Ocean Science Journal, Vol. 42 No. 2, 129-134(2007)

Article

Ocean Science Journal

http://osj.kordi.re.kr

Characterization of Proteins in the Muscle of *Limanda yokohamae* from the Masan Bay, Korea

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Received 21 May 2007; Revised 3 June 2007; Accepted 18 June 2007

Abstract – Increasing industrial development in the Masan Bay area of Korea over the past decades increased the risk for the survival of marine organisms in the bay area by the deterioration of the water quality. Since living organisms have the ability to adapt contamination-associated stimuli by the alteration of gene expression, changes in proteins can be used as an important criterion for assessing the levels of environmental conditions. In this study, therefore, alterations of the expression of proteins in the muscle of *Limanda yokohamae* from Dukdong and Dotsum in the bay area were surveyed and characterized as compared with Haegumgang, which served as a control site. The results demonstrated that the twenty spots detected from Dukdong and Dotsum were similar to each other. Fifteen proteins were found to be predicted or undefined proteins, while five proteins were identified as heavy polypeptide 11 of myosin, apolipoprotein A-I, fibroblast growth factor 17b precursor, G protein-coupled receptor kinase 1 b and bonnie and clyde. These data suggest that local fish in the bay area have dysfunction in muscle physiology including contraction, lipid metabolism, proliferation and differentiation and nervous system.

Key words – proteomics, environmental contamination, gene expression, Masan Bay, *Limanda yokohamae*

1. Introduction

The Masan Bay is located in the industrial area of the southern part of Korea. Increasing industrial development and population in the bay area over the past decades have increased the risks for pollution of the bay and deterioration of the water quality. A recent annual report from the Ministry of Maritime Affairs and Fisheries (MOMAF) (2006) of Korea shows that the high levels of environmental pollution in the bay area severely affects the maintenance of homeostasis among marine organisms in the bay area.

A variety of chemical pollutants, such as polychlorinated biphyls (PCBs), dichlorodipheyltrichloroethanes (DDTs), hexachlrocyclohexanes (HCHs), chloradene related compounds (CHLs), and polycyclic aromatic hydrocarbons (PAHs) in the sediments of the bay area also were detected in the bay area (Hong *et al.* 2003; Yim *et al.* 2005). These chemical pollutants are found to be implicated in many disease processes causing autophagy, apoptosis or necrosis in aquatic animals (Moore *et al.* 2006). Endocrine disrupting chemicals (EDCs) are representative pollutants in the bay area and are known to disturb hormone actions in fish (Goksoyr 2006), which causes the dysfunction of protein expression in mammals (Fenton 2006). The exposure of environmental chemical mixtures also induces neoplasms in various organs, including muscle in the Japanese medaka (Toussaint *et al.* 1999).

It is therefore hypothesized that fish in the bay undergo alterations of gene expression in response to the contaminated environment. In order to understand the biological effects of the bay conditions on marine organisms, this study was designed to characterize proteins in the coast-settled fish *Limanda yokohamae* (*L. yokohamae*) from the Masan Bay in Korea.

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2. Materials and Methods

Sample preparation

Adult *L. yokohamae* weighing 90-120 g were collected from Dotsum and Dukdong in the Masan Bay and from Haegumgang, which served as a control site, during the period from April to June 2005 (Fig. 1) (n=7-8 per site). Muscle of *L. yokohamae*, was immediately isolated, dissected and frozen in liquid nitrogen and then stored at -70 °C for 2-dimentional electrophoresis (2-DE). Muscle samples were homogenized in lysis buffer [7 M urea (Sigma-Aldrich, MO), 2 M thiourea (Sigma Aldrich), 4% (w/v) CHAPS (USB, OH), 0.5% (v/v) Pharmalytes pH 3-10 (Amersham Biosciences, NJ), 100 mM DTT (Amersham Biosciences), and 1.5 mg/mL complete Protease Inhibitor Cocktail (Sigma Aldrich)] and incubated for 1 hr at 4 °C while mildly shaking. The lysate was then centrifuged at 13,000 rpm for 30 min and supernatant was transferred to a fresh centrifugation tube. Protein concentration was determined by using a BIO-RAD Protein Assay Kit and samples were stored in aliquots at -80 °C.

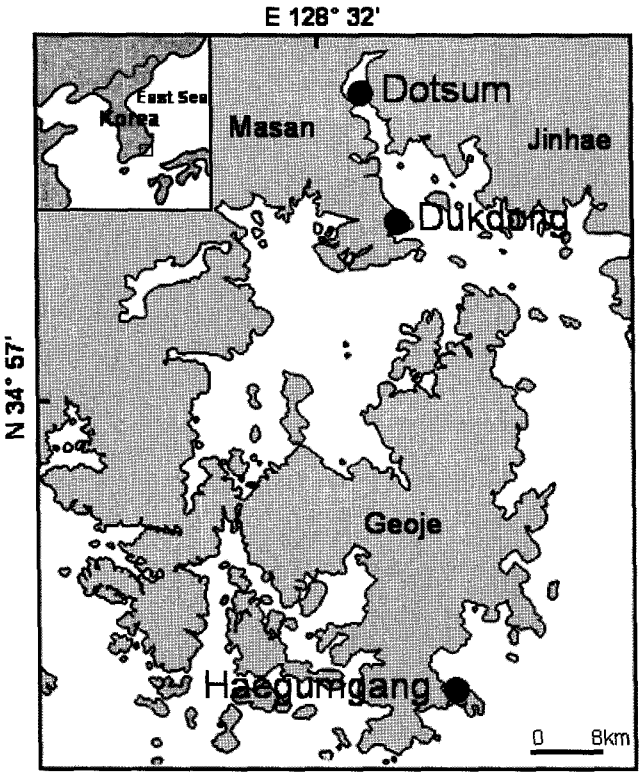

Fig. 1. Sampling sites of *L. yokohamae* from Dotsum and Dukdong in the Masan Bay and from Haegumgang in Geoje, which served as a control site.

2-DE analysis

18 cm Immobiline Dry Strips pH 3-10 (Amersham Biosciences) were rehydrated with DeStreak Rehydration Solution (Amersham Biosciences) with 0.5% IPG buffer (Amersham Biosciences) for 12 hr at room temperature. Rehydrated Strips were transferred to Ettan IPGphor System (Amersham Biosciences) and then were positioned sample cups with 500 μg protein samples. Isoelectric focusing (IEF) was carried out at Step and Hold mode at 500 V for 1 hr, Gradient mode at 1,000 V for 1 hr, Gradient mode at 8,000 V at 1 hr and Step and Hold mode at 8,000 V for 3 hr up to a total of 38,000 Vhr. The focused strips were equilibrated sodium dodecyl sulphate (SDS) equilibration solution [6 M urea, 30% (v/v) glycerol (Amersham Biosciences), 2% SDS, 50 mM Tris-HCl pH 8.8] with DTT and then idoacetamide (Amersham Biosciences). Equilibrated strips were placed on the top of 12.5% SDS-polyacrylamide gels (SDS-PAGE) and sealed with 0.5% (w/v) agarose (Promega Bioscience, WI). SDS-PAGE was separated using Ettan Dalt six system (Amersham Biosciences). For visualization, the separated gels were incubated overnight in fixing solution (45% methanol, 5% phosphoric acid) and then were stained with staining solution [0.1% (w/v) Coomassie brilliant blue G250 (Bio-Rad Laboratories, CA) (Neuhoff *et al.* 1988), 17% (w/v) ammonium sulfate, 3.6% phosphoric acid, 34% methanol] for 1 day. Over dyed gels were destained washing solution (1% acetic acid, 15% methanol).

Analysis of 2-DE gel images

The stained gels were scanned using ImageMaster Scanner (Amersham Biosciences), and then analyzed with ImageMaster 2D Platinum 6.0 software (GE healthcare) for spot detection, quantification, and matching automatically and manually. Protein spot abundance was determined by area of the spot multiplied by the density and referred to as the volume. Background was removed and spot volume was normalized to the total protein detected on each gel by program automatically. A 50% decreased or 50% increased difference in protein abundance was taken as showing a difference between two groups, Haegumgang and Dukdong or Dotsum. The difference was analyzed with student's t-test in which p values of <0.05 were considered the level of statistical significance.

Identification of proteins

Protein spots having difference as described above were subjected to mass spectrometry (MS) analysis for identification. Selected protein spots were excised from the gels and were destained with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate pH 8.0 three times for 15 min. The spots were dehydrated with 100% ACN. For in-gel digestion, the spots were rehydrated in 15 ml trypsin solution [10 μg/mL of sequencing-grade modified trypsin (Promega Biosciences, CA) in 25 mM ammonium bicarbonate

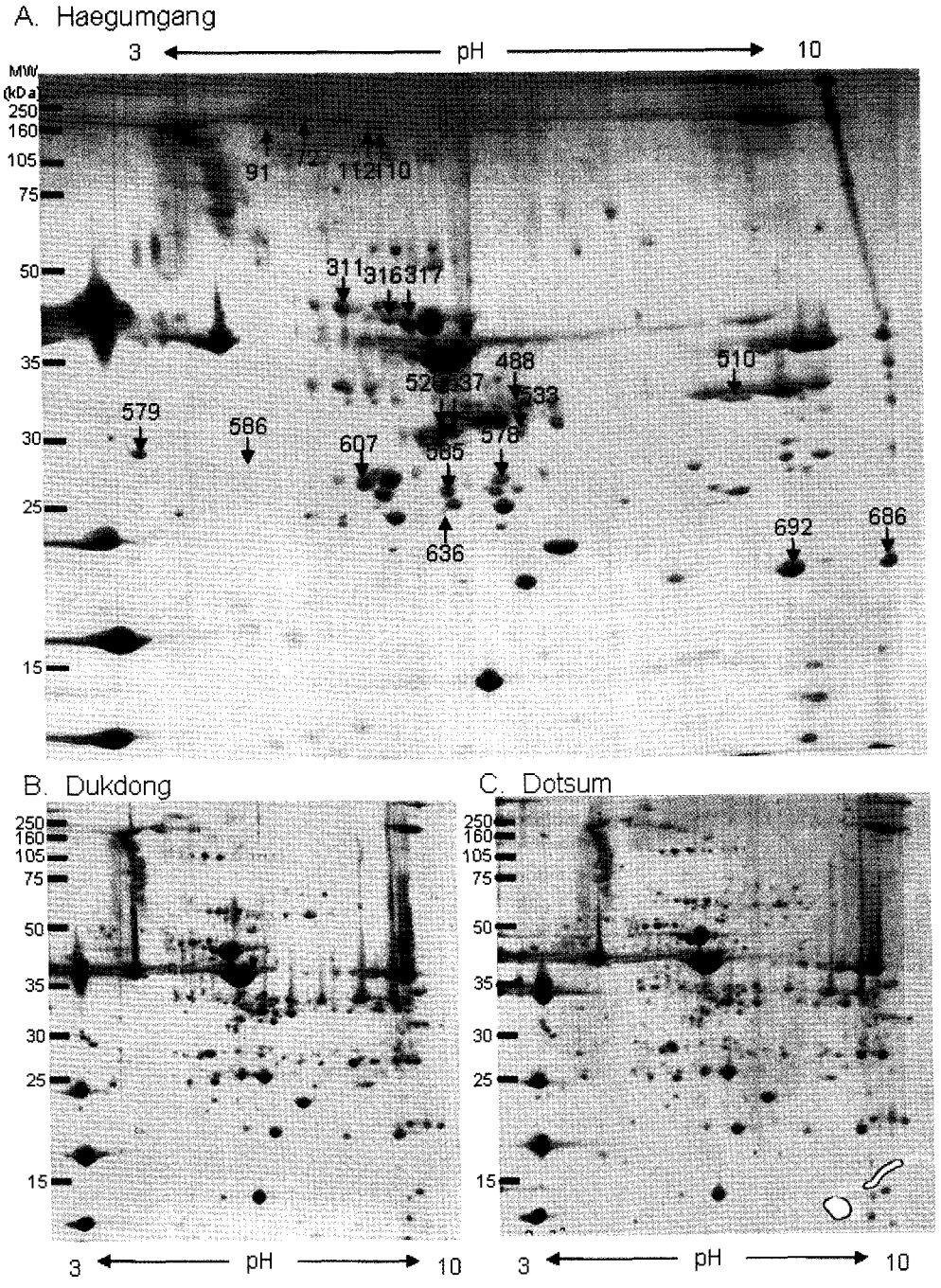

Fig. 2. 2-DE gel images in the muscle of *L. yokohamae* from Heagumgang (A), Dukdong (B) and Dotsum (C). The total proteins (500 μg) extracted from the muscle of *L. yokohamae* were separated on pH 3-10 IPG strip, electrophoresis on 12.5% SDS-PAGE gel and staining with colloidal coomassie blue G250. The numbered spots in the figure were excised for trypsin digestion and then identified using MALDI-TOF MS analysis.

pH 8.0] and were incubated at 37 °C overnight for digestion. The supernatants were collected and the peptides were extracted with 5% trifluoroacetic acid (TFA) in 50% ACN and dried with vacuum. The extracted peptides were desalted using ZipTips C18 resin (Millipore, MA) and eluted with matrix solution (α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA). Peptide mass spectra were acquired on Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, CA). Identified proteins were characterized using the web site http://www.ebi.uniprot.org/ or http://www.expasy.org/. In general, a mass tolerance of ± 50 ppm to ± 100 ppm, one missed trypsin cleavage, and fixed modification of carboxyamidomethyl cysteine were selected as matching parameters.

3. Results and Discussion

The protein spots detected on each gel varied from 900 to 1,000 (Fig. 2). Altered twenty protein spots were characterized in this study. The expression pattern of altered twenty proteins from Dukdong and Dotsum in the bay area were very similar to each other. The thirteen protein spots in Dukdong and Dotsum were significantly decreased, while seven protein spots were significantly increased as compared to Haegumgang (Table 1, Fig. 3).

The altered spots were analyzed for the identification of proteins using MS-Fit program with NCBI or SWISS PROT database in which zebra fish database is mainly used for the identification of proteins.

The results were shown in Table 2 and Fig. 3, in where the pattern of proteins in Dukdong and Dotsum in the bay area has close similarity to that of Haegumgang. Fifteen proteins identified in the bay area were found to be predicted or undefined proteins. While five proteins are identified as heavy polypeptide 11 of myosin, apolipoprotein A-I, fibroblast growth factor 17b precursor, G protein-coupled receptor kinase 1 b, and bonnie and clyde. The former three proteins were significantly decreased in

Table 1. Statistical analysis of altered spots in the muscle of *Limanda yokohamae* from the Masan Bay, Korea

Altered Spot No.	Ratio			Value			Relative		
	Hae	Duk	Dot	Hae	Duk	Dot	Hae	Duk	Dot
Decreased									
72	1	0.553261	0.418712	0.0768328	0.0425086	0.0321708	0	-0.0343242	-0.044662
110	1	0.352892	0.614854	0.071954	0.025392	0.0442412	0	-0.046562	-0.0277128
112	1	0.408021	0.580465	0.0488016	0.0199121	0.0283276	0	-0.0288895	-0.020474
488	1	0.38691	0.545738	0.119787	0.0463468	0.0653723	0	-0.0734402	-0.0544147
528	1	0.233285	0.363391	0.402024	0.0937863	0.146092	0	-0.308238	-0.255932
537	1	0.250848	0.313034	0.372241	0.0933759	0.116524	0	-0.278865	-0.255717
578	1	0.330351	0.336375	0.155793	0.0514663	0.0524048	0	-0.104327	-0.103388
579	1	0.61108	0.353646	0.227206	0.138841	0.0803506	0	-0.088365	-0.146855
585	1	0.310479	0.302718	0.163067	0.0506288	0.0493633	0	-0.112438	-0.113704
586	1	0.551195	0.547567	0.0391139	0.0215594	0.0214175	0	-0.0175545	-0.0176964
607	1	0.309353	0.346982	0.451698	0.139734	0.156731	0	-0.311964	-0.294967
686	1	0.281675	0.373828	0.418578	0.117903	0.156476	0	-0.300675	-0.262102
692	1	0.483235	0.530042	0.90616	0.437888	0.480303	0	-0.468272	-0.425857
Increased									
91	1	1.52979	0.990573	0.0445851	0.0682057	0.0441648	0	0.0236206	-0.0004203
311	1	1.4063	1.75182	0.0502474	0.0706628	0.0880242	0	0.0204154	0.0377768
316	1	1.82247	1.96033	0.155634	0.283639	0.305094	0	0.128005	0.14946
317	1	1.54941	1.7328	0.6173	0.95645	1.06966	0	0.33915	0.45236
510	1	2.48423	3.82311	0.0817882	0.203181	0.312685	0	0.121393	0.230897
533	1	3.23319	1.46594	0.0607886	0.196541	0.0891123	0	0.135752	0.0283237
636	1	13.1258	10.8623	0.0375025	0.492249	0.407365	0	0.454747	0.369862

Spot numbers are same as shown in Fig. 2. Ratio means this normalization divides all values by the central tendency and thus gives a ratio for all data. Value is raw spot value. Relative means this normalization places the central tendency values to 0. Hae, Haegumgang; Duk, Dukdong; Dot, Dotsum.

Table 2. Identification of proteins in the muscle of *Limanda yokohamae* from the Masan Bay, Korea

Altered Spot No.	Protein Name	MOWSE Score	Mr (kDa)/pI	Accession No.
Decreased				
72	myosin, heavy polypeptide 11, smooth muscle	4.47E+06	227/5.5	66773050 M
110	PREDICTED: similar to myosin phosphatase-Rho interacting protein isoform 2	140705	180/5.2	68357196
112	PREDICTED: similar to very large inducible GTPase 1, partial	16490	198/6.0	68441125
488	hypothetical protein LOC606499	13275	28/8.6	73611912 M
528	PREDICTED: similar to lectin C-type domain containing protein isoform 2	19410	28/6.1	68400244
537	PREDICTED: similar to 26S proteasome non-ATPase regulatory subunit 4 (26S proteasome regulatory subunit S5A) (Rpn10) (Multiubiquitin chain binding protein) (Antisecretory factor-1) (AF) (ASF)	12316	39/4.5	68357984
578	apolipoprotein A-I	16989	30/5.1	18858281 M
579	PREDICTED: hypothetical protein XP_693485	18466	28/5.2	68374131
585	Fibroblast growth factor 17b precursor (FGF-17b)	13789	25/10.7	Q6SJP8
586	PREDICTED: similar to Diacylglycerol kinase, zeta (Diglyceride kinase) (DGK-zeta) (DAG kinase zeta), partial	34567	30/5.2	68422942
607	PREDICTED: similar to Putative eukaryotic translation initiation factor 3 subunit (eIF-3)	40497	30/7.2	68356500
686	PREDICTED: similar to Mitochondrial ornithine transporter 1 (Solute carrier family 25, member 15)	10542	33/8.9	68359901
692	PREDICTED: similar to Zinc finger protein 180 (HHZ168), partial	71172	26/9.4	68439571
Increased				
91	novel protein similar to human talin 2 (TLN2)	55638	238/5.2	55962543
311	PREDICTED: similar to SWAP-70 protein isoform 2	25610	67/5.9	68389742
316	hypothetical protein LOC550243	13633	37/4.8	62955131 M
317	G protein-coupled receptor kinase 1 b	155052	63/6.4	62204631 M
510	novel protein	12397	27/5.2	56207319
533	Bonnie and clyde	14564	37/7.9	67678183 M
636	PREDICTED: similar to Myosin-1 (Myosin heavy chain D) (MHC D)	65150	33/6.0	68387108

Dukdong and Dotsum as compared with Haegumgang. In contrast, the latter two proteins were significantly increased in the bay area.

The spot number 72 was identified as a myosin heavy chain family protein found in smooth muscle in general which is crucial in the contractile apparatus. The spot number 578 was identified as apolipoprotein A-I, a major protein component of high density lipoprotein (HDL) that is relatively abundant in the plasma of mouse. The protein is known to bind to lipopolysaccharide or endotoxin, and plays a major role in the anti-endotoxin function of HDL (Ma *et al.* 2004). The spot number 585 was identified as fibroblast growth factor 17b precursor (FGF-17b), a family of growth factors involved in wound healing and regulation of cell proliferation, migration and differentiation (Ornitz *et al.* 2001). The spot number 317 was identified as G protein-coupled receptor kinase 1 b, which in general regulates a variety of G-protein-coupled receptor activities. Finally, the spot number 533 was identified as bonnie and clyde that encodes a Mix family homeodomain protein regulating the generation of endodermal precursors in zebra fish (Kikuchi *et al.* 2000). These data suggest that local fish in the bay area have dysfunction in muscle physiology including contraction, lipid metabolism, proliferation and differentiation. Since the protein families identified in this study are closely involved in physiological processes in response to environmental perturbations, these data may provide a useful criterion for assessing biological effects of marine environment in the Masan Bay of Korea.

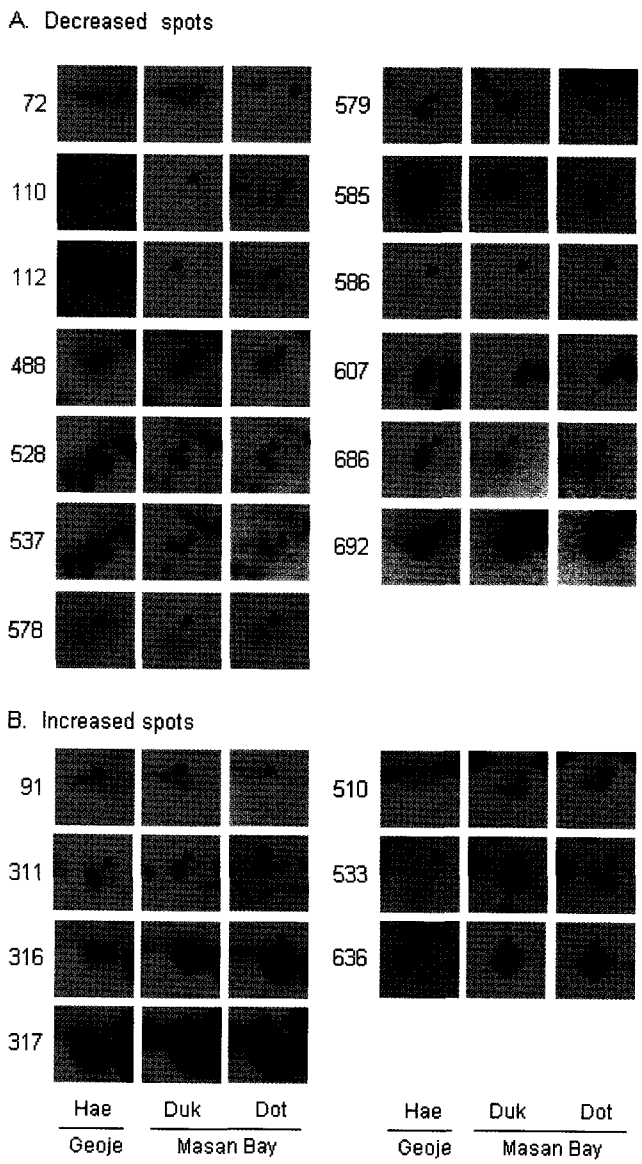

Fig. 3. Altered protein spots in the muscle of *L. yokohamae*. Each panel demonstrates enlarged images of gel spots in the Fig. 2. Hae, Haegumgang; Duk, Dukdong; Dot, Dotsum.

Acknowledgments

This work was supported for two years by Pusan National University Research Grant.

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