ENHANCED ANTI-PROTEASES IN *LABEO ROHITA* FED WITH DIET CONTAINING HERBAL INGREDIENTS

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ABSTRACT

Aqueous root extract of *Achyranthes aspera* was incorporated in the experimental diet of *Labeo rohita* (rohu). Control diet was prepared without root extract. Feeding of fishes with experimental diet has significantly ($p < 0.05$) enhanced the serum anti-proteases level than fishes fed with control diet.

KEY WORDS

Labeo rohita, *Achyranthes aspera*, Anti-proteases

INTRODUCTION

Anti-proteases are one of the components of non-specific immunity of the vertebrates. Several pathogens invade the host and obtain their nutrients by using their extracellular enzymes. The serum of vertebrates contains a number of substances, which have anti-enzyme activity, and therefore have a role in defending the body against pathogens by inhibiting their extracellular enzymes. The inhibition of serine proteinases by mammalian serum is mainly due to $\alpha 1$ -proteinase inhibitor (1). Fish plasma contains a number of protease inhibitors, principally $\alpha 1$ -anti-protease, $\alpha 2$ -anti-plasmin and $\alpha 2$ macroglobulin, which play a role in restricting the ability of bacteria to invade and grow *in vivo* (2). Anti-proteases analogous to $\alpha 1$ -anti-protease ($\alpha 1$ -anti-trypsin) have been identified and isolated from cod *Gadus morhua* (3). A homologue of the human $\alpha 2$ -macroglobulin was demonstrated in the plasma of plaice *Pleuronectes platessa* (4) and in rainbow trout *Oncorhynchus mykiss* (5). $\alpha 2$ -macroglobulin was isolated and characterized in plaice (6). A trypsin proteinase inhibitor has been purified from the skeletal muscle of white croaker, *Micropogon opercularis* (7). Inhibition of trypsin activity is the most convenient way of measuring anti-protease activity of the serum.

The plant *Achyranthes aspera* (Family:

Amaranthaceae) has the ability to stimulate the immunity in mice (8). An experimental fish diet was prepared by incorporating the root extract (0.5%) of *A. aspera*. The efficacy of this diet on the immunity of Indian major carp *Labeo rohita* was tested in the present study.

MATERIALS AND METHODS

Animals

Labeo rohita, rohu of same age were obtained from fish market, Delhi and were acclimatized in outdoor condition for three weeks. Fish (average weight: 200 ± 17 g) were divided into different groups, under two feeding schemes, with four fishes in each group. Water temperature ranged from 26 to 30°C and dissolved oxygen level was maintained 5 mg/L throughout the culture period.

Normal diet and experimental diet

Normal artificial diet (30% protein) was prepared using fishmeal, wheat flour, cod liver oil and vitamin premix as ingredients. In experimental diet, aqueous root extract of *Achyranthes aspera* (0.5%) was incorporated, in addition to the above mentioned normal diet (Table 1). Test groups of fishes were fed with experimental diet and control groups were fed with normal diet. Feeding started from four weeks prior to the immunization and continued till the end of the experiment at the rate of 1% of body weight.

Immunization of fish

All groups of fish were injected intraperitoneally with 500 μ l of 20% suspension of c-RBC in phosphate buffered saline using a syringe with 28G needle.

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Table 1 Composition of artificial diets

Ingredients/ 100 g of feed	Experimental diet	Control diet
Fish meal (g)	32.88	32.88
Wheat flour (g)	55.12	55.12
Cod liver oil (g)	10	10
Vitamin and Mineral premix (g)	2	2
Plant root extract (0.5%)	Present	Absent

Sampling

Fishes were anaesthetized with MS-222 (tricaine methanesulfonate, Sigma chemicals) 1:10,000 in dechlorinated water. Blood was collected from fishes by cutting the tail on first, second, third and fourth week, i.e. 7th, 14th, 21st and 28th day after immunization and allowed to clot at room temperature. Serum was obtained by centrifugation and was stored in plastic vials at -20°C for further use.

Assay of serum anti-trypsin activity

Serum anti-protease activity was performed (3) by incubating 10 µl of serum with 20 µg of trypsin dissolved in 100 µl of Tris-HCl (50 mM, pH 8.2). In serum blank, 100 µl of Tris-HCl was added to 10 µl of serum, instead of trypsin in Tris-HCl, and in the positive control no serum was added to trypsin. All tubes were made upto 200 µl with Tris-HCl and incubated for 1 hour at room temperature. After the incubation, 2 ml of 0.1 mM substrate BAPNA (Na-benzoyl-DL-arginine-*p*-nitroanilide HCl, Sigma chemicals), dissolved in Tris-HCl (containing 20 mM calcium chloride), was added to all tubes and incubated for further 15 minutes. At the end of incubation, the reaction was stopped by adding 500 µl of 30% acetic acid. The optical density was measured at 410 nm by using UV-Visible spectrophotometer (Shimadzu UV-1601). The percentage trypsin inhibition was calculated from the following formula:

Trypsin inhibition (%) = $(A1-A2/A1) \times 100$; where A1 = control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum.

RESULTS AND DISCUSSION

The percentage inhibition of trypsin activity was shown in Fig. 1. After immunization, the levels of

Fig. 1. Fish were immunized with chicken RBC. Control groups of fish were fed with normal diet and test groups were fed with experimental diet, four weeks prior to immunization till the end of the experiment. Serum was collected from individual fishes for four weeks after immunization. The percentage trypsin inhibition for each individual serum was determined. The values represented were the mean $\pm$ SE of four fishes. * $P < 0.01$

protease inhibitors were high on the first week (test group: 91.73 ± 0.052 ; control group: 89.675 ± 0.274) and then the levels were gradually decreased towards the fourth week (test group: 87.565 ± 1.499 ; control group: 79.906 ± 2.038). Though the serum of test groups exhibited more anti-protease activity than the control groups throughout the study period, the difference was significant on first week and third week ($p < 0.05$). In control group, the serum anti-protease activity was 89.68% on first week after immunization and it drastically decreased to 79.91% on the fourth week, whereas in test group the serum anti-protease activity was 91.73% on the first week and slightly decreased (87.56%) on the fourth week. This indicated that the serum of the test group of fishes, which were fed with experimental diet containing *A. aspera*, maintained high level of protease inhibitors even after four weeks of infection (antigen injection) than the control group. The specific immunity of fishes was also enhanced when fed with the experimental diet containing *A. aspera* than fed with the normal diet. The antigen-specific antibody levels, serum globulin levels and RNA/DNA ratios were enhanced in *L. rohita* when fed with the experimental diet. These results collectively

confirmed that along with the specific-immunity the non-specific immunity was also enhanced in *L. rohita* when fed with experimental diet containing *A. aspera*. Similar results were found on oral administration of oxytetracycline to turbot *Scophthalmus maximus*, which produced a significant increase in the plasma bactericidal activity and total protein concentration (9). Similarly both the non-specific and antigen-specific defense mechanisms were elevated in common carp *Cyprinus carpio* (10) and in rainbow trout *Oncorhynchus mykiss* (11) when fed with levamisole through diet. Evidence showed that protease inhibitors can selectively arrest replication of microbial pathogen without untoward toxicity to the host (12). When fed with the *Achyranthes aspera* mixed diet, the protease inhibitor levels were enhanced in *Labeo rohita*, thus the host can defend more strongly against invading pathogens.

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