Fish & Shellfish Immunology 36 (2014) 320–323

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Protective effect of aquacultured flounder fish-derived peptide against oxidative stress in zebrafish

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ARTICLE INFO

Article history:
Received 22 July 2013
Received in revised form
24 September 2013
Accepted 26 November 2013
Available online 11 December 2013

Keywords:
Zebrafish
Oxidative stress
Antioxidative peptide
In vivo model
AAPH

ABSTRACT

This study investigates the protective effect of aquacultured flounder fish-derived peptide (AFFP) against 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH)-induced oxidative damage in a zebrafish model. Zebrafish embryos were evaluated for the protective effect by heartbeat rate, survival rate, ROS generation, lipid peroxidation, and cell death. In the results, the AAPH group showed a low survival rate, whereas the AFFP and AAPH co-treated group increased a survival rate. Also, AFFP dose-dependently reduced AAPH-induced intracellular ROS and lipid peroxidation, and decreased cell death in AAPH-induced zebrafish. These results revealed that AFFP could be used as a natural antioxidant, and that the zebrafish provides an alternative *in vivo* model to efficiently evaluate the antioxidative effects of peptides on fishes.

1. Introduction

The zebrafish (*Danio rerio*) has been widely used as an animal model in studies on molecular genetics, development biology, drug discovery, and toxicology [2–5]. The advantages of the zebrafish are its size, fecundity, large clutches, low cost, physiological similarity to mammals, and rapid embryonic development *in vivo*, which facilitates morphological monitoring [6,7]. In particular, its embryos and juveniles are useful for imaging studies, because their transparencies allow the visualization of specific cells, tissues, and organs [8]. For these reasons, the zebrafish has been recently used as an *in vivo* model of oxidative stress [9].

Aerobic life forms are associated with the generation of reactive oxygen species (ROS). Oxidative stress arises due to imbalance between the production of ROS and antioxidant scavenging activity. ROS can cause oxidative damage to biological macromolecules like DNA, lipids and proteins. In particular, lipid peroxidation may impair membrane functions, increase permeability, reduce membrane fluidity, inhibit signal transduction over the membrane and cause immune dysfunction [10–12]. Lipid peroxidation of zebrafish embryo was easily induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which generates peroxyl radicals ($ROO^{\cdot}$), which in turn attack lipids [13].

In recent years, many bioactive peptides have been used to develop new drugs and health foods. Bioactive peptides from marine animal protein hydrolysates have been reported to possess nutritional, antioxidative, antihypertensive, antimicrobial, anti-inflammatory, and immunomodulatory properties [14,15]. In our previous study, we found that a Cys-Ala-Ala-Pro peptide (AFFP) from aquacultured flounders had antioxidative effects [1].

The purpose of this study was to investigate the protective effects of AFFP against AAPH-induced oxidative stress in zebrafish embryo.

2. Materials and methods

2.1. Materials and reagents

AFFP (Cys-Ala-Ala-Pro, purity: >95%, Fig. 1) as antioxidative peptide synthesized from Peptron, Inc. (Yuseong-gu, Daejeon,

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http://dx.doi.org/10.1016/j.fsi.2013.11.018

Korea). 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), diphenyl-1-pyrenylphosphine (DPPP), acridine orange, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). The other reagents used were of analytical grade.

2.2. Origin and maintenance of zebrafish

Adult zebrafish were purchased from a commercial dealer (Seoul aquarium, Seoul, Korea) and were kept in a 3 L acrylic tank at 28.5 °C with a 14 h/10 h light–dark cycle. The zebrafish were fed 3 times/day, 6 days/week, with tetramin flake food supplemented with live *Artemia salina*. Embryos were obtained from natural spawning that was induced in the morning by turning on the light. The collection of embryos was completed within 30 min.

2.3. Waterborne exposure of embryos to CAAP

Contemporized embryos were collected and randomly arrayed by pipetting, 25 embryos/wells in 24 well plates containing 475 μl embryo medium. We divided six test groups: 1) non-treated control group, 2) only treated 10 mM AAPH group, 3) and 6) groups were pretreated with 25, 50, and 100 μg/ml of AFFP with the treated AAPH, respectively. AFFP was dissolved in distill water, and then added to the embryo medium from 3 h post-fertilization (hpf) for 1 h. Then 10 mM AAPH was treated for up to 120 hpf. After treating with 10 mM AAPH for 16 h, the embryo media was changed.

2.4. Measurement of heartbeat and survival rate

The heart-beating rate of both atrium and ventricle were measured at 48 hpf after AAPH induction [16]. Counting and recording of atrial and ventricular contraction were performed for 1 min under the microscope, and results are represented as the average heartbeat rate per min. The survival rate was measured at 72 hpf after AAPH induction by zebrafish embryos mortality.

2.5. Estimation of intracellular ROS generation, lipid peroxidation and cell death image analysis in zebrafish embryos

Generation of ROS production in zebrafish embryos were determined by an oxidation – sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). In the presence of cellular ROS, peroxides convert non-fluorescent dichlorofluorescein (DCFH-DA) into highly fluorescent dichlorofluorescein (DCFH) through the oxidation [17]. Lipid peroxidation was evaluated by a fluorescent probe dye diphenyl-1-pyrenylphosphine (DPPP) [18]. DPPP is non-fluorescent, but it becomes fluorescent for detection when the cell membrane lipid is peroxidized. Cell death was detected in zebrafish using staining with acridine orange, a nucleic acid specific metachromatic dye. It interacts with DNA and RNA by intercalation or electrostatic attractions and visualized necrotic or apoptotic cells. At 2 days post-fertilization (dpf), the embryos were transferred into a 96 well plates and treated with DCFH-DA for 1 h (20 μg/ml), DPPP for 1 h (25 μg/ml) and acridine orange for 30 min (7 μg/ml) in the dark at 28.5 °C, respectively. Then, the zebrafish embryos were rinsed in fresh embryo medium and anaesthetized before visualization. Individual zebrafish embryo fluorescence intensity was quantified using a Perkin–Elmer LS-5B spectrofluorometer. The images of the stained embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

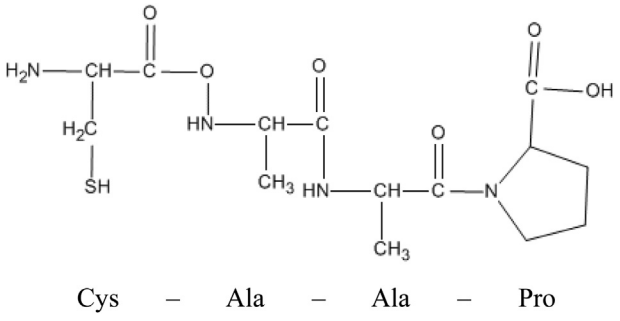

Fig. 1. Structure of aquacultural flounder fish-derived peptide (AFFP).

2.6. Statistical analysis

All experiments were conducted in triplicate ($n = 3$) and an ANOVA test (using SPSS 11.5 statistical software) was used to analyze the data. Significant differences between the means of parameters were determined by using the Tukey test to analyze the difference. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Protective effects of AFFP on AAPH-induced heartbeat rate increases and survival rates in zebrafish

In order to examine AAPH-induced toxicity in zebrafish, we measured heartbeat and survival rates. As shown in Fig. 2, heartbeat rates of the AAPH treated group (AAPH group) were significantly increased compared with the non-treated group (control group). Interestingly, the AFFP and AAPH treated group (AFFP/AAPH group) showed a dose-dependent decrease of heartbeat rates. Regarding survival rates, the AAPH group showed a 45% survival rate, whereas the AFFP/AAPH group increased a survival rate to 80–90%.

3.2. Inhibitory effects of AFFP on AAPH-induced ROS generation, lipid peroxidation and cell death

The inhibitory effects of AFFP on AAPH-induced ROS generation, lipid peroxidation and cell death were investigated using DCF-DA, DPPP and acridine orange dye in zebrafish embryos (Fig. 3). AFFP was found to reduce total ROS levels dose-dependently. Fluorescence intensity in the AAPH group was 201.46% versus the control group, but the AFFP/AAPH groups dose-dependently reduced fluorescence intensity (148.92%, 122.39%, and 107.18%, respectively). In the result of lipid peroxidation, the AAPH group showed a higher lipid peroxidation intensity of 164.23% than that of the control group. But lipid peroxidation intensities of the AFFP/AAPH group were significantly decreased compared with the AAPH group. In particular, the AFFP/AAPH group showed the strongest protective activity at 100 μg/ml concentration. In the result that dead cells in zebrafish are stained as red color by acridine orange, the control group had a clear image free of fluorescence, whereas the AAPH group exhibited to significantly increase cell death through the fluorescence intensity (145.62%). However, all AFFP/AAPH groups dramatically decreased cell deaths in a dose-dependent manner. Therefore, these results implied that AFFP provided strong protection against AAPH-induced ROS generation, lipid peroxidation and cell death in the zebrafish embryo.