Fish & Shellfish Immunology 43 (2015) 510–519

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Oxidative damage of hepatopancreas induced by pollution depresses humoral immunity response in the freshwater crayfish *Procambarus clarkii*

Keqiang Wei [a, *], Junxian Yang [b]

[a] School of Life Science, Shanxi University, Taiyuan 030006, People's Republic of China
[b] School of Economics and Management, Shanxi University, Taiyuan 030006, People's Republic of China

ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form
12 January 2015
Accepted 22 January 2015
Available online 2 February 2015

Keywords:
Hemocyanin
Hepatopancreas
Phenoloxidase
Procambarus clarkii
Protein oxidation

ABSTRACT

Previous studies provide evidences for the possible oxidative damage of toxic environmental pollutants to tissue protein in fish and amphibian, but little information is available about their effects on immunity response in crustacean. In the present study, we evaluated the relationship between oxidative damage and immune response induced by both typical pollutants (viz. copper and beta-cypermethrin), by exposing the freshwater *Procambarus clarkii* to sub-lethal concentrations (1/40, 1/20, 1/10 and 1/5 of the 96 h LC_{50}) up to 96 h. Five biomarkers of oxidative stress, i.e. reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and protein carbonyl in hepatopancreas, and two immune factors, i.e. phenoloxidase (PO) and hemocyanin in haemolymph were determined. The results indicated that there was a significant increase ($P < 0.05$) in the contents of ROS, MDA and protein carbonyl accompanied by markedly decreased ($P < 0.05$) PO and hemocyanin levels in a dose and time dependent manner. The significant and positive correlation ($P < 0.01$) between protein carbonyls induction and MDA formation was observed in crayfish hepatopancreas at 96 h. The production of these protein carbonyls could significantly depress ($P < 0.01$) the levels of phenoloxidase and hemocyanin in hemolymph. Higher contents of ROS enhanced the risk of lipid peroxidation, protein carbonylation and immunosuppression of crayfish, and hepatopancreas might play an important role in immune system of crustaceans. Protein oxidation may be one of the main mechanisms for pollution-induced immunotoxicity in *P. clarkii*.

1. Introduction

There is an increasing concern that large numbers of agricultural and industrial chemicals are entering the aquatic environment and being taken up into tissues of aquatic organisms. Especially, heavy metal and pesticide pollution have been shown to cause serious health risks as they are immunotoxic to fish, crab and shrimp even at low concentrations [1]. The 2012 Report on the State of the Fishery Eco-Environment in China emphasized that copper (Cu) is one of the most common pollutant in the important fishery areas [2]. Cu concentrations ranging from 50 mg/L to >560 mg/L have been reported in polluted freshwater areas all over the world. Beta-cypermethrin (MF: $C_{22}H_{19}CL_2NO_3$) is widely used and most effective pesticide based on pyrethroid preparations. It is usually detected at levels of 0.01–9.8 μg/L in the water, but its concentration can reach up to 194 μg/L in some agricultural runoff [3]. Copper is an essential trace element for most life forms, acting as a cofactor in many vital enzymes such as cytochrome *c* oxidase (COX) and superoxide dismutase (SOD). But excess copper could catalyze the production of reactive oxygen species (ROS) through the Haber–Weiss reaction [4]. Due to the lipophilicity in nature, cypermethrin has a high rate of gill absorption thereby rendering crayfish as most sensitive to the pesticides. Cypermethrin has been found to cause neurotoxicity and disrupt the balance between the production and scavenging of ROS [5]. Higher ROS concentrations could enhance the risk of DNA damage, lipid peroxidation and protein carbonylation. Studies have indicated that these ROS play a role in modulating immune response [6]. Therefore, oxidative

* Corresponding author. School of Life Science, Shanxi University, 92 Wucheng Road, Taiyuan 030006, People's Republic of China. Tel.: +86 351 7018206.
E-mail addresses: kqwei88@aliyun.com, kqwei@sxu.edu.cn (K. Wei).

http://dx.doi.org/10.1016/j.fsi.2015.01.013
1050-4648/

stress is acknowledged as the most important mechanisms for pollutant-induced toxicity in aquatic organisms [7,8].

Proteins are the most abundant cell components (70%) and primarily responsible for most functional processes within cells. They are possibly the most critical target for ROS assault. It has been estimated that proteins can scavenge majority (50%–75%) of free radicals generated. Alterations of protein carbonylation levels could reflect severe tissue damage or metabolic disorders triggered by contamination [9]. For aquatic organisms, elevated protein carbonylation was firstly found in blue mussels *Mytilus edulis* in 2005 by McDonagh et al., who revealed digestive gland is the major site of carbonylation in response to oxidative stress [10]. In recent years, Cu-induced carbonylation of protein has been confirmed in corkwing wrasse *Symphodus melops* as well as model organisms yeast *Saccharomyces cerevisiae* and zebrafish *Danio rerio* [11]. Protein carbonyls have been proposed as a molecular biomarker of ROS mediated oxidative damage in freshwater fish *Channa punctata* and the black tiger shrimp *Penaeus monodon* exposed to deltamethrin, endosulfan and paraquat [9,12]. The latest advances in proteomics tools have made it possible to identify oxidized proteins along with specific sites of oxidative damage and the consequences of protein oxidation [13]. Research showed protein oxidation can lead to peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid and ultimately influences the structure, function and integrity of protein. The relative early formation and the chemical stability of carbonylated proteins makes them suitable markers for oxidative stress in comparison with other oxidation products. Lipid peroxidation products are detoxified within minutes, whereas cells degrade oxidized proteins within hours and days.

Increased levels of protein carbonyls have been observed in various human diseases, although the relationship among protein oxidation, immunity dysfunction and diseases remains largely unclear. It has been reported that environmental pollutants in the aquatic ecosystem could weaken immunocompetence of organisms, which are caused by or related to the generation of ROS [14]. Pesticides such as cypermethrin and trichlorfon were found to impair immune system and induce abnormal immune response in the giant freshwater prawn *Macrobrachium rosenbergii* and rainbow trout *Oncorhynchus mykiss*, copper in the marine mussel *M. edulis* and carp *Cyprinus carpio* L., finally causing the increase of susceptibility to pathogens [15–17]. The host defense of invertebrates rests entirely with innate immune system, including both humoral and cellular immune responses. The prophenoloxidase-activating system (proPO-AS), residing in hemocyte granules, is considered the most important component of the defense system in crustaceans [17]. Traditionally, hemocyanin, produced in hepatopancreas and transported in haemolymph, serves as an oxygen carrier. But several studies have reported that it might be a novel immune molecule in mollusks and arthropods [18–20], because hemocyanin could be functionally converted into phenoloxidase-like enzyme, antiviral agent, antimicrobial protein, agglutinin and hemolysin [20]. Hepatopancreas is the site of multiple oxidative reactions and therefore may be a site of substantial free radical generation. To date, little is known about its role in protecting crustaceans from disease. However, most of the immune-related genes including encoding prophenoloxidase and hemocyanin would mainly express on hepatopancreas tissue [18,21]. Gross et al. (2001) postulated that an organ of hemolymph filtration and detoxification could play an important part in both humoral and cellular immunity [22].

The red swamp crayfish *Procambarus clarkii* is native to northeastern Mexico and south-central USA (Louisiana). Now it has been the most common crayfish species being cultured commercially in China, and the farmed production reached 370,000 tons in 2010. Its long life-cycle, wide distribution, and sedentary lifestyle make it use as a sensitive bioindicator to assess the effects of contaminants in freshwater ecosystems [23]. However, very few reports are available regarding protein damage in *P. clarkii* hepatopancreas when exposed to Cu and commercial formulation of beta-cypermethrin. In the present study, we evaluated the relationship between oxidative stress and immune response induced by both typical pollutants, by measuring ROS content, SOD and CAT activity as well as MDA and protein carbonyl level in hepatopancreas, a potential immune organ, and phenoloxidase and hemocyanin concentration in haemolymph, the circulating fluid of immune component. The results will provide the evidence for the possible toxicological mechanisms of pollution on aquatic organisms.

2. Materials and methods

2.1. Crayfish

Adult crayfish *P. clarkii* (8.6 ± 0.5 cm in length, 22 ± 0.6 g in wet weight) were purchased from a local aquatic product market. Prior to experiment, they were maintained in glass aquaria (45 cm × 30 cm × 30 cm) containing aerated city tap water (22 ± 1 °C) with a photoperiod 12 h:12 h for 10d. The animals were fed once daily with commercial pellets. Only apparently healthy crayfish in the intermoult stage were used. No mortality was observed during either the acclimation period or throughout the exposure duration.

2.2. Chemicals

All the chemicals were analytical grade, obtained from Sigma (St. Louis, MO, USA) unless stated otherwise. A commercial formulation of beta-cypermethrin was supplied by Institute of Plant Protection, Shanxi Academy of Agricultural Sciences, Taiyuan, China. The formulation was an emulsifiable concentrate that contained 4.5% active ingredient. Assay kits for SOD and CAT activities, and MDA and protein contents were from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). For preparation of the stock solution, $CuSO_4 \cdot 5H_2O$ (99% purity) was dissolved in deionized water. Beta-cypermethrin was solubilized with appropriate amount of distilled water containing 0.5% acetone [24]. The stock solutions were stored at 4 °C until use. Measurements were conducted on Spectra Max M5 multifunctional microplate reader (MDC, Sunnyvale, CA, USA).

2.3. Exposure

After acclimatization, crayfish were randomly divided into nine groups of fifteen animals each. The tests were carried out using three replicates per group. The 96 h LC_{50} values (95% confidence level) previously estimated for crayfish of the same size were considered [25,26]. The animals were exposed to four sublethal concentrations corresponding to 1/40 LC_{50}, 1/20 LC_{50}, 1/10 LC_{50} and 1/5 LC_{50}, respectively. The treatment protocol was: Group 1 (0.75 mg/L of Cu^{2+}), Group 2 (1.5 mg/L of Cu^{2+}), Group 3 (3.0 mg/L of Cu^{2+}), Group 4 (6.0 mg/L of Cu^{2+}), Group 5 (0.005 μg/L of beta-cypermethrin), Group 6 (0.01 μg/L of beta-cypermethrin), Group 7 (0.02 μg/L of beta-cypermethrin) and Group 8 (0.04 μg/L of beta-cypermethrin). Previous experiments showed that pesticide emulsifier and acetone at a concentration used in the dilution of the maximum cypermethrin concentration did not induce toxic effects on crayfish *P. clarkii* [24,25]. Therefore, dechlorinated tap water received no chemicals and was used as a control. According to Chinese National Water Quality Standard for drinking water (GB