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# EFFECTS OF HEAT EXTRACT FROM FIREFLY SQUID, *Watasenia scintillans*, ON THE NONSPECIFIC DEFENCE MECHANISMS AND PROTECTION AGAINST FURUNCULOSIS IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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A B S T R A C T. Influence of heat extract from firefly squid (FSE-1) on the nonspecific cellular and humoral defence mechanisms in rainbow trout experimentally infected with *Aeromonas salmonicida* was examined. After application of FSE-1 by intraperitoneal injection or immersion, phagocytic ability of neutrophils, respiratory burst activity and potential killing activity of blood phagocytes, lysozyme activity and total Ig level in plasma were measured. One week after these fishes were inoculated with live and virulent *Aeromonas salmonicida* by intraperitoneal injection, the same kinds of parameters were measured and compared with the control. The results showed that FSE-1 applicated by immersion or injection significantly ($p<0.05$) increased the phagocytic ability of neutrophils, respiratory burst activity and potential killing activity of phagocytes, lysozyme activity and total Ig level in serum, compared to the control. After artificial infection, all of the immunological parameters of both injected or immersed fishes with FSE-1 significantly increased compared with the control. These results indicate that FSE-1 administration accelerates nonspecific cellular and humoral defence mechanisms and protection against furunculosis in rainbow trout.

Key words: FIREFLY SQUID, DEFENCE MECHANISMS, PHAGOCYTES, LYSOZYME, RAINBOW TROUT

## INTRODUCTION

The use of immunostimulants in intensive fish culture for the prevention of infectious diseases is a promising new development in aquaculture (Anderson et al. 1989, Siwicki 1989, Siwicki and Anderson 1990, Anderson 1992). The classic chemotherapeutics include antibiotics for treating fish diseases, are therefore frequently used to control the bacterial diseases, an approach that is expensive, provides only short term

benefit, and risks generating antibiotic-resistant strains of the causative bacterium in water. While each chemotherapeutant is at least partially effective in the treatment of a particular disease, problems arise with accumulation of these substances in the environment as well as the emergence of resistant pathogenic strains when using antibiotics. These shortcomings, combined with a strong popular sentiment against the use of antibiotics in aquaculture or in food fish continue to make the developing of an effective nonspecific protection by immunostimulants or specific protection by vaccines quite important. But some of the protection after application of vaccines are not effective in the practice (Ellis 1988). The immunostimulants comprise a group of natural and synthetic compounds that enhance (modulate) the nonspecific cellular and humoral defence mechanisms and protection against diseases. Several type of immunostimulants such as levamisole (Siwicki 1987, Siwicki and Anderson 1990), beta-glucans (Yano et al. 1989), trace mineral and vitamin combinations (Anderson 1992), and various products derived from many plant and animal sources (Anderson 1992, Siwicki et al. 1994, Siwicki et al. 1996) are stimulating the cellular and humoral defence mechanisms and protection against viral and bacterial diseases when given alone, without benefit of a vaccine.

Although the development of specific humoral immune response for *Aeromonas salmonicida* is a typical response to exposure to the pathogen, the presence of these factors by antibodies is not necessarily associated with protection. Protection against *A. salmonicida* in fish is more closely associated with the cellular defence mechanisms than with the humoral specific immune response (Smith et al. 1980).

In the present study , we continue the experimental investigation on the influence of heat extract from firefly squid (*Watasenia scintillans*) on the cellular and humoral defence mechanisms against furunculosis in rainbow trout.

## MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) from a disease-free population, mean body weight 100 g, were used in this experimental study. The fish were kept in plastic tanks with running water, at temperature 12°C, and were fed once daily with commercial pellets containing 42% protein. During the 2 weeks acclimation period the fish were divided into six groups 50 individuals in each.

The heat extract from firefly squid (FSE-1) was purchased from Ise Sea Food Company Namerikawa (Japan). FSE-1 was prepared for intraperitoneal injection and im-

mersion by dr Tougo Miyazaki from TFRI Namerikawa (Japan). Four groups of fishes (50 fish per group) were held in separate tanks and given either a single intraperitoneal injection of FSE-1 (0.5 ml/fish), immersion in 25 % solution of FSE-1 for 30 min, or intraperitoneal injection of PBS (0.5 ml/fish) and immersion in FSE-1-free water as the control respectively. The days 7 after administration of FSE-1, the immunological parameters were examined in 10 fishes from each experimental or control group, and 20 fishes in each group were given single intraperitoneal injection of 0.2 ml of live and virulent *Aeromonas salmonicida* suspension in PBS ($1 \times 10^7$ bacteria/ml). The immunological parameters were examined at day 7 after the experimental infection.

The phagocytic ability of neutrophils was examined using the NBT-spectrophotometric oxidative radical production assay with modification of the method as outlined by Sigma and adapted to fish cells by Siwicki et al. (1993). Respiratory burst responses have been measured in peripheral blood phagocytic cells, isolated by adherence, by modification of the method presented by Chung and Secombes (1988). For the detection of intracellular $0_2^-$, the NBT is dissolved in RPMI-1640 medium (Sigma), and phorbol myristate acetate (PMA, Sigma) added at 1 µl/ml of NBT solution. A modification by Siwicki and Anderson (1993) of the technique presented by Rook et al. (1985) was used to measure potential killing activity of blood phagocytic cells. After removal of the non-adherence cells, a 0.2 % NBT in PBS solution containing *Enterococcus seriolicida* cells ($1 \times 10^7$ bacteria/ml) was used for this study.

The lysozyme activity in plasma was measured using the turbidimetric assay (Studnicka et al. 1986) and total plasma immunoglobulin (Ig) was determined by spectrophotometric assay based on the burette method which detects amount of nitrogen in amino acids, with modification of micro-methods by Siwicki and Anderson (1993).

For statistical analysis, means and standard deviations for all test values were calculated and Student's *t*-test was used to determine whether there were differences between control and experimental groups (statistically significant at $P<0.05$).

## RESULTS AND DISCUSSION

In the first part of this study, 7 days after application of FSE-1 by injection or immersion, the immunostimulating effects on the nonspecific cellular and humoral parameters were observed. The influence of FSE-1 administered by injection or immersion on neutrophil oxidative radical production (NBT reduction) are presented in Fig. 1, on respiratory burst activity of blood phagocytes - in Fig. 2, on potential killing activity of blo-

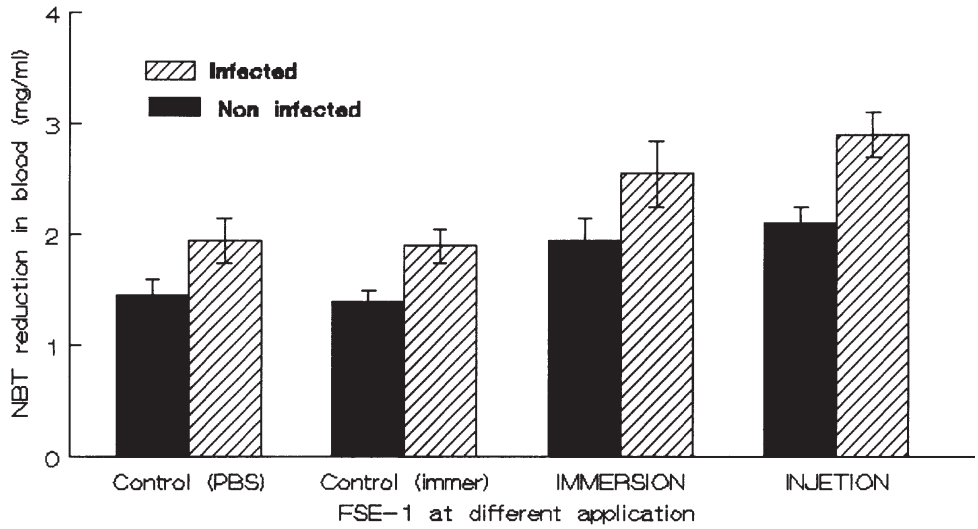


Fig. 1. Effects of FSE-I applicated by immersion or injection on the NBT reduction in blood of non-infected and infected rainbow trout (mean and SD; n=10)

od phagocytes - in Fig. 3, on lysozyme activity in plasma in Fig. 4, and on total Ig level in plasma - in Fig. 5. The application of FSE-1 by immersion and injection statistically significantly increased all immunological parameter, compared to the control groups, but the higher activity of cellular and humoral parameters after administration of FSE-1 by injection was observed.

In the second part of this study, wherein fishes of each group were experimentally infected, the significantly higher nonspecific cellular and humoral immune response were observed in FSE-1 groups of fish (immersion and injection) compared to the control. Production of oxygen radicals by neutrophils as measured by NBT reduction (Fig. 1), respiratory burst activity of blood phagocytes (Fig. 2) and potential killing activity of phagocytic cells (Fig. 3) were significantly higher in FSE-1 applied groups by immersion and injection than the control groups. Also the lysozyme activity (Fig. 4) and total Ig levels (Fig. 5) were statistically significantly elevated in FSE-1 applied groups by immersion or injection compared to the fish from control groups. But the injection again showed the greatest increases.

In our previous *in vitro* study, we tried to observe immunomodulatory influence of FSE-1 and DFSE-1 on the two major functions of the immune system in fish, *i.e.* the mechanisms of lymphocyte proliferation and the response of phagocytes (Siwicki et al. 1996). Both FSE-1 and DFSE-1 showed immunomodulatory influen-

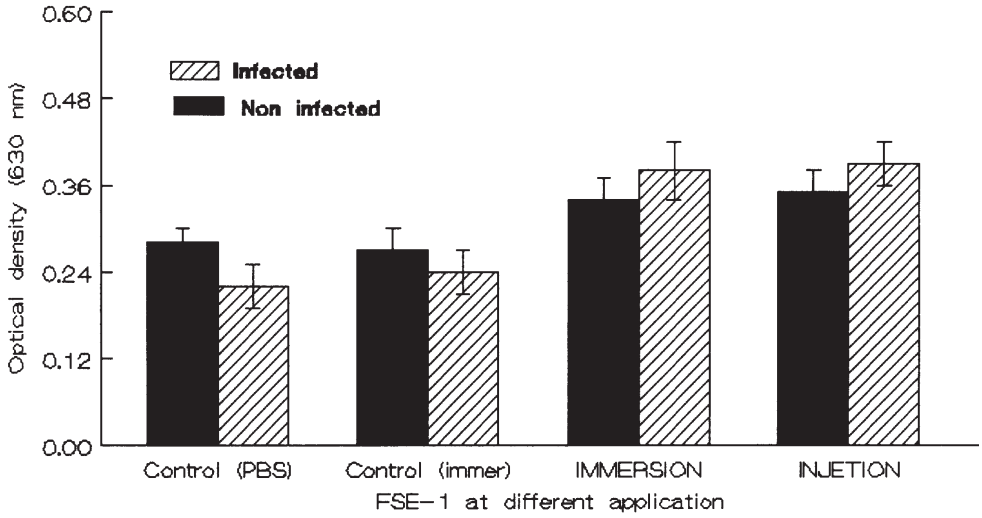


Fig. 2. Effects of FSE-1 applicated by immersion or injection on the respiratory burst activity of blood phagocytes in non-infected and infected rainbow trout (mean and SD; n=10)

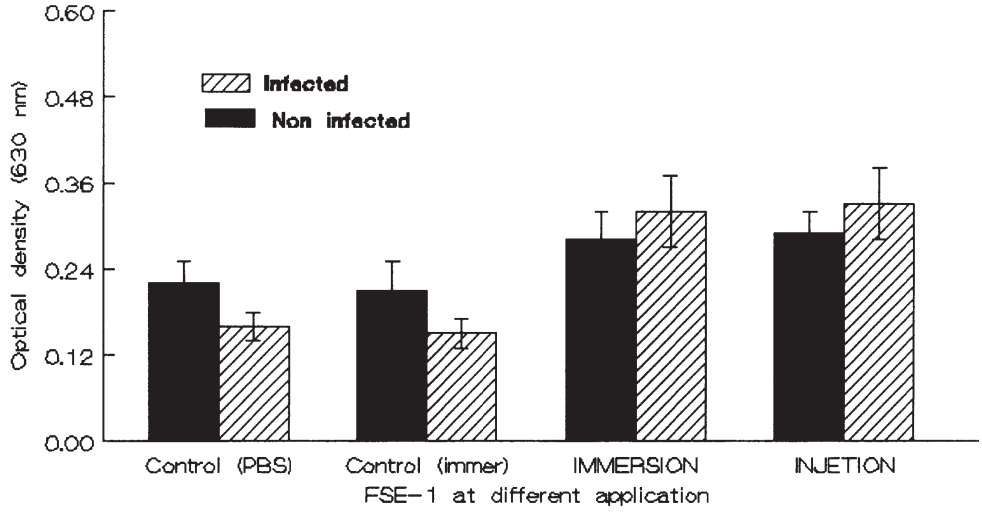


Fig. 3. Effects ofFSE-I applicated by immersion or injection on the potential killing activity of blood phagocytes in non-infected and infected rainbow trout (mean and SD; n=10)

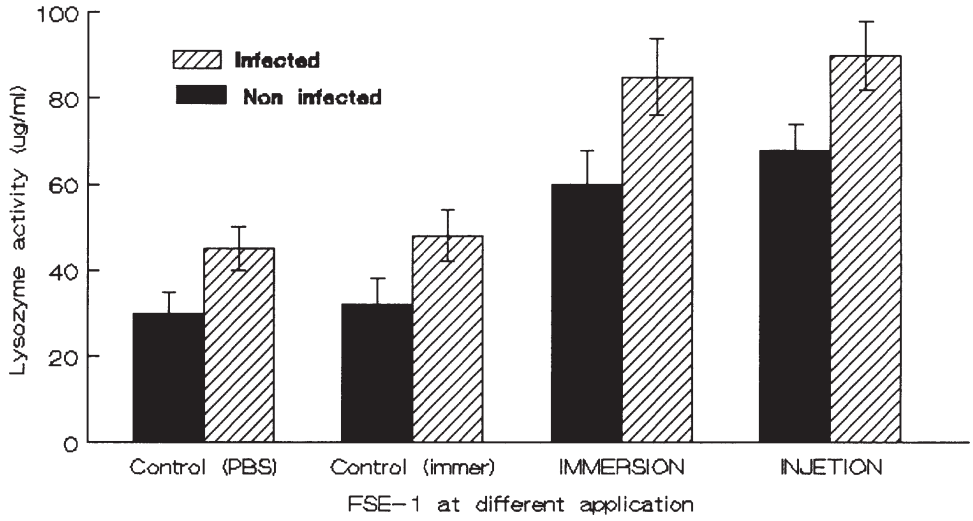


Fig. 4. Effects of FSE-1 applicated by immersion or injection on the lysozyme activity in plasma of non-infected and infected rainbow trout (mean and SD; n=10)

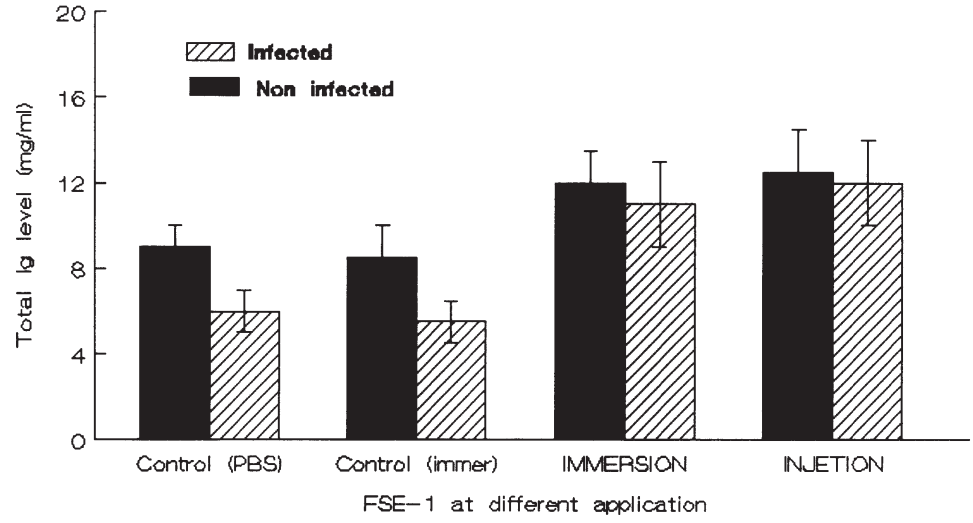


Fig. 5. Effects ofFSE-1 applicated by immersion or injection on the total Ig levels in serum of non-infected and infected rainbow trout (mean and SD; n=10)

ce *in vitro* on blood phagocytes. Also the stimulatory influence of FSE-1 and DFSE-1 at different concentrations on the lymphoblastic transformation were observed after stimulation with T and B lymphocyte mitogens. *In vitro* results showed that FSE-1 and DFSE-1 may be utilised *in vivo* for the enhancement of defence mechanisms in fish.

In the present *in vivo* study, we show that FSE-1 stimulates the nonspecific cellular and humoral defence mechanisms and increases or potentiates the nonspecific immune response after experimental infection with *A. salmonicida*. This observation suggests that this natural product may be applied to for enhance the protection against bacterial and viral diseases in fish.

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## STRESZCZENIE

WPŁYW GORĄCEGO EKSTRAKTU ZE ŚWIETLIKA KAŁAMARNICY, *Watasenia scintillans*, NA MECHANIZMY OBRONNE I ODPORNOŚĆ PRZECIWKO FURUNKULOZIE U PSTRĄGA TĘCZOWEGO (*Oncorhynchus mykiss*)

Celem badań było określenie wpływu ekstraktu uzyskanego ze świetlika kałamarnicy na nieswoiste komórkowe i humoralne mechanizmy obronne i odporność na zakażenie patogennymi bakteriami *Aeromonas salmonicida* u pstrąga tęczowego. Ekstrakt z kałamarnicy (FSE-1) podawano rybom w iniekcji dootrzewnowo oraz w immersji przez zanurzenie w roztworze FSE-1. Uzyskane wyniki badań wykazały że FSE-1 podany zarówno w iniekcji, jak i w kąpieli aktywuje komórki fagocyterne oraz powoduje wzrost poziomu lizozymu i całkowitego poziomu immunoglobulin w surowicy. Równocześnie obserwowano wzrost odporności nieswoistej na zakażenie patogennymi bakteriami *A. salmonicida* u pstrąga tęczowego .

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