using phorbol mysristate acetate (PMA) to stimulate the respiratory burst (Pick and Mizel, 1981; Secombes et al. 1988). All reactions performed in quadruplicate, take place at 20℃ and the absorbance of the reaction was red spectrophotometer at 550 nm at periodically over the course of 2 h using the reaction mixture contained, SOD as a reference. The values observed were adjusted for 2×10^5 $well^{-1}$ (Secombes, 1990). The nmol of O_2^- produced per 2×10^5 cells was calculated by multiplying the adjusted value by a conversion factor of 15.87 (Pick and Mizel, 1981). This factor derives from the extinction coefficient for the absorption at 550 nm of reduced minus oxidised cytochrome c in a 96-well microtiter plate.

Intracellular superoxide anion production

Intracellular production of superoxide anions by trout head kidney macrophages was quantified spectrophotometriaclly by the reduction of nitroblue tetrazolium (NBT) to formazan, using PMA to stimulate the respiratory burst (Chung and Secombes, 1988).

Phagocytosis assay

Macrophage monolayers in the chamber slides were incubated with purified 26 kDa substance overnight (diluted at 85, 17, 3.4, and 0.68 ng well^{-1}). After the cells were washed twice with L-15 and the number of adherent macrophages determined (Secombes, 1990). Dried yeast (Sigma), were dissolved in saline (0.85% NaCl) (0.1 g/mℓ), and opsonised with 10% non-immune rainbow trout serum for 30 min, washed three times with fresh saline by centrifuging at 1000 g for 20 min. The yeast was resuspended in PBS and the concentration of the yeast in the suspension determined with a haemocytometer. Macrophages were incubated with the yeast cells at a macrophage: yeast cell ratio of 1 : 40, and phagocytosis allowed to proceed for 60 min at 20℃. After phagocytosis, monolayers were washed four times with saline, fixed and stained with a Rapi-Diff Staining Kit (Diachem®). The number of yeast cell in 100 macrophage per fish was determined under oil immersion. The phagocytic ratio (PR) for each fish was determined.

$$PR = \frac{\text{Number of macropahges with one or more ingested yeast cell}}{100} \times 100$$

Statistical analysis

Bacterial attachment and cell toxicity were analysed using a one-way analysis of variance or a student *t*-test. Multiple comparisons among means were made with a Turkey pairwise comparison using Minitab 11 programme where variance was consider significant at $p < 0.05$.

RESULT

Non-specific activity of the 26 kDa band

The non-specific activity of the 26 kDa band on nitrocellulose membranes containing whole cell *Ph. d.* subsp. *damsela* with a variety of substance was examined and attempts were made to block this activity when biotin/streptavidin amplication was applied to the membrane (Fig. 1). Total carbohydrate determination (lane 2) showed slightly different with lane 3, which did not performed oxidation. While streptavidin-alkaline phospatase was only used, strong band appeared (lane 4). The non specific reaction was observed by either biotin labelled anti-mouse IgG (lane 5) or biotin labelled anti-rabbit IgG (lane 6) followed by streptavidin-HRP, as well as streptavidin-HRP only (lane 7). Biotin (0.5 M) (lane 8) were able to block the activity when streptavidin-HRP was applied continually.

Cell attachment blocking test

The attachment of isolate, Sp92144, to the cell line CHSE-214 was significantly blocked by anti-*Ph. d.* subsp. *piscicdia* mouse and rabbit sera,

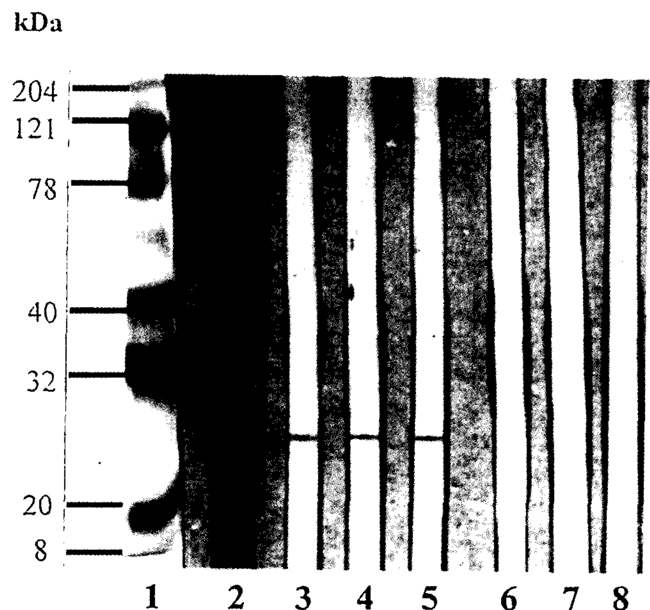

Fig. 1. Non-specific reactivity of 26 kDa band of *Ph. d.* subsp. *piscicida* in Western blot analysis.
Lanes: (1) Molecular weight standard markers; (2) Total carbohydrate determination using Glycoprotein determination kit; (3) Biotinlated anti-mouse, then streptavidin-HRP; (4) Biotinlated anti-rabbit, then streptavidin-HRP; (5) streptavidin-HRP; (6) Anti-mouse HRP; (7) Anti-rabbit HRP; (8) Biotin (0.5 M), then sterptavidin-HRP.

while these antisera did not affect the attachment of isolate I736 (Fig. 2). Both sera were raised against isolate I736. Biotin did not appear to effect the blocking of isolate I736 to the cells, but attachment of Sp92144 has a reduced ability attachment when treated with biotin. The attachment of both of strains was enhanced affects treatment with Poly-L-lysine.

Purification of the 26 kDa material from the ECP of Ph. d. subsp. damsela

Before purification, the non-specific material present at 26 kDa in concentrated ECPs on SDS PAGE was confirmed using streptavidin HRP in western blot analysis (Fig. 3). Band substance was eluted from the 2-imminobiotin beads by changing the pH of the buffer. It was not possible to detect the eluted substance at 280 nm with normal protein determination. Therefore, an ELISA was used to establish when fractions contained the eluted material. The results of the ELISA are shown Fig. 4 which represent the absorbance of each fraction in the ELISA. The main peak of activity was located between fractions 23 and 25. These factions were then pooled for further analysis.

Silver staining and the glycoprotein determination

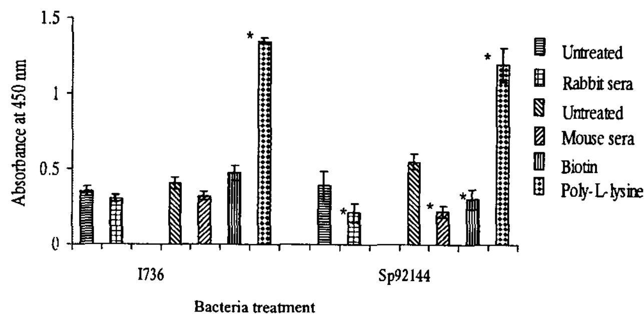

Fig. 2. The effects of pre-treatment of *Ph. d.* subsp. *piscicida* on their attachment to fish cell using a modified ELISA method. Bacteria were reacted with anti- *Ph. d.* subsp. *piscicida* rabbit sera and, while the attachment ability was measured with anti- *Ph. d.* subsp. *piscicida* mouse sera and vice versa. Biotin and Poly-L-lysin reacted bacteria was measured with anti- *Ph. d.* subsp. *piscicida* rabbit sera. Values represent means ± SD of 5 (I736) and 4 (Sp 92144) wells. Asterisks indicate significant differences between treated one and untreated bacteria at $p < 0.05$.

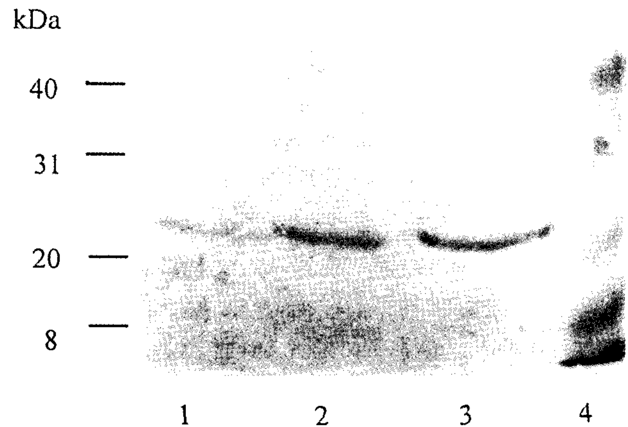

Fig. 3. Confirmation of the non-specific binding substance present in bacteria culture supernatants. Lanes (1) Concentrated supernatants 3 $\mu g\ m\ell^{-1}$; (2) 6 $\mu g\ m\ell^{-1}$; (3) 12 $\mu g\ m\ell^{-1}$; (4) Molecular Weight markers. The weights determined by a protein determination kit.

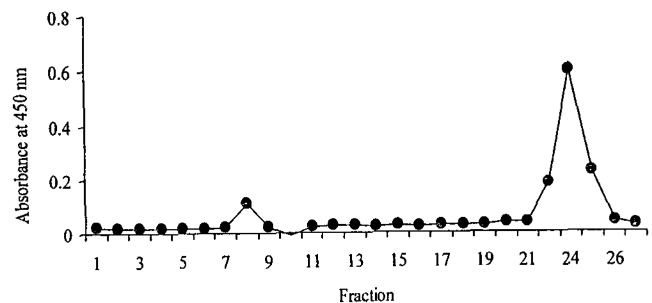

Fig. 4. ELISA to determine which fraction contained eluted material from 2-imminobiotin affinity chromatography.

kit were used to confirm the purity of the preparation. Four bands were present silver staining, the SDS-PAGE gel contained the purified substance (Fig. 5a). The upper band appeared to have molecular weight of 26 kDa. Carbohydrate determination of the glycoprotein showed the presence of only one band in the purified substance (Fig. 5b) with a molecular weight lower than 26 kDa. The concentrated ECPs did not exhibit the same molecular band as the bacteria which had a strong band at 26 kDa. The molecular weight of the purified substance were much lower than both that of the whole cell and of the concentrated ECP, with a band estimated at 12 kDa.

Cell toxicity

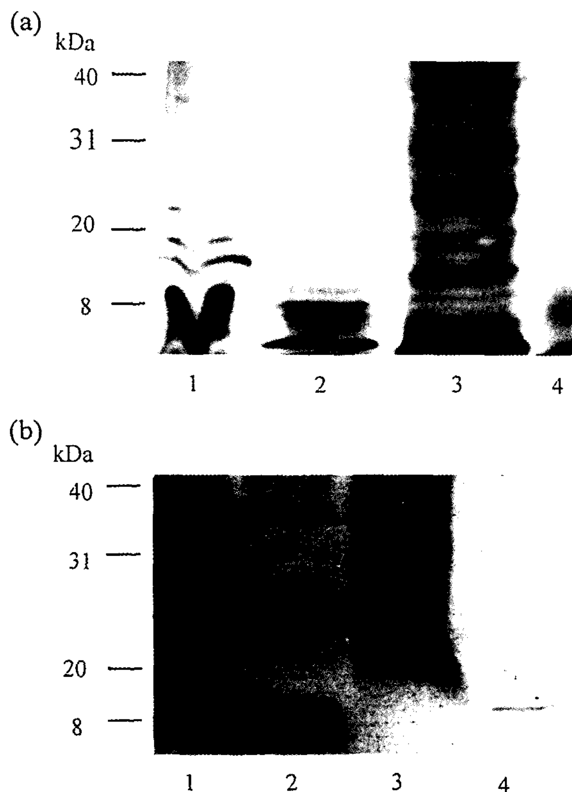

Fig. 5(a). Silver staining of substance eluted from affinity column. Lanes: (1) Purified substance (85 ng 0.1 mℓ$^{-1}$: carbohydrate concentration); (2) Concentrated supernatants (10 μg 0.1 mℓ$^{-1}$: protein concentration); (3) whole bacteria (2×10^{8} c.f.u mℓ$^{-1}$); (4) Molecular Weight markers.

(b). Carbohydrate determination of substance purified from affinity column, Lanes: (1) Molecular Weight markers; (2) whole bacteria (2×10^{8} c.f.u mℓ$^{-1}$); (3) Concen-trated ECPs (10 μg 0.1 mℓ$^{-1}$: protein concentration); (4) Purified substance (85 ng 0.1 mℓ$^{-1}$: carbohydrate concentration).

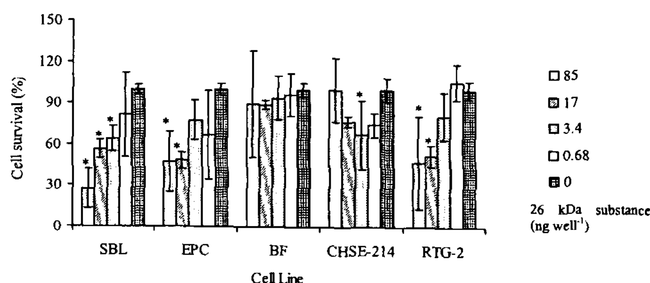

Fig. 6. Cell toxicity test (% survival) of the purified 26 kDa substance. Values represent means ± SD of 4 wells. Asterisks indicate significant differences from control well at $p < 0.05$. SBL; sea bass larvae, EPC; epithelioma papulosum cyprini, BF-2; bluegill sunfish, CHSE-214; chinook salmon embryo, RTG-2; rainbow trout gonad.

Five different cell lines were used to examine any cytotoxicity of the purified substance. Significant cell toxicity was found with cell line SBL, RTG-2 and EPC showed at 85 and 17 ng/well, while CHE-214 cell line was more sensitive to the substance with a significant cytotoxic effect observed with at 3.4 ng/well (Fig. 6). However, no toxic effect was observed with BF-2 cell line at any concentration of the substance.

Effect of substance on macrophage activity

No significant differences were observed in respiratory bust activity with rainbow trout macrophages with different concentration of the purified substance. Intracellular superoxide anion production showed similar decline with extracellular superoxide anion production. (Fig. 7a). Significant differences were observed on extracellular superoxide anion generation by the reduction of cytochrome c test. Only the concentration (lowest 0.67 ng well^{-1}) of the purified substance showed such a difference after 1 hour with negative control (medium only) and positive control (MAF). But the other concentration was not found any differences over the periods (Fig. 7b).

The head kidney macrophages phagocytosis heated with the substance were used to phagocytose more yeast cells as the concentration of the subsistence decreased. The lowest concentration, 0.68 ng well^{-1}, stimulated the macrophages to phagocytosis the highest number of yeast cells, while the highest concentration of the substance, 85 ng well^{-1}, showed levels similar to that of the control (Fig. 8).

Discussion

At present study, *Ph. d.* subsp. *damsela* was identified to react non-specifically with streptavidin in Western blotting, moreover, this substance showed a variety of characteristics in terms of cytotoxic effects and immunostimulant effects.

The band of 26 kDa was already shown by

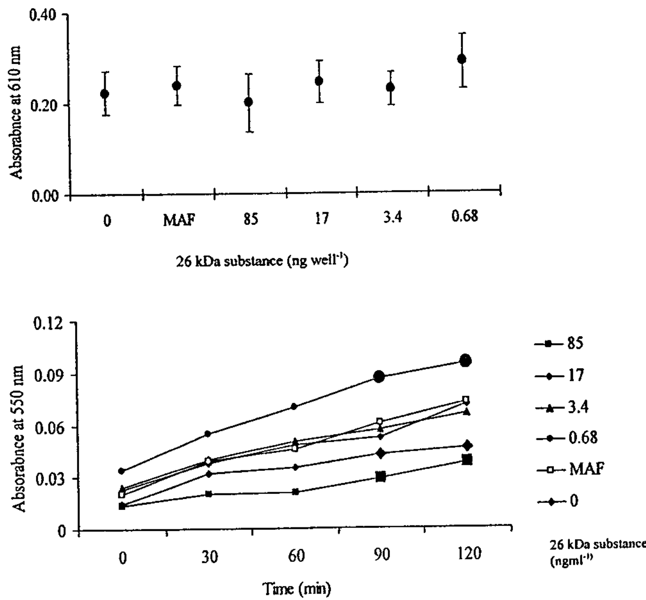

Fig. 7(a). Nitroblue tetrazolium (NBT) reduction by rainbow trout head kidney macrophages stimulated with purified 26 kDa substance. Values represent the means of 4 wells ± SD. MAF is macrophage activation factor. Wells untreated the substance were used as a negative control and wells with MAF were used as positive control.

(b). Reduction of cytochrome c by rainbow trout head kidney macrophages stimulated with 26 kDa purified substance. Values represent means of 3 wells. MAF is macrophage activation factor. Wells untreated the substance were used as a negative control and wells with MAF were used as positive control.

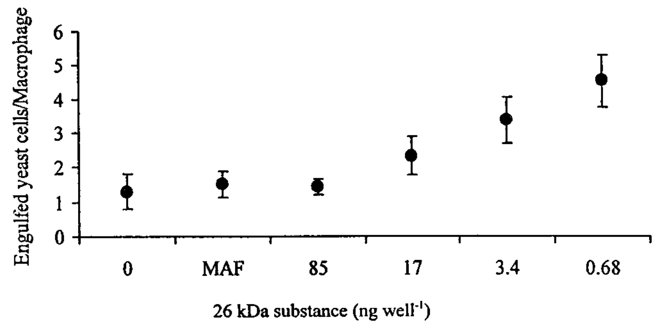

Fig. 8. Evaluation of the ability of rainbow trout macrophages to phagocytose yeast after incubation with the 26 kDa substance. Valure were the number of engulfed yeast cell in 100 macrophage 4 samples. Concentration 3.4 and 0.68 ng well^{-1} showed significant difference with maintanance medium and MAF ($p < 0.05$). MAF is macrophage activation factor.

Bakopoulos et al. (1999c), when the authors performed Western blot analysis with anti- *Ph. d.* subsp. *piscicida* sea bass sera raised against dead bacteria immunization by use of ABC system. The band was, however, recognised as one of specific band of sea bass sera against dead bacteria. It is identified a non-specific band reacted with avidin at present study.

As far as we know, no bacterial pathogen has been reported to interact with streptavidin non-specifically in fish diseases. However, the presence of endogenous biotin in retinal Mueller cells of gold fish (*Carassius auratus*) and salamaner (Bhattacharejee et al. 1997), and presence of avidin-interacting proteins in Spisula oocytes (Haneji and Koide, 1988) and cuticle of the blue crab (*Callinectes sapidus*) (Shafer et al. 1991) were reported to interact with avidine in piscine world.

Biotin has been known to be as Vitamin B6 and Coenzyme. It is a water-soluble vitamin and member of the B-complex family R (Bonjour, 1991). Avidin, a glycoprotein, found in raw egg white. It combined stoichiometrically with biotin. This egg-white protein and it's bacterial counterpart, streptavidin, have since become standard reagents for diverse detection schemes (Wilchek and Bayer, 1990).

A non-specific reaction band with avidin-HRP was appeared at estimating 26 kDa, and compared with two different conjugates, streptavidin-alkaline phospatase and sterptavidin-HRP. Initially, this band was found with Glycoprotein Determination kit (BIORAD®) (Jung et al. 2000). The relation between sialic acid band and this nonspecific band remains to be elucidated for the future.

The differences of intensity between lane 2 in parallel with biotin blocking, and then streptavidin conjugate application made it possible to suspect that a certain level of blocking was occurred but not completely. Regardless of using previous reagents, such as biotinlated anti-mouse and biotinlated anti-rabbit, the bands were appeared when streptavidin-HRP applied con-tinually.

In an attempt to block this band, three reagents

were applied to the blotted papers Poly-L-lysine (0.01%) (data not shown) and biotin (0.5 M). Poly-L-lysine was often used to pretreatment of the microplates in order to increase the electrostatic potential (Kennet, 1980). It was thought that the Poly-L-lysine could bind this band, therefore it made a structural change that the non-specific reaction would be disappeared. The bacteria capacity of binding streptavidin strongly might be involved in the pathogenesis of this bacteria as a secondary effect since avidin was known to cause vitamin B deficiency in mammalian (Boas, 1924). avidin-biotin complex was also known to be non-digestable complex so that it was not absorbed from the intestine (Eakin et al. 1941).

Two representative isolates, I736 and Sp92144, were used to examine cell attachment test. Both anti- *Ph. d.* subsp. *piscicida* rabbit sera and anti- *Ph. d.* subsp. *piscicida* mouse sera reduced very slightly the attachment ability of I736. On the contrary, biotin and Poly-L-lysin showed enhanced attachment ability. A Japanese strain, Sp92144, was reduced significantly with anti- *Ph. d.* subsp. *piscicida* rabbit sera, anti- *Ph. d.* subsp. *piscicida* mouse sera and biotin except poly-L-lysin. The sera used in this study produced from Mediterranean isolate, it might give some influences to the attachment ability between two strains, it might in turn produce such differences between strains. Biotin incubation had differences between two strains, it might be caused by differences of sialic acid structure (Jung et al. 2000). Biotin incubation also decreased a certain level of attachment for Sp 92144 but not completely. The role of biotin blocking in relation with Figure 1 made it possible to suspect that the band (26 kDa) seemed to play a certain role in attaching cells. But the other factor could not rule out, because anti- *Ph. d.* subsp. *piscicida* mouse sera could not recognise this band but decreased cell attachment ability. Poly-L-lysin assumed to evoke largely electric charge changes, this changes made both of strain enhance the attachment ability to fish cells.

The importance of extracellular polysaccharide was reported other fish bacterial pathogens, such as the polysaccharide of ECPs of *Aeromonas salmonicida* could protect against experimental challenge by virulent *A. salmonicida* (Bricknell et al. 1997), and polysaccharides from *Renibacterium salmoninarum* regarded as a major components to consider as a vaccine even if it was poor immunogens (Lynch et al. 1997). Those bacteria are able to secret or slough off the substances, this fact was made to examine whether or not the non-specific substance contained in ECPs. Surprisingly, it was able to confirm in the ECPs using sterpavidin-HRP non-specific binding. The non-specific substance is able to purify from the ECPs.

The other purpose of blocking test was to find any corresponding substance on this band which would be useful for purifying this substance. Avidin appeared to bind very tightly with the substance, however, the avidin-biotin complex has an extremely low dissociation constant so that it was very difficult to dissociate at bench (Green, 1975). Biotin, even if it seemed not to block completely, was thought to be an alternative substance instead of avidin columns. Because 2-iminobitin is used the interacts to purify (Fudem-Goldin and Orr, 1990). After passing through the column with concentrated ECPs, however, it was impossible to detect any peak using normal protein measuring methods. The substance was already understood to bind with strepavidin-HRP, this characteristics made it to change the way of detecting by ELISA for the fractions. The substance was showed peak around 24th tube. A small peak was found around 8th tube but the small peak thought to be no significant peak.

Coomassiae brilliant staining, silver staining and total carbohydrate determination was performed to elucidate the purified substance. Coomassie brilliant staining resulted in being impossible to find any bands at purified substance lane, which might be caused by insufficient concentration. Otherwise the purified substance might not consist of proteins. The results of silver staining and carbohydrate determination were able to suspect that the purified substance was made up of at least 4 different molecules which was cleaved in electrophoresis procedure, moreover one of them was contained carbohydrate branch.

The toxicity of *Ph. d.* subsp. *piscicida* ECPs for cell lines was reported by Magarinos et al. (1992). Fish and homiothermic cell lines produced, in general, degenerative changes manifested by

pyknotic nucli, clusters of round cells, shrinking, dendritic elongations and finally cell detachment. But they assayed with total ECPs, at the present study, the purified substance showed selectively different cytotoxic effects for 5 different fish originated cell lines. The sensitive cell lines also showed clusters of round cells, shrinking and cell detachment. The purified substance might have specificity for different cells. This may explain why sea bass produced antibody against in vivo grown bacteria ECPs (Jung et al. 2000), SBL showed strong toxic effects.

A number of reports was described the relation between macrophage and *Ph. d.* subsp. *damsela*. The capacity of *Ph. d.* subsp. *damsela* strains to survive contact with macrophages obtained from rainbow trout, sea bass and gilthead sea bream was evaluated using an *in vitro* assay (Skarmeta et al. 1995). Then the increased production of superoxide anion (O_2^-) by rainbow trout macrophages infected with *Ph. d.* subsp. *damsela* coinciding with the highest bactericidal activity (5 h incubation) suggested that the O_2^- could be involved in the killing of *Ph. d.* subsp. *damsela*. An increased phagocytic effect was observed after injecting O-antigen to turbot in 48 hours (Santarem et al. 1995). Figueras et al. (1997) reported a medium concentration (100 μg) of O-antigen exhibited the optimum phagocytic activity, specific antibody alone and specific antibody plus complement produced a significant results in turbot. Kawakami et al. (1997) also pointed the involvement of superoxide anion in vaccination with lipopolysaccharide-mixed chloroform-killed cells in yellowtail. Arijo et al. (1998) examined the effects of capsular bacteria for gilthead seabream and reported significant differences in phagocytosis between noncapsulated and capsulated bacteria.

On the other hand, a number of experiments were reported about immune response caused by ECPs or bacteria related substances, such as an ECPs of *Aeromonas salmonicida* (Espenes et al. 1996) and supernatants of *Vibrio anguillarum* (Joosten et al. 1996), both of the ECPs were reported to evoke immune response. The addition of *Ph. d.* subsp. *damsela* ECPs to vaccine also increased resistance for pasteurelliosis (Magarinos et al. 1994).

For the purpose of understanding the effects of the purified substance to the immune responsible cells, respiratory bust activity and yeast phagocytosis capacity was performed with rainbow trout head kidney macrophage. Fish pahgocytes have been shown to have a major role in cell mediated immunity and also capable of generating oxygen (Secombes and Fletcher 1992; Secombes, 1994). The stimulated macrophages showed high capacity to kill bacterial pathogen using reactive oxigen (Sharp and Secombes, 1993).

Reduction of NBT was focused on the ability to produce oxygen radicals (O_2^-), the quantified numbers of NBT assay might indicate the capacity of killing phagocytosed bacteria in macrophages. However, the NBT assay resulted in no significant differences between different concentrations, while extracellular superoxide anion generation measured by the reduction of cytochrome c assay showed significant difference after 1 hour incubation at the lowest concentration. This difference might be caused by the purified substance, it was soluble substance so that it might give more effective stimulation to extracellular than intracellular superoxide anion generation. But between reduction of NBT and reduction of cytochrome c assay appeared similar patterns of reaction, e. g. the highest concentration showed less activity than control but the lowest concentration exhibited the highest reaction.

The result of yeast phagocytosis confirmed that the purified substance stimulated the phagocytosis activity. This result, in parallel with NBT and Cytochrome c test, macrophages were stimulated at low concentration. When this experiment performed, the concentration was selected in arbitrary so that the appropriate concentration might not appear at this study. Rainbow trout has been known non-host fish of *Ph. d.* subsp. *Damsela,* one of reasons would be the purified substance which stimulated the non-specific immune reaction to the rainbow trout, the bacteria is in turn removed quickly.

The *Ph. d.* subsp. *damsela* had a capacity to bind with streptavidin non-specifically. The non-specific binding substance was secreted in ECPs. And the purified substance showed cytotoxic effects selectively for piscine cell lines, as well as it stimulated rainbow head kidney macrophage.

Acknowledgement: Natalio Garcia Garbi who gave advice in carrying macrophage isolation and measuring the effect of substance on macrophage activity

References

Arijo, S., J.J. Borrego, I. Zorilla, M.C. Balebona and M.A. Morinigo. 1998. Role of the capsule of *Photobacterium damsela* subsp. *piscicida* in protection against phagocytosis and killing by gilt-head seabream (*Sparus aurata,* L) macrophages. Fish and Shellfish Immunology 8, 63~72.

Bakopoulos, V., A. Adams and R.H. Richards. 1997a. The effect of iron limitation growth conditions on the cell and extracellular components of the fish pathogen *Pasteurella piscicida.* Journal of Fish Diseases 20, 297~305.

Bakopoulos, V., D. Volpatti, A. Adams, M. Galeotti and R.H. Richards. 1997c. Qualitative differences in the immune response of rabbit, mouse and sea bass, *Dicentrarchus labrax,* L. to *Photobacterium damsela* subsp. *piscicida,* the causative agent of fish Pasteurellosis. Fish and Shellfish Immunology 7, 161~174.

Bhattacharjee, J., B.N. Cardozo, W. Kamphuis, M. Kamermans and G.F. Vrensen. 1997. Pseudo-immunolabelling with the avidin-biotin-peroxidase complex (ABC) due to the presence of endogenous biotin in retinal Mueller cells of goldfish and salamander. Journal of Neuroscience Methods 77, 75~82.

Boas, M. 1924. An observation on the value of egg white as the sole source of nitrogen for young growing rats. Biochemical Journal. 18, 422~424.

Bonjour, J.P. 1991. Biotin. In *Handbook of Vitamins* (L.J. Machlin, ed) pp. 393~427. Marcel Dekker, Inc. New York.

Braun Nesje, K. Bertheussen, G. Kaplan and R. Seljelid. 19 81. Salmonid macrophages: separation, *in vitro* culture and characterisation. Journal of Fish Diseases 4, 141~151.

Braunbeck, T. 1993. Cytological alterations in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) exposed *in vitro* to 4-chloroaniline. Aquatic Toxicology 25, 83~110.

Bricknell, I.R., T.J. Bowden, J. Lomax and A.E. Ellis. 1997. Antibody response and protection of Atlantic salmon (*Salmo salar*) immunised with an extracellular polysaccharide of *Aeromonas salmonicida.* Fish and shellfish Immunology 7, 1~16.

Chung, S. and C.J. Secombes. 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comparative Biochemistry and Physiology 89, 539~544.

Diamandis, E.P. and T.K. Christopoulos. 1991. The biotin-(strept)avidin system: Principles and applications in biotechnology. Clinical Chemistry 37, 625~636.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric methods for determination of sugars and related substances. Analytical Chemistry 28, 350~356.

Eakin, R., E. Snell and R. Williams. 1941. The concentration and assay of avidin, the injury producing protein in raw egg white. Journal of Biological Chemistry 140, 535~543.

Espenes, A., C.M. Press, L.J. Reitan and T. Landsverk. 1996. The trapping of intravenously injected extracellular products from *Aeromonas salmonicida* in head kidney and spleen of vaccinated and nonvaccinated Atlantic salmon, *Salmo salar* L. Fish and Shellfish Immunology 6, 413~426.

Figureas, A., M.M. Santarem and B. Nova. 1997. Phagocytic activity of turbot (*Scophthalmus maximus*) leucocytes: opsonic effects of antibody and complemnt. Fish and Shellfish Imunology 7, 621~624.

Fudem-Goldin, B. and G.A. Orr. 1990. 2-Iminobiotin-containing reagent and affinity columns. Methods in Enzymology 184, 167~73.

Graham, S. and C.J. Secombes. 1988. The production of macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. Immunology 65, 293~297.

Green, N.M. and E.J. Toms. 1972. The dissociation of avidin-biotin complexes by guanidinium chloride. Biochemical Journal 130, 707~711.

Haneji, T. and S.S. Koide. 1988. Identification of avidin-interacting proteins in Spisula oocytes: Correlation with germinal vesicle breakdown. Journal of Experimental Zoology 246, 216~222.

Hofmann, K., S.W. Wood, C.C. Brinton, J.A. Montibeller and F.M. Finn. 1980. Iminobiotin affinity columns and their application to retrieval of streptavidin. Proceedings of the National Academy of Sciences of the USA 77, 4666~46 68.

Jones, C.J., S.M. Mosley, I.J. Jeffrey and R.W. Stoddart. 1987. Elimination of the non-specific binding of avidin to tissue sections. Histochemical Journal 19, 264~268.

Jung, T.S., K.D.Thompson and A. Adams. 2000. Identification of sialic acid on *Photobacterium damsela* subspecies *pisicida*-possible role in cell adhesion and survival in the fish host. Fish & Shellfish Immunology (in press) 10, 285.

Kawakami, H., N. Shinohara, Y. Fukuda, H. Yamashita, H. Kihara and M. Sakai. 1997. The efficacy of lipopolysaccharide mixed chloroform-killed cell (LPS-CKC) bacterin of *Pasteurella piscicida* on Yellowtail, *Seriola quinqueradiata.* Aquaculture 154, 95~104.

Kennett, R.H. 1980. Monoclonal Antibody. (R.H. Kennett, T. J. McKearn & K.B. Bechtol, eds). Plenum New York.

Kusuda, R. and K. Kawai. 1998. Bacterial Diseases of Cultural Marine Fish in Japan. Fish Pathology 33, 221~227.

Lynch, W.H., T. Barton and S.G. Griffiths. 1997. Extracellular products associated with Renibacterium salmoninarum, the causative agent of BKD in salmonids. In Proceedings of the Hunstman Marine Science Centre Symposium Coldwater Aquaculture to the Year 2000 (M.D.B. Burt & S.L. Waddy, eds).

Magarinos, B., Y. Santos, J.L. Romalde, C. Rivas, J. Barja and A.E. Toranzo. 1992. Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida.* Journal of General Microbiology

138, 2491~2498.

Magarinos, B., J.L. Romalde, Y. Santos, J.F. Casal, J.L. Barja and A.E. Toranzo. 1994. Vaccination trials on gilthead seabream (*Sparus aurata*) against *Pasteurella piscicida* Aquaculture 120, 201~208.

Noya, M., B. Magarinos, A. Toranzo and J. Lamas. 1995. Sequential pathology of experimental pasteurellosis in gilthead seabream *Sparus aurata*. A light- and electron-microscopic study. Disease of Aquatic Organisms 21, 177~186.

Pick, E. and D. Mizel. 1981. A rapid microassays for the measurement of superoxide and hidrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. Journal of Immunological Methods 46, 211~226.

Rao, A.J. and R. Chakraborti. 1989. Studies on non-specific interference due to serum in the avidin-biotin microELISA for monkey chorionic gonadotropin and a method for its elimination. Journal of Immunological Methods 125, 261~164.

Rodgers, C.J. and M.D. Furones. 1998. Disease Problems in Cultured Marine Fish in the Mediterranean. Fish Pathology 33, 157~164.

Santarem, M.M. and A. Figueras. 1995. Leucocyte numbers and phagocytic activity in turbot Scophthalmus maximus following immunization with Vibrio damsela and Pasteurella piscicida O-antigen bacterins. Disease of Aquatic Organisms 23, 213~220.

Secombes, C.J. 1990. Isolation of salmonid macrophages and analysis of their killing activity. In *Techniques in Fish Immunology*. (J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Robertson & W.B. Van Muiswinkel, eds) pp. 137~154. SOS Publications, Fair Haven, USA.

Secombes, C.J., S. Chung and A.H. Jeffries. 1988. Superoxide anion production by rainbow trout macrophages detected by the reduction of ferricytochrome c. Developmental and Comparative Immunology 12, 201~206.

Secombes, C.J. 1994. Enhancement of fish phagocyte activity. Fish and Shellfish Immunology 4, 421~4636.

Secombes, C.J. and T.C. Fletcher. 1992. The role of phagocytes in the protective mechanisms of fish. Annual Review of Fish Diseases 2, 53~71.

Shafer, T.H., R.D. Roer and R.M. Dillaman. 1991. Changes in glycoprotein composition of the exoskeleton of Callinectes sapidus at the time of ecdysis. American Zoologist 31, 119.

Sharp, G.J.E. and C.J. Secombes. 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish and Shellfish Immunology 3, 119~129.

Skarmeta, A.M., I. Bandin, Y. Santos and A.E. Toranzo. 1995. In vitro killing of *Pasteurella piscicida* by fish macrophages. Disease of Aquatic Organisms 23, 51~57.

Snieszko, S.F., G.L. Bullock, E. Hollis and J.G. Boone. 1964. *Pasteurella* sp. from epizootic of which perch (*Roccus americanus*) in Chesapeake Bay tidewater areas. Journal of Bacteriology 88, 1814.

Wilchek, M. and E.A. Bayer. 1990. Avidin-biotin mediated immunoassays: overview. Methods in Enzymology. 184, 467~469.