Journal of Food and Nutrition Research, 2017, Vol. 5, No. 3, 156-159
Available online at http://pubs.sciepub.com/jfnr/5/3/3

DOI:10.12691/jfnr-5-3-3

Identification of Molecular Biomarkers for Food Quality Assessment of Oysters Exposed to Hypoxia

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Abstract Hypoxia, corresponding to a low oxygen concentration of less than 2.8 mg O_2/L (91.4 mM), may cause serious problems in marine environments. In this study, we applied differential display PCR and fatty acid analysis to investigate molecular biomarkers for assessing hypoxic effects using the oyster, *Crassostrea gigas*, as a model organism. Oysters were exposed to normoxic (7.6 mg O_2/L) or hypoxic (1.8 mg O_2/L) concentrations of dissolved oxygen for 2 days. We found that glutamine synthetase (GS) gene expression decreased and glutathione S-transferase (GST) gene expression increased in oysters exposed to hypoxia. In addition, linoleic acid content significantly decreased following hypoxic exposure compared with controls. Collectively, our findings indicate that GS and GST expression levels and linoleic acid content are potentially good biomarkers for analyzing the effects of hypoxia.

Keywords: *hypoxia, biomarkers, DD-PCR, fatty acid, oyster*

Cite This Article: So Yun Park, Jinik Hwang, Juyun Lee, Youngjae Chung, Donggiun Kim, and Taek-Kyun Lee, "Identification of Molecular Biomarkers for Food Quality Assessment of Oysters Exposed to Hypoxia." *Journal of Food and Nutrition Research*, vol. 5, no. 3 (2017): 156-159. doi: 10.12691/jfnr-5-3-3.

1. Introduction

There has been a recent increase in the use of marine organisms for the biological assessment of water quality, and such applications are continuing to grow in number. Biological approaches are recognized as indispensable for the rapid and simple assessment of the effects of various types of water pollution and for safeguarding the stability of marine ecosystems and human health [1].

Biomarkers—biological entities that exhibit a change in status in response to external influences—are useful indicators of the effects of pollution in the marine environment. Researchers can predict changes in a population or colony of organisms caused by various environmental factors by quantifying biochemical changes at cellular, organismal, and community levels [2]. Whereas conventional toxicity approaches study the impact of pollutants on organisms by administering contaminants of interest to an organism at different concentrations and observing their effects or by determining their lethal concentrations, biomarkers can be used to quantify the effects of pollution through molecular and physiological methods, and thus gauge the level of environmental stress—something that is not feasible with toxicity experiments. There is an extensive body of research on health assessment indexes that make use of biomarkers, encompassing the health assessment index, biomarker index, integrated biomarker response index, bio-nutritional assessment index, and the generalized linear model [3]. However, studies that have used biomarkers to assess the effects of exposure of marine organisms to environmental toxins have been limited to organisms that inhabit specific regions, especially fish and benthic invertebrates [4,5].

Previous studies showing the effects of hypoxia on marine organisms have primarily drawn on bio-behavioral experimental methods. For example, under hypoxic conditions, jellyfish, which are highly resistant to hypoxia, reduce their respiration [6]; fish increase their rate of gill movement [7]; and bivalves increase their respiration and reduce growth [8]. Studies using mammalian cells have shown that exposure to hypoxic conditions activates the transcription factor, hypoxia-inducible factor 1 (HIF-1), and that activated HIF-1 binds to hypoxia-response elements (HRE) in hundreds of gene promoters to induce the expression of genes that are required for adapting to hypoxic conditions [9]. Several studies have reported the effects of HIF-1 on gene expression under hypoxic conditions in fish species. Results obtained using the marine medaka (*Oryzias melastigma*) fish model suggest that HIF-1, activated under hypoxic conditions, induces an increase in the expression of telomerase reverse transcriptase (TERT) in the testes and the liver [10]. In addition, there is an increase in the expression of leptin receptor (LepR), which is associated with energy metabolism in the liver, heart, and gills of marine medaka exposed to hypoxia [11]. The identification of biomarkers that are sensitive to growth inhibition and endocrine disruption, both of which occur in marine organisms under

hypoxic conditions, is an active area of research that involves the identification of genes whose expression is up- or downregulated under hypoxic conditions, such as TERT and LepR. However, the molecular response of marine organisms to hypoxic stress is still poorly understood.

The aim of this study was to develop new biomarkers in oysters exposed to hypoxic stress using differential display PCR (DD-PCR) and the analysis of fatty acid content, and to use these markers to assess the health of marine organisms.

2. Materials and Methods

2.1. Oyster Culture and Exposure to Hypoxic Stress

Oysters used in these experiments belong to the species *Crassostrea gigas*, found along the entire coast of South Korea. Individual oysters were purchased from an oyster farm in Geoje. They were fed microalgae and cultured in a tank located in a farm building of the South Sea Institute. The natural seawater required for cultivation was filtered prior to use. After a 7-day acclimatization period, hypoxia exposure experiments were conducted in a hypoxia chamber designed to maintain a specified concentration of dissolved oxygen (Figure 1). The dissolved oxygen concentration was held at 7.6 mg O_2/L under control conditions and at 1.8 mg O_2/L during experimentally induced hypoxia.

Oysters were subjected to hypoxic stress in a chamber designed to maintain a constant concentration of dissolved oxygen using nitrogen flow unit (Aquacontroller pro base unit, Neptune, USA). The specified oxygen concentration was achieved by adjusting the flow of nitrogen through the chamber by means of an automatic, power-controlled regulator on the nitrogen tank. To identify genes whose expression was characteristically altered in oysters under hypoxic stress, we subjected oysters to experimental hypoxia for 6, 12, and 24 hours. All experiments were conducted using 5 oysters per experimental condition and time point. Oyster samples were collected at each time point, and DD-PCR was performed on each sample using 80 types of ACP primers. Genes whose expression varied with the length of exposure to hypoxic stress were identified, and confirmatory experiments were conducted prior to cloning into the TA vector for nucleotide sequencing.

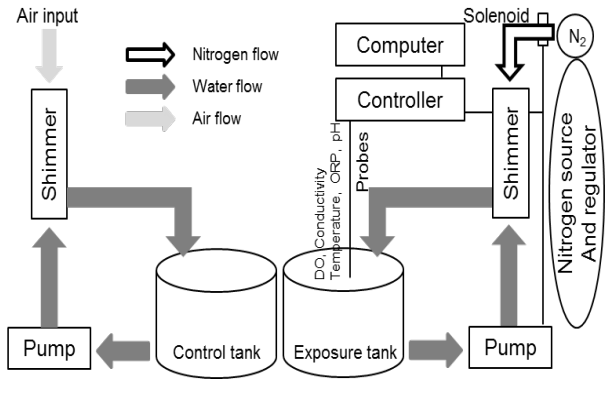

Figure 1. Schematic diagram of the hypoxia chamber

2.2. Oyster RNA Extraction and Differential Display PCR

As a first step toward detecting genes whose expression is characteristically altered under hypoxic conditions, total oyster RNA was extracted and reverse transcribed to cDNA. Differentially expressed genes (DEGs) were subsequently identified by regulating the annealing specificity of random PCR in the context of AC technology using the GeneFishing DEG kit (Seegene, Seoul, South Korea). Differential expression of identified genes was verified by comparing gene expression at different degrees of hypoxic stress.

2.3. Total Lipid Extraction and Fatty Acid Analysis

Lipid extraction was performed based on the method of Folch et al. [12]. In particular, 5 mL of $CHCl_3$:MeOH (2:1) was added to 5 g of pine needle powder and the sample was sonicated for 20 min. Then, 5 mL of 0.58% sodium chloride was added and the sample was sonicated for an additional 10 min. The sample was then centrifuged for 5 min at 3000 rpm, the upper layer was removed, and the lower layer was transferred to another tube using a Pasteur pipette, followed by drying with nitrogen gas. A known amount of heneicosanoic acid (C21:0) was used as an internal standard. Next, 0.5 mL of toluene and 2 mL of 0.5 N NaOH were added to the dried sample, which was then incubated for 5 min in a heated bath and then cooled. BF_3MeOH was then added, and the sample was heated in the bath for 3 min and cooled again. In the final step, 15 mL of petroleum ether and 20 mL of H_2O were added, the sample was sonicated, and the supernatant was isolated and dried using nitrogen gas. Lipid extraction followed the procedure described previously by Folch et al. [12]. Briefly, 0.5 mL of pellets obtained by centrifuging cultured oysters was mixed with 5 mL of chloroform:methanol (2:1) and sonicated twice for 20 min each; 5 mL of 0.58 % sodium chloride was then added and sonication was repeated for 10 min. The mixture was centrifuged at 2000–3000 rpm for 5 min, after which the upper fraction was removed and the liquid at the bottom was transferred to another tube with a Pasteur pipet and dried under nitrogen gas. A known quantity of heneicosanoic acid (C21:0) was used as an internal standard. The dried sample was dissolved in 0.5 mL of toluene and 2 mL of 0.5 N NaOH, and immersed in a cooling bath for 5 min. The solution was mixed with boron trifluoride-methanol (BF_3MeOH) and immersed in a cooling bath for 3 min. The solution was then mixed with 15 mL of petroleum ether and 20 mL of H_2O, sonicated, and allowed to stand to let the solid components settle. The supernatant was extracted and dried again under nitrogen gas.

The fatty acid content of oysters exposed to hypoxia was analyzed by following the method used by Suh et al. [13]. Fatty acid methyl esters (FAMEs) were analyzed with a Varian CP-3800 gas chromatograph equipped with a HP-Innowax silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas, and the retention times of fatty acids were compared to those of 37 standard fatty acid methyl esters

(FAME, Supelco). Fatty acids that accounted for 0.1 % or more of the total fatty acid content were determined and their respective percentages were recorded.

2.4. Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS). The normality and homogeneity of data were checked by analysis of variance (ANOVA), and differences between experimental groups were evaluated by one-way ANOVA and Duncan's multiple range test.

3. Results and Discussion

3.1. Genome Analysis of Oysters Exposed to Hypoxic Stress

Cloning and sequencing of genes identified by comparing gene expression under experimental conditions (1.8 mg dissolved O_2/L) and normoxic control conditions (7.6 mg dissolved O_2/L) showed that most differentially expressed genes encoded ribosomal proteins. In addition, expression of glutamine synthetase (GS, ACP-9) decreased at 24 h, and expression of glutathione S-transferase (GST, ACP-44) tended to increase with the duration of exposure to hypoxia (Figure 2, Table 1).

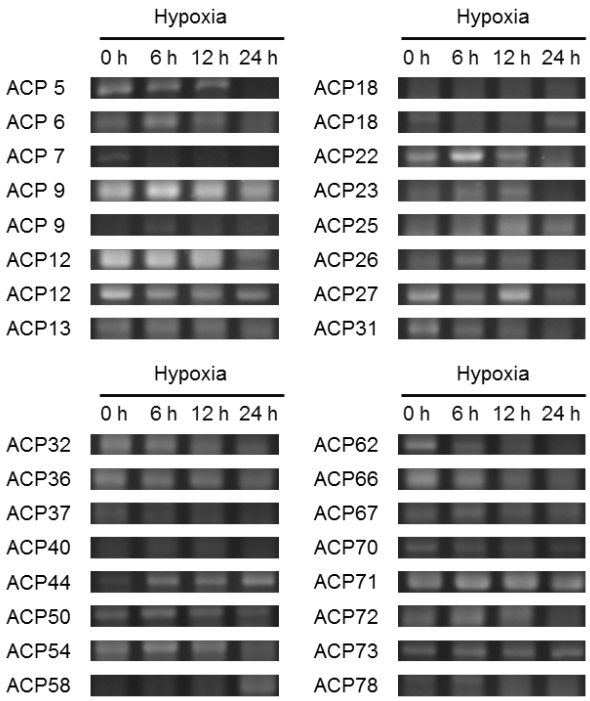

Figure 2. Expression of hypoxia-responsive genes

The decrease in GS expression is probably a response to oxidative stress induced by hypoxia [14,15] and arises in particular because the free radicals induced by hypoxia suppress the reaction between metal ions and GS, which is necessary for GS activity. The increase in GST expression was probably caused by increased intracellular antioxidant activity [16] and is likely closely associated with increased expression of antioxidant enzymes generally, which are induced to remove the free radicals generated by hypoxia.

Table 1. Oysters genes differentially expressed in response to hypoxic stress

ACP NO.	Gene	GenBank accession No.
ACP 7	Ribosomal proteins L12	CX069140
ACP 9	Glutamine synthetase	CG1753
ACP12	Ribosomal proteins L7	CX069138
ACP25	Ribosomal protein S3a	CF369245
ACP31	No similarity	-
ACP36	No similarity	-
ACP44	Glutathione S-transferase	CB617447
ACP58	No similarity	-
ACP62	Ribosomal protein S5	CB617370
ACP66	Ribosomal protein S4	CX069145
ACP72	No similarity	-

3.2. Fatty Acid Analysis of Oysters Exposed To Hypoxic Stress

An analysis of changes in oyster fatty acid content showed that, compared with controls, oysters under hypoxic conditions had a markedly higher content of stearic acid (C18:0), linoleic acid (C18:3n3), linolelaidic acid (C18:2n6t), arachidic acid (C20:0), γ-linolenic acid (C20:3n6), erucic acid (C22:1n9), and lignoceric acid (C24:0). In contrast, linoleic acid (C18:2n6c) tended to decrease with the duration of exposure to hypoxia (Figure 3).

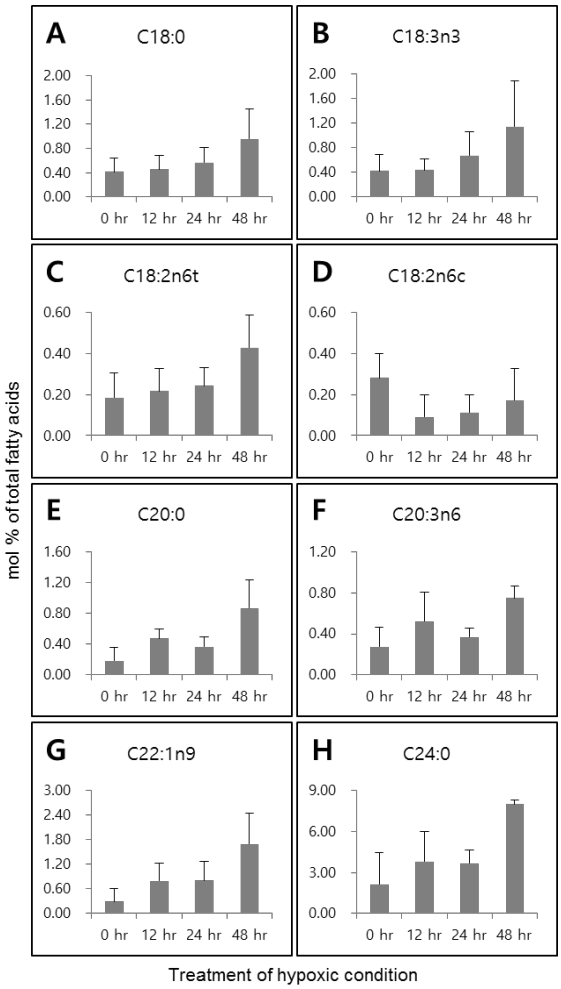

Figure 3. Changes in the content of major fatty acids in oysters exposed to hypoxia

Polyunsaturated fatty acids are essential, but are exogenous fatty acids that require biosynthesis from a precursor [17]. Polyunsaturated fatty acids affect the growth of larvae and spat of oysters and other bivalves. In the present study, oyster tissues exposed to hypoxia exhibited an increased content of key polyunsaturated fatty acids, but showed a marked decrease in linoleic acid. Linoleic acid has been reported to play a role in protecting cells against the decrease in mitochondrial and transport functions induced by hypoxia [18], and the decrease in linoleic acid content observed in the present study is likely associated with the formation of cis-epoxyoctadecenoic acids and dihydroxy octadecenoic acids [19], which are known to reduce the adverse effects of hypoxia on cells.

4. Conclusions

The results presented here are applicable to marine environmental risk assessments that rely on differential hypoxia biomarkers, and thus move beyond biological impact assessments based on basic ecological or contaminant bioaccumulation surveys. It is expected that our findings will be utilized in seawater and wastewater toxicity assessments and environmental assessments for special marine management areas. Considering the worldwide paucity of research on health assessments of marine organisms, this study provides valuable findings for live oyster farming by developing new biomarkers for hypoxic stress impact assessments based on DD-PCR and fatty acid content analysis.

Acknowledgements

This research was supported by the Public Welfare & Safety Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013M3A2A1067529).

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