

Lysozyme activity of grass carp (*Ctenopharingodon idella*) following exposure to sublethal concentrations of organophosphate, diazinon

Soltani, M.^{*1}, Pourgholam, R.²

¹Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran- Iran. ²Fisheries Research Institute of Iran, Tehran-Iran.

Abstract: Lysozyme activity of grass carp (*Ctenopharingodon idella*) weighing 850 g were assessed following exposing fish to various concentrations of diazinon at 1, 2 and 4mg/L provided as a bath for 12 hours at 20–22°C. Compared with the control fish there was no significant difference in levels of lysozyme in tissues of spleen and kidney of experimental fish after one day post-exposing to the toxicant ($p>0.05$), whilst lysozyme contents in sera of experimental fish were significantly lower than control fish ($p<0.05$). After 7 day-post-exposing fish to toxicant, the lysozyme contents in both spleen and kidney of fish exposed to 2 and 4 mg/L of toxicant were significantly higher than control fish ($p<0.05$). Also, compared with control group, no significant difference was observed in lysozyme content in sera and tissues of kidney and spleen of experimental fish 15, 30 and 45 days post-exposure ($p>0.05$). These results show that diazinon at sublethal concentrations stimulate some non-specific immune defence mechanisms of grass carp through enhancing of lysozyme content in hematopoietic tissues and blood serum. *J.Vet.Res.* 62,2:49–52,2007.

Key words: Diazinon, grass carp, lysozyme.

Introduction

Nonspecific defence mechanisms are eminently important in fish because of possessing fewer complex specific immune capabilities than higher vertebrates (Ingram, 1980; Rijkers, 1982; Anderson and Zeeman, 1995; Kozinenko *et al.*, 1999; Rice, 2001). Adequate works have been carried out to study the antibacterial and opsonin stimulation properties of lysozyme as a major factor of natural resistance or innate immunity in fish (Fletcher and White, 1976; Murrery and Fletcher, 1976; Studnicka *et al.*, 1986; Siwicki and Studnicka, 1987; Grinde, 1988; Grinde *et al.*, 1988; Mock and Peters, 1990; Itami *et al.*, 1992; Paulsen *et al.*, 2001; Mikryakov *et al.*, 2002). However, limited data are available concerning the role of this enzyme to unfavourable environmental

conditions or in pathological and adaptive processes under such stresses. Diazinon is one of major herbicidal/insecticidal chemicals currently used in both North and South Iran where grass carp are mainly grown. The aim of this study was to determine the effect of various concentrations of diazinon on the lysozyme activity of grass carp in order to evaluate the fish immunity after exposing to such toxic chemical.

Materials and Methods

Fish: One hundred twenty grass carp (*Ctenopharingodon idella*) weighting 850 g from Mazandran province fish farms were used for the experiments. Fish were held in 1200L tanks containing 15 fish each tank with continuing water flow at 4L/minute at 20–22°C, dissolved oxygen of 7.7 mg/L and pH 7.5. Fish were acclimated to new conditions one week prior to the experiments. Fish were fed with fresh

*Corresponding author's email: msoltani@ut.ac.ir, Tel: 021-66427517, Fax: 021- 66933222



vegetables consisting of lucerne, clover and lettuce.

Application of Diazinon: Diazinon (Maccidal EC 600) in the form of 60% emulsion was applied to the water of each tank at concentrations of 1, 2 and 4 mg/L as a bath for 12 hours at 20–22°C. Fish were then transferred to clean water and were kept separately for 45 days. Control group were kept in clean water separately.

Sample Collection and Assay: Samples were collected after 1, 7, 15, 30 and 45 days post exposure to the toxicant. Five fish per treatment were used at each sampling time. Blood was obtained by cutting fish tail after stunning with a sharp blow to the head and removing the scales and scraping the tail with alcohol.

In order to assay lysozyme content, 3–4 ml blood was collected into sterile tubes, after 1 hour at room temperature, blood samples were centrifuged at 7000g for 10 minutes and separated sera were frozen at -20°C until used for lysozyme assay within 3 days post sampling. Weighted amounts of <1.0 g of tissue samples consisting of spleen and kidney were immediately obtained, homogenized with one part (W/V) of sterile sodium phosphate buffer (PBS) (0.004M, pH 6.2), incubated at room temperature for one hour and subjected to three cycles of freeze-thaw to extract additional lysozyme from the samples. The tissue samples were then centrifuged at 7000g for 30 minutes and the supernatants were used for lysozyme assay. Lysozyme level in blood and tissue samples was determined by the turbidimetric assay according to the method described by Ellis (1990) with slight modifications. Briefly, aliquots (1.75 ml) of *Micrococcus lysodeikticus* suspension (Sigma) (0.375 mg/ml, 0.05 M sodium phosphate buffer, pH 6.2) was mixed with 250 µL of each sample and the optical density was measured after 15 and 180 seconds by spectrophotometer (Biophotometer Eppendorf) at 670 nm wavelength. PBS was used as the blank and results were expressed in amounts of lysozyme (µg) per one milligram of sample calibrated using a standard curve determined with hens egg white lysozyme (Sigma) in PBS.

Data were statistically analysed using Spss and Anova software (t-test) and values were significant as $p < 0.05$.

Results

Results of lysozyme contents in sera and tissues of spleen and kidney samples of fish exposed to various concentrations of diazinon are shown in Table 1. Compared with control fish the level of lysozyme in tissues of spleen and kidney was significantly higher in fish exposed to 2 and 4 mg/L diazinon 7 days post-exposure ($p < 0.05$). Also, significantly lower level of lysozyme was detected in sera of fish exposed to all concentrations of toxicant only one day post-exposure ($p < 0.05$). Also, compared with control group, no significant difference was observed in lysozyme content in sera and tissues of kidney and spleen of experimental fish 15, 30 and 45 days post-exposure ($p > 0.05$). The highest and the lowest reactions to diazinon on the average level of lysozyme were recorded in spleen and sera samples of fish exposed to 4 mg/L of toxicant 7 and 1 day post-exposure, respectively.

Discussion

Lysozyme available in the lysosomes of neutrophils and macrophages, is secreted into the blood by these cells (Goldstein *et al.*, 1975; Murray *et al.*, 1976; Gallin, 1982; Mock *et al.*, 1990) and can function in the blood or intercellular spaces of the tissue following their destruction possibly under the influence of stressor-localized factors as reported in mammals (Fänge, 1984; 1986; Weeks and Warinner, 1984; Florensov and Pestova, 1990; Zapata *et al.*, 1996, Kozinenko *et al.*, 1999). The highest level of lysozyme in spleen and kidney tissues following 7 days of exposing grass carp to sublethal concentrations of diazinon may be indicative of an increase in the percentage of segmented forms of neutrophils in the blood as shown by differential counts of leucocytes (data not shown). The strong reaction in spleen tissue in response to the toxicants was considered in studies on the influence of sublethal concentrations of some heavy metal salts consisting of Hg, Cd and Cu on the contents of



Table 1. Lysozyme contents ($\mu\text{g}/\text{mg}$ or ml) in spleen, kidney and serum of grass carp exposed to diazinon a $20\pm 2^\circ\text{C}$, ($n=5$, Mean \pm SEM).

Organ sampled	Sampling time (day)	Diazinon concentration (mg/l)			Control
		1	2	4	
Spleen	1	6.15 – 0.06	6.22 – 0.13	5.95 – 0.07	6 – 0.28
	7	7.95 – 0.06	8.6 – 0.08 *	9.35 – 0.11 *	7.09 – 0.2
	15	6.4 – 0.15	6.74 – 0.05	6.8 – 0.02	6.9 – 0.05
	30	5.5 – 0.03	6.1 – 0.04	6 – 0.04	6.1 – 0.16
	45	6.5 – 0.16	6.4 – 0.02	6.5 – 0.03	6.5 – 0.05
Kidney	1	3.9 – 0.04	3.8 – 0.02	3.8 – 0.02	4 – 0.28
	7	4.9 – 0.06	6.1 – 0.08 *	6.1 – 0.13 *	4 – 0.28
	15	4.8 – 0.04	4.9 – 0.02	4.9 – 0.03	5 – 0.04
	30	5.02 – 0.03	4.5 – 0.04	4.8 – 0.1	5 – 0.1
	45	6 – 0.04	5 – 0.24	6.2 – 0.05	6 – 0.05
Serum	1	2 – 0.2 *	1.47 – 0.02 *	1.07 – 0.04 *	10.54 – 1.2
	7	12.25 – 0.02	12 – 0.05	11.77 – 0.1	12.34 – 0.06
	15	13.94 – 0.13	14.4 – 0.07	14.6 – 0.07	14.56 – 0.98
	30	12.2 – 0.02	11.72 – 0.25	12.07 – 0.16	12.32 – 0.06
	45	12.07 – 0.04	12.1 – 0.04	12.13 – 0.03	12.2 – 0.05

* Indicating values are significant at $p < 0.05$.

lysozyme in the tissues of sturgeon fingerlings (*Acipenser baeri*) by Mikryakov *et al.*, (2002). Moreover, the rise in the levels of lysozyme in spleen tissues of fish may be due to an increasing number of lysozyme producing cells of the myeloid tissues, in particular non-segmented and segmented neutrophils as were demonstrated by other authors (e.g. Mikryakov and Lapirova (1997). It is assumed that this enzyme participates in the support of homeostasis during adaptation to unexpected environmental changes (Marc *et al.*, 1995; Charles and Rice, 2001). A high activity of this enzyme has been found in lymphomyeloid tissue of cartilaginous fish as well as in lymph and plasma of bony fishes (Fange, 1984; Øysein *et al.*, 1989; Anderson and Zeeman, 1995). In mammals, during periods of high stress, lysosome exocytosis from macrophages and neutrophils can be altered by action of the stress hormones, in particular hydrocortisone as mentioned by Mock *et al.*, (1990). The present results propose that the discharge of different amounts of lysozyme in response to toxic stress, possibly mediated via hydrocortisone, may also occur in fish. Also, Mock *et al.*, (1990) concluded that the activity of this enzyme presents the modulatory action of the defence system of the organism, relying not only on the nature, but

also concentration or strength of stress factor. Therefore, weak influences occur, for example 30 minute handling can result in both decrease and increase in the level of serum lysozyme, while a strong stress, for example the acute exposure of fish to ammonia ions, decreases the level of this enzyme. Therefore, the comparison of these data on concentrations of lysozyme in tissues and serum of grass carp shows that lysozyme level depends on the effect of various factors including diazinon concentrations. Also, these data show that the lysozyme content in serum and tissues of grass carp were variable and the fluctuations were defined by duration of sampling and concentration of toxicant.

Acknowledgment

This work was financially supported by the research council of University of Tehran and Iranian Fisheries Research Institute.

References

1. Charles, D., Rice, C.D. (2001) Fish immunotoxicology, understanding mechanisms of action. In: Target Organ Toxicity in Marine and Freshwater Teleosts, volume Z- systems; Edited by: Daniel Schlenk and William H. Benson. pp. 97-108.



2. Ellis, A. E. (1990) Lysozyme Assays. Techniques in Fish Immunology, 101-103.
3. Fange, R.(1984) Lymphomyeloid tissues in fishes. Vidensk. Meddor. Dansk. Naturh. Foren. 145: 143-162.
4. Fange, R.(1986) Lymphoid organs in sturgeons (Acipenseridae). Vet. Immunol. Immunopathol. 12: 153-161.
5. Fletcher, T.C. and White, A. (1976) The lysozyme of the plaice *Pleuronectes platessa* L. Comparative Biochemistry and Physiology. 55B: 207- 210.
6. Florensov, V.A. and Pestova, I.M.(1990) Manual of Evolutionary Immunomorphology. Irkutsk state University, Irkutsk. pp. 72-75.
7. Gallin, J. I. (1982) Role of neutrophil lysosomal granules in the evolution of the inflammatory response. In phagocytosis past and future (Karnovsky, M.L. and Bolis, L., eds). pp. 519-541.
8. Goldstein, I.M., Hoffstein, S.T. and Weissmann, G.(1975) Mechanisms of lysosomal enzyme release from human polymorphonuclear leucocytes. J. Cell Biol. 66: 647-652.
9. Grinde, B. (1988) Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. J. Fish Diseases. 12: 95- 104.
10. Grinde, B., Lie, O., Poppe, T. and Salte, R. (1988) Species and individual variation in lysozyme activity in fish of interest in aquaculture. Aquaculture. 68: 299-304.
11. Ingram, G.A. (1980) Substances involved in the natural resistance of fish to infection-a review. Fish. Biol. 16: 23-60.
12. Itami, T., Takchara, A., Nagano, Y., Suetsuna, K., Mitsutani, A., Takesue, K. and Takahashi, Y. (1992) Purification and characterization of lysozyme from an ayu skinmucus. Nippon Suisan Gakharshi. 58: 1937-1944.
13. Kozinenko, I.I., Isaeva, N.M., Balakhnin, I.A. (1999) Humoral factors of nonspecific defense of fish. J. Problems Ichthiol. 39: 394- 400.
14. Marc, A.M., Quentel, C., Severe, A., Le Bail, P.Y. and Boeuf, G. (1995) Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. J. Fish Biol. 46: 1065-108.
15. Mikryakov, V.R., Lapirova, T.B. (1997) Influence of salts of some heavy metals on picture of white blood cells of Siberian sturgeon fingerling *Acipenser baeri* Brandt. J. Problems Ichthyol. 37: 538-542.
16. Mikryakov, V.R., Lapirova, T.B., Soltani, M., Mavrin, A.S. and Vinogradov, G.A. (2002) Influence of sublethal concentrations of some heavy metal salts (Hg, Cd and Cu) on the contents of lysozyme in tissues of sturgeon fingerlings (*Acipenser baeri*). Bull. Eur. Ass. Fish. Pathol. 22:10.
17. Mock, A. and Peters, G. (1990) Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling transport and water pollution. J. Fish Biol. 37: 873-885.
18. Murray, C.K., Fletcher, T.C. (1976) The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa*). Tissue. J. Fish Biol. 9: 329 - 334.
19. Øystein, L., Øystein, E., Sørensen, A., Frøysdal, E. (1989) Study on lysozyme activity in some fish species, Diseases of Aquatic Organisms. 6: 1- 5.
20. Paulsen, S.M., Engstad, R.E., Robersten, B. (2001) Enhanced lysozyme production in Atlantic Salmon (*Salmo salar* L.) macrophages treated with yeast β -glucan and bacterial lipopolysaccharide, Fish and Shellfish Immunol. 11:23 -37.
21. Rijkers, G.T. (1982) Non-lymphoid defense mechanisms in fish. Dev. Comp. Immun. 6:1-13.
22. Siwicki, A. and Studnicka, M. (1987) The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, *Cyprinus carpio* L. J. Fish Biol. 31 (Supplement A): 57- 60.
23. Studnicka, M., Siwicki, A. and Ryka, B. (1986) Lysozyme level in carp (*Cyprinus carpio* L.), Bamidgeh. 1: 22 -25.
24. Weeks, B.A. and Warinner, J.F. (1984) Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. Marine Environm. Res. 24:327-335.
25. Zapata, A.G., Chiba, A. and Varas, A. (1996) cells and tissues of the immune system of fish. In: Iwama, G. and Nakanishi, T. (eds) The fish Immune system. Academic Press, SD, pp.18 -19 and 31-36.

