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ELISA of polyclonal antibody to fish MT and study on heavy metal tolerance in fish

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Abstract—On the basis of the purification of metallothionein (MT) from fish, the purified polyclonal antibody of rabbit against fish MT was prepared. Antibody was labeled with horseradish peroxide (HRP) and then the enzyme linked immunosorbent (ELISA) for MT in fish tissues was established, the minimal, detection level being 0.1—1.0 ng/g FW. Induction of fish tolerance against heavy metals indicated that the contents of MT, SH - group and Cd increase with the accumulation doses of Cd in the water. The investigations from 7 species of fresh - water fish showed that Cd and MT amounts in their liver and kidney are very low and, particularly, the muscle is lowest, approximately to be zero. So, it is considered that the degree of the heavy metal pollution in water body in which fish live is light and fish is permission to consume.

Keywords: heavy metal tolerance; fish; polyclonal antibody; metalothionein (MT).

1 Introduction

It has been reported that fish exposed to heavy metals repeatedly because of water pollution develops a tolerance toward subsequent exposure to them and an induction of tolerance may be partially due to the increased production of metallothionein (MT), a protein which is low molecular weight, 1/3 of its amino acid is cysteine and selectively binds and deactivates Cd(II), Zn (II) and certain other metals (Kagi, 1984; Run, 1991; Kito, 1982a; Benson, 1987; Kito, 1982b). In fish , however, there is little information on the relationship between MT, SH - group and metals in organism and water. As fish is an important source of protein for people and animals and it becomes an indicator of the heavy metal pollution in water (Micheal, 1987). An enzyme linked immunosorbent assay (ELISA) for polyclonal antibody of rabbit against Cd(II)-MT form crucian carp (*Carassius auratus*) would be established because of its sensitivity, rapid-

ness and simplicity (Chatterjee, 1987). By ELISA, the tolerance induction of fish to Cd(II) following progressive sublethal exposure to the metals and the contents of Cd(II) and MT of liver and kidney from 7 species of the freshwater fish will be observed.

2 Materials and methods

2.1 Materials

7 species of freshwater fish were purchased from Wan Zhuang Fishery in Beijing. Rabbits were obtained from Department of Biology of Peking University.

Tris G. R. Merck German. Tween 20 BioRad Co., USA. 2,4-DNFB (2,4-dinitrofluorobenzene), TMB (3,3′,5,5′-tetramethylbenzidine) and HRP (horseradish peroxidase, RZ 3.0), Sigma Co., USA. Fetus bovine serum Tianjin Pharmaceutical Factory, China. Sephacryl S-100, -200 and -300RH, and DEAE-Sepharose FF ion exchange Pharmacia Co., Sweden. $CdCl_2$, $(NH_4)_2SO_4$, $NaIO_3$, Glycol, Na_2CO_3, H_2O_2, $(NH_4)_2CO_3$, Phosphate, EDTA etc. used in this experiment are A. R. products made in China. BCC (Bacille calmatte-Guerin) Beijing Institute of Bioproducts, China.

System of gel filtration Pharmacia Co., Sweden. 96 well polystyrene ELISA plate Tianjin Plastic Factory, China. 202K High-speed ice centrifugator Sigma Co., USA. Vaccum drying system Savant Instrument, USA. Minireader II Dynatech Lab. Co., USA. Model 9200 Atomic Spectrometer Philip Co., England.

2.2 Methods

Induction of fish MT Crucian carp was transported to water tank with tap water to acclimate for a week. During the experiment, water temperature was kept at 20°C and aerated, dissolved oxygen concentration ranged from 7.0 to 8.2 mg/L and pH varied from 7.4 to 7.6. After acclimating, the induction of fish MT was conducted by the following two groups. In MT preparation group, fishes were injected intraperitoneally with $CdCl_2$ in fish saline. The dose of Cd(II) was 2.0, 4.0, 6.8, 8.0 mg/kg bw (body weight) after 1, 4, 7 and 10 days, respectively. On the eleventh day fishes were killed and livers were collected from fish to supply MT preparation. In MT tolerance group, it is 1 fish per glass, 20-liter tank with tap water. The induction of fish MT was performed in the same conditions as above through progressive increasions in Cd(II) dose, i.e., fishes were maintained at 22.0, 42.0, 82.0 and 122.0 μg/L for 16, 20, 28 and 36 days, respectively. And the livers in each accumulation dose were collected from fish killed at day 16, 20, 28 and 36 to provide the tolerance observation. Besides, fishes tested in this investigation were killed as soon as obtained from fishery and quickly the livers were taken out for analysis of MT and other indicators.

2.2.1 Isolation and purification of MT

The liver tissues used to MT preparation were rinsed, homogenated and centrifuged at 106000 g for 60 min at 4°C. The collecting supernatants were heated at 80°C for 30 min to remove the high molecular fractions. Heated fractions were centrifuged at 10000 g for 10 min at 4°C. The supernatants were applied to a column of Sephacryl S-200 (2.6 × 100 cm) equilibrated with 0.01 mol/L Tris-HCl buffer (pH 8.6); fractions (5 ml) were collected. The fractions

of high UV absorbance (254 nm) and major Cd(II) binding were pooled and applied to a column of DEAE Sepharose FF ion exchange (2.4 × 30 cm) preequilibrated with Tris buffer above. The column was eluted with one liter of linear gradient (0.01—0.05 mol/L Tris - HCl buffer, pH 8.6). The effluents containing MT were pooled and then concentrated by vacuum drying system. The desaltizations of the concentrated sample were conducted on a column of Sephacryl S -100 (1.6 × 120 cm). Finally, the effluents to be collected were lyophilized to obtain the freeze - dried MT from crucian carp livers (Ren, in press).

2.2 **Preparation of polyclonal antibody**

The milky suspensions were prepared from 10 mg BCC with the equal volume of the mixture of 5.0 mg/ml fish MT solutions and complete Freund's adjuvants (Yu, 1982), which was made from 10 mg inactivation BCC adding to 1 ml incomplete Freund's adjuvants. Rabbits were injected subcutaneously at two plantar metatarsus with 2 ml suspensions above and, 2 week later, were boosted at swollen poples lymphnodes of two lower climbs with three subcutaneous injections using 0.5, 1.0 and 2.0 ml mixtures of fish MT (3.0 mg/ml) and incomplete Freund's adjuvants (1:1) in a week. Bleeding was made by incubating the blood for 1.0 h at 37°C, stored at 4°C overnight and finally centrifuged at 5000g for 20 min. Antibody to obtain were diluted with equal volume of cold PBS and then added with a drop of equal volume of saturated $(NH_4)_2SO_4$ solution and then the resulting precipitates were gathered up. The same procedure was repeated three times. Through dialysis against 0.01 mol/L phosphate buffer and lyophilization, the purified fish polyclonal antibody was prepared and stored in −30°C. Titer of antibody was tested to be 10^{-11}g/ml.

Labeling of antibody with HRP 5.0 mg were dissolved in 1.0 ml of 0.3 mol/L $NaHCO_3$ solution, pH 8.1. In the conditions of stirring, HRP solutions were supplemented, in turn, with 0.1 ml ethanol solutions of 1% 2,4- DTFB, 1.0 ml 0.06 mol/L $NaIO_3$ and 1.0 ml 0.16 mol/L glycol. The mixture was dialyzed against 0.1 mol/L Na_2CO_3 buffer, pH 9.5 (three exchanges, 1.0 liter) at 4°C overnight. The resulting HRP solution in 0.1 mol/L carbonate buffer added by 5.0 mg of fish polyclonal antibody was incubated for 3 h at room temperature and then dialyzed against normal saline overnight (three exchanges). The dialysate added by $NaBH_4$ was dialyzed again against saline overnight. Antibody labeled HRP was separated on Sephacryl A 300HR column . The eluate of the conjugated was dropped with equal volume of saturated $(NH_4)_2SO_4$ solution. After centrifuging at 5000 g for 15 min, the precipitate was dialyzed against 0.01 mol/L PBS, pH 7.4, overnight (several exchanges). Finally, the purified HRP - fish MT polyclonal antibody was prepared and its titer was tested to be 10^{-9} g/ml.

ELISA for MT 10 μg/ml of crucian carp MT solutions were prepared with PBS. 100 μl of MT solutions were added to each well of 96 well microliter plates and diluted, in turn, by 10 fold using PBS. The plates were incubated for 2 h at 37°C and then washed three times with 0.01% Tween 20 in PSB. 100 μl of 10% fetus bovine serum in PBS were added to block non - specific adsorption and allowed to incubate for 30 min at room temperature. Next, 100 μl of the various concentrations of HRP - polyclonal antibody were placed to each well and incubated for 1 h at 37°C, followed by wash with PBS - Tween 20. 50 μl of the enzyme substrate solutions sodium

phosphate pH 6.0:TMB (5.0 mg/ml DMSO): H_2O_2= 100:1.0:0.15 were added to reach well and, followed by 15—30 min incubation at 37℃, the reaction was stopped by addition of 2 mol/L H_2SO_4 and then OD_{450} value read out on Miniread II. In addition, the negative control well lacking any of the antigen was set (Yu, 1982).

2.2.3 **Determination of sample**

The tissues of fish observed for Cd(II)-tolerance and purchased from Fishery were rinsed, homogenated and then the supernatant was made by centrifuging at 106000 g for 30 min, to obtain MT-contained extract. The contents of MT were determined on 96 well plates to be added with the tissue extracts using the ELISA procedure described above.

Caluculation of MT

$$\text{MT (mg/g Fw)} = C \times \frac{V_1}{V_2} \times D \times \frac{1}{W},$$

where C is MT contents (mg) tested from 96 well plates; V_1 is volume (ml) of tissue sample solution; V_2 is volume (ml) of dropped sample solution; D is total times of the dilution of tissue sample solution; W is weight (g) of tissue sample.

2.2.4 **Detection of metal and SH-group**

The Cd(II) and Zn(II) contents were determined with an atomic absorption spectrophotometer. SH-group contents were detected with a method of Ellman's reagent (Ren, in press).

3 Results and discussion

ELISA of HRP-labeled fish MT polyclonal antibody raised in rabbit has been established for assaying fish MT. Fig. 1 shows the relationship between the fish MT contents in crucian carp or carp (*Cyprinus carpio*) and OD values at maximal absorbance wavelength 450 nm determined by ELISA mentioned above, exhibiting that the curve of crucian carp MT has analogy to carp's. The results indicated that the minimal, detection level of this technique is 0.1 ng/g Fw and its detection range is from 0.1 to 100 ng/g Fw, similar to the data reported by Justine *et al.* using

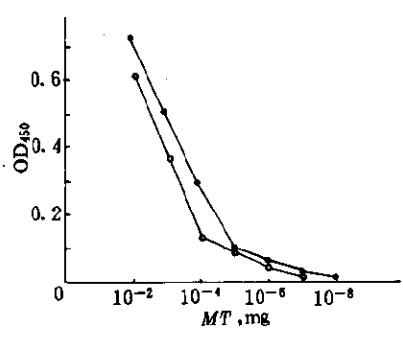

Fig. 1 Relationship between fish and absorption at 450 nm
. crucian carp 。carp

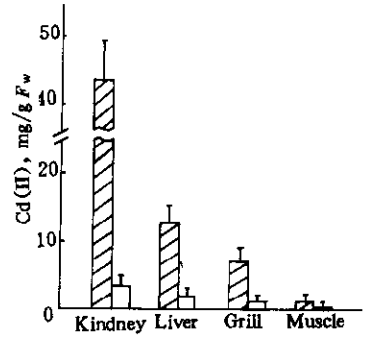

Fig. 2 Distribution of Cd(II) in fish tissues
▭ Cd(II)-exposure group Cd(II) dose 14.0 mg/kg bw, IP, for 11 days;
□ Control no Cd(II) expose.

ELISA of the polyclonal antibody to mammalian MT (Justine, 1987). In addition, the polyclonal antibody for crucian carp Cd(II)-MT cross-reacted well with MT from crucian carp and carp, i.e., cross-reactivities of both are similar, reflecting that crucian carp is the same as carp in the basic characteristics of antigen, probably to contribute to the same family - Cyprinidae.

The distribution of Cd(II) in fish tissues induced by $CdCl_2$ are shown in Fig. 2. The Cd(II) concentrations in the tissues induced and non-induced both decreased as the order of kidney, liver, gill and muscle. However, Cd(II) concentrations of the fish tissues induced increase over one of the tissues non-induced, particularly, the renal Cd(II) concentrations of the former exceed the latter by a factor of about ten. Thus, the kidney among fish tissues is of highest affinity to heavy metal, the liver is second, the gill is third and the muscle lowest to be almost zero. Therefore, the muscle of fish is harmlessly eatable even though it exposed repeatedly in water body polluted lightly by heavy metals. Besides, the results demonstrate that both kidney and liver are the appropriate indicators of the tissue to observe MT changes.

Table 1 MT, SH-group and metal contents of liver, kidney and gill from crucian carp exposed by Cd(II)

Cd(II)	Accumulation dose, μg/L	0	22	42	82	122
	Exposure time, d	0	16	20	28	36
Liver	MT, mg/Fw	0.01	0.093	0.353	0.781	1.346
	SH, μmol/ml	0.215	0.469	0.747	1.534	1.892
	Cd, μg/g Fw	0.067	0.703	0.784	9.401	10.487
	Zn, μg/g Fw	0.361	0.137	0.084	0.027	0.011
Kidney	MT, mg/Fw	0.013	0.075	0.419	0.986	1.408
	SH, μmol/ml	0.197	0.075	0.986	1.777	2.103
	Cd, μg/g Fw	0.036	0.473	0.918	20.295	43.355
	Zn, μg/g Fw	0.193	0.116	0.091	0.031	0.012
Gill	MT, mg/Fw	0.007	0.043	0.113	0.167	0.247
	SH, μmol/ml	0.098	0.207	0.401	0.609	0.973
	Cd, μg/g Fw	0.027	0.703	0.985	3.195	6.650
	Zn, μg/g Fw	0.570	0.490	0.241	0.094	0.053

Fw: fresh weight of tissue

Cd(II) accumulation doses (days) in water and Cd(II) and Zn(II) concentrations, SH-group contents and MT amounts in liver, kidney and gill from fish exposed in water progressively added by Cd(II) are presented in Table 1. The increases of metal ion, SH-group and MT in the whole fish body are parallel with the increases of Cd(II) doses (days) in water. Thus, the more SH-group (cystein) MT contain, the greater MT bind to heavy metals, resulting in the enhance of the ability of fish protection from the hazard of the heavy metals. In the sublethal dose used in this experiment, moreover, Cd(II) accumulations in liver and kidney performed as MT. This is because that the inducible synthesis of MT is enhanced in liver, and while MT is transported into the kidney by glomerular filtration of the hepatic MT and even the progressively accumulations of MT continue in tubule by reabsorption from filtrate until kidney "sutured" when renal damage occurs. As a result, the contents of MT are highest in kidney, second in liver and lower in gill.

Finally, the fact that the substitution extent of Cd(II) to Zn(II) in MT increases with the raising of Cd(II) dose in water shows that Zn(II) in MT was progressively replaced by Cd(II). It suggested that there is another path of MT detoxification in tissues except for the way through the increasion of the synthesis of apothionein. Indeed, the tolerance induction of fish against metals is, in parts, contributed to the production of MT, more and all, SH-group (Kito, 1982b).

Table 2 Cd(II) and MT contents of liver and kidney from 7 species freshwater fish

Species	Weight, g	Size, cm	Liver		Kidney	
			Cd, μg/gFw	MT, mg/gFw	Cd, μg/gFw	MT, mg/gFw
Crucian carp (*Carassois airatus*)	50—200	14—18	0.018	0.011	0.017	0.010
Carp (*Cyprinus carpio*)	500—600	30—35	0.000	0.009	0.000	0.007
Silver carp (*Hypophthalmichthys molitrix*)	700—800	40—45	0.009	0.006	0.010	0.009
Bighead (*Aristichthys nobilis*)	700—800	40—45	0.088	0.028	0.093	0.031
Grass carp (*Ctenopharyngodon idella*)	1000—1200	55—65	0.000	0.003	0.002	0.005
Snakehead (*Ophicephalus argus*)	400—500	25—30	0.000	0.001	0.000	0.002
Triangular bream (*Megalobrama terminalis*)	1200—1600	60—70	0.000	0.003	0.001	0.004

Fw: fresh weight of tissue

Cd(II) and MT contents of liver and kidney, and weight and size of the body from 7 species of fish, obtained from fishery, are given in Table 2. The results demonstrated that the increasing tendency of Cd(II) and MT contents in the tissues are parallel each other, i. e., the more Cd (II) the tissues contain, the greater MT become. Next, the concentrations of Cd(II) and MT in kidney are higher than those in liver, particularly, when higher level of Cd(II) in water, being as same as the data reported above. The contents of Cd(II) and MT in the tissues from 7 species fish, in the magnitude, are dighead, crucian carp, silver carp, carp, grass carp, triangular bream and snakebead. And thus kidney is analogous to liver in the tendency of Cd(II) and MT magnitude in various species fish. Finally, according to the data in this experiment, the relationship between the contents of Cd(II) and SH-group in the tissue and weight and size of the body from 7 species fish is not significant, the reason having yet to be researched. In a word, two points have been suggested that the muscle of fish, most of all, is eatable so that it is harmless for the public and the water body for fish culture is considered to be nearly no heavy metal pollution.

In summary, ELISA technique of HRP labeled polyclonal antibody for fish MT was successfully established. The fact that polyclonal antibody of crucian carp MT cross reacts with other fish MT indicated that the antigenic nature of MT from 7 species fish is quite similar each other. The practical application further shown that this technique is of rapidness, simplicity and more sensitivity, but exists a disadvantage of lacking strong specific of fish MT so that the detection

capability is quite limited. Therefore, the establishment of a new ELISA technique of monoclonal antibody will be conducted in the future to improve sensitivity and deductibility. The experimental results also revealed that MT plays a vital role in the detoxification for heavy metals i. e., complex and inactivate nonessential elements such as Cd(II), Pb(II) etc. (Webb, 1975). However, the phenomenon that the essential elements such as zinc, copper etc. were substituted from MT during the increased tolerance of fish to protect from metals progressively induced by Cd (II), should not be negligible because the non-essential elements disturb the homeostasis of trace metals and influence the normal physiological function. A study of the mechanism of MT detoxification in detail is essential to control this phenomenon. In sum, the information presented in this work is favorable in understanding the toxicological and biochemical ecology of aquatic life, assessing water qualify and adopting the effective measures of the protection to heavy metal pollution.

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