Form 3

Annual Report

LaMer, Ehime University

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To Director of LaMer

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Name in print MARIA CLARET TSUCHIYA

Include the report on the result of the project/meeting in a separate sheet.

1. Project / Meeting title

Proteome screening of pollutant biomarkers in tilapia and giant toads from the Philippines

2. Members of project / meeting

Name	Affiliation	Position	Contribution part
PI: Maria Claret Tsuchiya	University of the Philippines Los Banos	Assistant Professor	Research concept and experimental design, Conduct of experiment, Data analysis, Writing of report
Members: Charisse Leanne Legaspi	University of the Philippines Los Banos	Graduate Student (M1)	Conduct of experiment, Data analysis, Writing of report
LaMer Faculty member in charge: Hisato Iwata	CMES, Ehime University	Professor	Research concept and experimental design, Data analysis, Writing of report

3 Contents (separate sheets)

AIM

To date, there is still yet to be a study or a publication on a CYP profile of a species from the Philippines. Thus, the objective of this study is to determine the effect of environmental pollutants in Philippine local species by biochemical and proteome analysis. The specific objectives are to determine the CYP content and their activities in the liver microsomes of Philippine local species, and to analyze the hepatic proteome including CYPs by mass spectrometry-based proteomics approach. Findings in this study are the first reports on the toxicoproteomics of Philippine local species.

PROCEDURE

Sample collection Tilapia and giant toads were collected from February 22 – March 4, 2013 in Sta. Rosa and Los Banos, Laguna, and in Bilar, Bohol, Philippines. The animals were immediately dissected on board after measurement of biometry (body length, body weight, etc.). Organ (liver, spleen, muscle, and kidney) samples were removed and total organ weight was measured. The subsamples were frozen in liquid nitrogen, transported to Ehime University, Japan, and stored at – 80°C.

Preparation of microsomal fraction Microsomes was prepared as reported by Hirakawa et al (2007). Tissue (~6 g) from liver was homogenized in five volumes of cold homogenization buffer (50 M Tris-HCl, 0.15M KCl, pH 7.4–7.5) with a teflon-glass homogenizer (10 passes), and centrifuged for 10 min at 750 Å~ g. The supernatant will then be centrifuged at 12,000 Å~ g for 10 min. The supernate was further centrifuged at 105,000 g for 70 min. The supernatant (cytosol) fraction was removed, and microsomal pellets was resuspended in one volume of resuspension buffer [50mM Tris-HCl, 1mM EDTA, 1mM dithiothreitol, 20% (vol/vol) glycerol, pH 7.4–7.5]. All the microsomal fractions was immediately frozen in liquid nitrogen, and stored at – 80°C until further CYP enzyme assays and immunoblotting.

Protein concentrations in microsomal fractions was determined by the bicinchoninic acid method (Smith et al., 1985). BCA Protein Assay Reagent (Pierce, Rockford, IL) was used for the protein assay and bovine serum albumin as a standard. Absorbance at 560 nm was measured using a multiwell plate reader (SpectraFluor Plus, Tecan Austria GmbH, Groedig, Austria).

CYP contents and enzymatic activities The content of hepatic microsomal CYP was determined from dithionite difference spectra of CO-treated samples (Omura and Sato, 1964)with a spectrophotometer. Measurements of methoxyresorufin O-demethylase (MROD), EROD, pentoxyresorufin O-depenthylase (PROD) and benzyloxyresorufin O-debenzylase (BROD) in microsomal fractions was performed as described previously with slight modification (Iwata et al., 2002; Kubota et al., 2005, 2006). EROD activity was measured with 0.002mM substrate and 1.33mM NADPH concentrations using a multiwell plate reader (SpectraFluor Plus, Tecan Austria GmbH) at 37°C. Measurements of MROD, PROD, and BROD was perfomed similarly with 0.005mM substrate and 1.33mM NADPH concentrations. Resorufin formed by CYP enzymatic activity was excited at 535 nm wavelength and detected at 595 nm wavelengths.

Protein Digestion, iTRAQ® labeling, and Fractionation Protein solubilization and trypsin digestion was done using FASP Protein Digestion kit (Expedeon). Briefly, 100µg total protein lysate was mixed with urea sample solution in the spin filter and centrifuged at 14000g for 15 min. IAA solution was added, incubated in the dark at RT for 20 min, and centrifuged. The spin filter was added with urea sample and centrifuged. 75µl of digestion solution containing trypsin (enzyme to protein ratio 1:100) was added and incubated overnight with shaking at 37°C. 50mM ammonium bicarbonate was added to elute the digested peptides. The peptide mixture was desalted, and mixed with iTRAQ® reagents for 2h at RT. All labeled peptides was combined and fractionated using iCAT® cation exchange chromatograph.

Protein Identification by nano-LC-MALDI TOF/TOF MS and Homology search The labeled peptide mixture was desalted using C18 Spin Tips (Thermo Fisher Scientific, USA) and resuspended in 2% ACN, 0.1% TFA. 20ul was injected to the nano-LC for elution into MALDI sample plate with the matrix solution (10 mg/mL α-cyano-carboxycinnamic acid in 50% ACN/0.1% TFA) and then analysed on MALDI TOF/TOF mass spectrometer (5800 Plus MALDI TOF/TOF™, AbSciex). Peptide identification was analyzed using the Protein Pilot software against the Uniprot ORENI (*Oreochromis niloticus*) database.

RESULTS

Total Protein and Microsome Content

The total protein content and microsome content was measured using the BCA assay. Table 1 shows the total protein and microsome concentration of the liver samples obtained from the three sampling sites.

Table 1. Total protein and microsome concentrations in Nile tilapia liver from three sampling sites in the Philippines.

Content	Los Banos	Sta. Rosa	Bohol
Total Protein (µg/g liver)	21.3 ± 4.5	16.2 ± 1.56	13.4 ± 0.69
Microsome (µg/g liver)	9.28 ± 0.97	6.37 ± 0.49	4.96 ± 0.38

The sampling site with the highest total protein and microsome concentration was Los Baños (21.287 ± 4.531 µg/g liver and 9.280 ± 0.965 µg/g liver, respectively). Microsome content of the tilapia in the three sampling sites were significantly different from each other (p = 0.0003). Compared to the Bohol, the reference site, Los Banos had a 1.9x fold-increase while Sta Rosa showed 1.7x fold-increase. Meanwhile, total protein content did not show significant differences among the three sampling sites (p = 0.230). However, in reference to Bohol, total protein content was 1.6-fold increased in Los Banos and 1.2-fold increased in Sta. Rosa.

Cytochrome P450 Content

Cytochrome P450 content was measured using the classical method proposed by Omura and Sato (1964). This method uses the difference between the oxidized and reduced CYP450 monooxygenases. In the study, among the three sampling sites, Nile tilapia in Los Banos had the highest CYP450 content. Sta Rosa and Bohol CYP450 content showed an almost same CYP450 content (Fig 1). In various studies, high CYP450 content had been highly correlated with the occurrence of pollutants in the environment (Bachman et al. 2015; Bainy et al. 1999; Don et al. 2015). CYP450 is the major biochemical marker in pollution studies as its expression is induced by the presence of endogenous and exogenous chemicals and drugs.

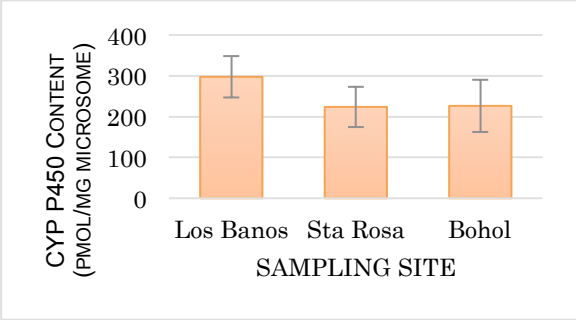

Figure 1. CYP450 content in Nile tilapia liver from three sampling sites in the Philippines.

Tilapia from Los Banos showed CYP450 content 1.3x higher than in Bohol. Bohol and Sta. Rosa samples did not have significant difference in each other (p = 0.420). Spectra from Sta. Rosa and Bohol yielded high 420nm reading thus giving a negative CYP450 content reading. The high 420nm reading was attributed by Guengerich et al. (2009) to either the high concentration of inactive and degraded CYP450 or through contamination of red blood cells which had a high absorbance at 420nm. Meanwhile, the high concentration of CYP450 content in the tilapia samples from Los Banos can be due to the presence of high polybrominated diphenylethers (PBDE). However, we still cannot fully establish this as a fact since it might be possible that the contaminants found in the each sampling sites can have antagonistic or synergistic effects on the organisms behavior towards these pollutants. The aquatic environment like the Laguna Lake receives daily substantial amounts of environmental pollutants that have the potential to cause oxidative stress in aquatic organisms through free radical and ROS mechanisms.

Nile Tilapia Liver proteome

All MS/MS spectra were processed using the ProteinPilot software. As shown in Table 2, iTRAQ analysis of zebrafish gill proteome showed 9260 queries in UNIPROT_ORENI database (27759 sequences) resulted in 867 protein hits (1591 proteins hits against all species database).

Table 2. Analysis of Nile tilapia liver proteome profile by iTRAQ.

Total Peptides	
ORENI UNIPROT database	43145
All Mammal Database	50134
Identified Peptides	
ORENI UNIPROT database	867
All Mammal Database	1591

Although 78.3% (679/867) were identified as uncharacterized proteins, further characterization of the uncharacterized proteins was done by checking the available annotated information such as molecular function, catalytic activity, Accession number, and Gene Ontology (GO) in UniProtKB. The identified proteins were categorized into diverse functional classes related to cellular process, metabolic process, oxidation-reduction process, biological regulation such as transcription and translation, and cellular component organization.

The differentially regulated proteins in each site are shown in Table 3. Upregulated proteins in male Nile tilapia included heat shock proteins, histones, aldehyde oxidase, ATP synthase and annexin. Thioredoxin was downregulated in the liver of tilapia in Sta Rosa site. Meanwhile, 16 proteins were significantly different between Los Banos and Sta. Rosa male Nile tilapia.

Among the 24 significantly regulated proteins in female tilapia, CYP2W1 was found to be upregulated in Los Banos Nile tilapia. CYP2J2 isoforms were identified but not significantly regulated. Glutathione S-transferase theta-2B were significantly downregulated in both Los Banos and Sta Rosa.

Table 3. Differentially regulated proteins in the liver of Nile tilapia from the Philippines.

Accession No.	Protein Name	Gene Name	Ratio	P-Value
LOS BANOS (MALE)				
Upregulated				
I3KY76	Histone H2A	HIST2H2AB	1.54	0.0366
I3KVL0	Histone H3	LOC106098523	1.41	0.0450
I3KL45	Uncharacterized protein	LOC100692314	1.69	0.0128
I3J7K9	Uncharacterized protein	LOC100695517	1.62	0.0366
I3JYR4	Uncharacterized protein	LOC100705870	2.37	0.0136
Downregulated				
I3KTT8	Uncharacterized protein	LOC100702802	0.08	0.0318
I3KDH3	Uncharacterized protein	fabp1	0.18	0.0021
I3K8S6	Uncharacterized protein	LOC100692070	0.23	0.0046
I3JK02	Uncharacterized protein	fasn	0.55	0.0016
I3IXL2	ATP-citrate synthase	acly	0.56	0.0055
I3JKE7	Uricase	LOC100711225	0.77	0.0367
STA. ROSA (MALE)				
Upregulated				
M1ZML5	Aldehyde oxidase beta		2.09	0.0491
I3JUY1	Annexin		1.71	0.0479
I3K1D1	ATP synthase subunit beta	LOC100693141	2.27	0.0423
I3JF31	Uncharacterized protein	hsp90ab1	1.18	0.0364
I3KHT2	Uncharacterized protein	LOC100534516	5.64	0.0013
I3K636	Uncharacterized protein	LOC106097863	2.95	0.0009
I3KQN5	Uncharacterized protein	scp2	1.82	0.0092
Downregulated				
I3K4K0	4-hydroxyphenylpyruvate dioxygenase	LOC100698438	0.59	0.0061
I3KBZ5	Galectin	LOC100702318	0.47	0.0222
I3J5R8	Protein disulfide-isomerase	LOC100704827	0.57	0.0151
I3K759	Pyruvate carboxylase	PC	0.64	0.0278
I3KH82	Ribosomal protein L19	rpl19	0.84	0.0468
I3K536	Thioredoxin	LOC100692805	0.52	0.0334
I3K5C3	Triosephosphate isomerase	tpi	0.71	0.0017
I3JKR2	Uncharacterized protein	LOC100699677	0.58	0.0363
I3KK43	Uncharacterized protein	PBLD	0.62	0.0382
I3KK90	Uncharacterized protein		0.53	0.0395
LOS BANOS (FEMALE)				
Upregulated				
I3KAY7	40S ribosomal protein S6	rps6	1.41	0.0072
I3JCK9	Uncharacterized protein	CYP2W1	1.43	0.0480
I3KAP1	Uncharacterized protein	LOC100705436	1.93	0.0149
Downregulated				
I3IXL2	ATP-citrate synthase	acly	0.62	0.0086
Q8UW60	Elongation factor 1-alpha	EF-1a	0.80	0.0058
I3KB31	Uncharacterized protein	bdh2	0.72	0.0287
I3JQS5	Uncharacterized protein	grhpr	0.55	0.0438
I3KP17	Uncharacterized protein	gstt2b	0.67	0.0160
STA. ROSA (FEMALE)				
Upregulated				
I3K1D1	ATP synthase subunit beta	LOC100693141	2.52	0.0470
I3JK02	Uncharacterized protein	fasn	3.12	0.0001
I3KSV3	Uncharacterized protein		2.93	0.0313
I3JUP5	Uncharacterized protein		2.64	0.0104
Downregulated				
I3KAY7	40S ribosomal protein S6	rps6	0.70	0.0341
I3KL67	Phosphoglycerate kinase	pgk1	0.61	0.0392
I3J5R8	Protein disulfide-isomerase	LOC100704827	0.46	0.0173
I3K759	Pyruvate carboxylase	PC	0.62	0.0085
I3K536	Thioredoxin	LOC100692805	0.57	0.0106
I3KDH3	Uncharacterized protein	fabp1	0.31	0.0007

I3KP17	Uncharacterized protein	gstt2b	0.55	0.0093
I3JIB1	Uncharacterized protein	LOC100707666	0.70	0.0450
I3KGB0	Uncharacterized protein	LOC100710204	0.73	0.0213
I3JCG5	Uncharacterized protein	LOC100710232	0.89	0.0469
I3KK43	Uncharacterized protein	PBLD	0.51	0.0377
I3JMZ2	Uncharacterized protein		0.74	0.0357

Between male and female Nile tilapia, transketolase and ATP-citrate synthase are both significantly upregulated in Sites 1 and 2. Transketolase, was also found to be upregulated in adult zebrafish exposed to BFRs (Wang et al, 2009). Transketolase is a key enzyme in pentose phosphate pathway, while ATP-citrate synthase is involved in cellular respiration and the production of the high-energy compound adenosine triphosphate (ATP) from glucose (Stryer, 1988). However, one enzyme (transaldolase) in reference site 3 is involved in carbohydrate metabolism but was found to be downregulated. Upregulation of trasketolase and ATP-citrate synthase may suggest that pollutants are present in Site 1 and 2 and may have activated hepatic glucose synthesis and gluconeogenesis.

Meanwhile, two proteins are commonly downregulated (RPL5 and thioredoxin) in both Site 1 and Site 3. RPL5 is a structural component of ribosome and plays a role in translation process. Thiroredoxin play an important role in the antioxidant defenses, either as a scavenger of ROS (Nordberg and Arnér, 2001) or co-factor of antioxidant enzymes (such as Prx) (Chae et al., 1998), and in reactivating proteins that have been damaged by oxidative insults (Fernando et al., 1992). Trx also selectively activates the DNA-binding site of a number of transcription factors. This interaction make this molecule a key regulator of pro-inflammatory cytokine expression, and an essential component in the initiation of the immune response, thus linking oxidative stress and the immune response.

Table 4. Differentially regulated proteins between male and female Nile tilapia in three sampling sites in the Philippines.

Accession	Protein Name	Gene Name	Ratio	P value
LOS BANOS				
I3K392	Transketolase	TK	1.44	0.0158
I3IXL2	ATP-citrate synthase	acly	1.30	0.0287
I3JPC8	Uncharacterized protein	hsp90b1	1.29	0.0065
I3JIB1	Uncharacterized protein	LOC100707666	1.24	0.0306
I3JQ64	Pyruvate dehydrogenase E1 component subunit alpha	LOC100705761	1.23	0.0471
I3KMQ8	Uncharacterized protein	LOC100695721	0.78	0.0299
I3KL67	Phosphoglycerate kinase	pgk1	0.74	0.0436
I3K1C7	Uncharacterized protein	LOC100692598	0.72	0.0146
I3KQD0	Alpha-amylase	LOC100701014	0.72	0.0413
I3KGB0	Uncharacterized protein	LOC100710204	0.69	0.0014
I3KSX1	Uncharacterized protein	RPL5	0.65	0.0156
I3JYR4	Uncharacterized protein	LOC100705870	0.60	0.0264
I3K536	Thioredoxin	LOC100692805	0.59	0.0340
STA ROSA				
I3J1Z5	Uncharacterized protein	fabp3	3.54	0.0001
I3KA28	Uncharacterized protein	rpl7	2.30	0.0301
I3JK02	Uncharacterized protein	fasn	1.99	0.0000
I3KSV3	Uncharacterized protein		1.94	0.0015
I3KI17	Uncharacterized protein	ssr1	1.93	0.0219
I3K392	Transketolase	TK	1.70	0.0001
I3K4T4	Peptidyl-prolyl cis-trans isomerase	ppib	1.58	0.0029
I3IXL2	ATP-citrate synthase	acly	1.57	0.0004
I3J5M6	Protein disulfide-isomerase	p4hb	1.47	0.0308
I3JF31	Uncharacterized protein	hsp90b1	0.92	0.0450
I3J232	Uncharacterized protein	LOC100703626	0.88	0.0358
I3KV66	Aconitate hydratase	aco1	0.84	0.0092
I3KGB1	Malate dehydrogenase	mdh1	0.84	0.0360
I3K759	Pyruvate carboxylase	PC	0.78	0.0007
I3KFV1	Uncharacterized protein	LOC100707972	0.77	0.0231

I3JVB3	Uncharacterized protein		0.74	0.0462
I3K7U3	Uncharacterized protein	vcp	0.73	0.0024
I3KHA1	Uncharacterized protein	LOC100711847	0.71	0.0492
I3IVP0	Alpha-1,4 glucan phosphorylase	pygl	0.71	0.0017
I3KP17	Uncharacterized protein	gstt2b	0.70	0.0068
I3K4Z4	Catalase	cat	0.69	0.0045
I3JMZ5	Uncharacterized protein	pck2	0.69	0.0317
I3J5R8	Protein disulfide-isomerase	LOC100704827	0.65	0.0262
I3JQN0	Uncharacterized protein	LOC100712095	0.53	0.0155
I3KHE7	Uncharacterized protein	LOC100698070	0.48	0.0076
I3KDH3	Uncharacterized protein	fabp1	0.45	0.0025
BOHOL				
I3J1Z5	Uncharacterized protein	fabp3	1.78	0.0014
I3KHL8	Isocitrate dehydrogenase [NADP]	idh1	1.66	0.0296
I3J175	Galectin	LGALS1	1.50	0.0230
I3IYZ1	Uncharacterized protein		1.39	0.0330
I3KB31	Uncharacterized protein	bdh2	0.88	0.0265
I3IYH3	Uncharacterized protein	ACTN4	0.87	0.0327
I3KAF1	Uncharacterized protein	hgd	0.87	0.0249
I3JEL2	Transaldolase	taldo1	0.86	0.0426
I3IZT7	Glycogen [starch] synthase	gys2	0.86	0.0061
I3KMV7	Uncharacterized protein	pah	0.82	0.0084
I3KSX1	Uncharacterized protein	RPL5	0.81	0.0315
I3KFV1	Uncharacterized protein	LOC100707972	0.80	0.0248
I3KRS2	Uncharacterized protein	prdx2	0.79	0.0391
I3J5R8	Protein disulfide-isomerase	LOC100704827	0.79	0.0159
I3KAP1	Uncharacterized protein	LOC100705436	0.76	0.0247
I3JUY1	Annexin		0.68	0.0294
I3K536	Thioredoxin	LOC100692805	0.66	0.0278
I3JMZ5	Uncharacterized protein	pck2	0.64	0.0418
I3KHE7	Uncharacterized protein	LOC100698070	0.47	0.0227

In this study, some proteins identified belong to a stress response domain in Nile tilapia. In general, proteins involved in carbohydrate, lipid metabolic processes, as well as in redox or antioxidant activity were identified and are potential biomarkers for Nile tilapia responses to chemical stress from pollutants in the environment.

FUTURE PERSPECTIVES

Pathway analysis and protein interaction among the differentially regulated proteins will elucidate more about the transduction process in the liver of Nile tilapia. Results from this study will be presented in conferences. Further studies to confirm the expression of proteins identified are recommended.

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