CHANGES IN RED TILAPIA (*Oreochromis sp.*) MOUTH MUCUS DURING MOUTHBROODING: A PROTEOMICS APPROACH

by

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TABLE OF CONTENTS

Acknowledgement	ii
Table of Contents	iii
List of Tables	vi
List of Figures	vii
List of Abbreviations	viii
Abstrak	x
Abstract	xi
CHAPTER 1 – INTRODUCTION	1
CHAPTER 2 - LITERATURE REVIEW	
2.1 Tilapia	3
2.2 Mouthbrooding	5
2.3 Fish Mucus	7
2.4 Biochemical Changes During Mouthbrooding	7
2.5 Extra-oral Survival of Mouthbrooding Embryos in Aquarium Water	9
2.6 Fish Mucus and Proteomics	9
2.7 Proteomics	11
2.8 Two-Dimensional Electrophoresis (2-DE)	12
2.9 Spots Visualization	13
2.10 Mass Spectrometry	14
2.11 MALDI-TOF and MALDI-TOF/TOF	15
CHAPTER 3 – MATERIALS AND MATHODS	
3.1 Fish Rearing and Observation	18

3.2 Mouth Mucus Collection 18

3.3 Sample Preparation 19

3.4 Protein Assay 19

3.5 Two-Dimensional Gel Electrophoresis 20

3.5.1 Rehydration 20

3.5.2 First-Dimensional Isoelectric Focusing 21

3.5.3 IPG Strip Equilibration 21

3.5.4 Second-Dimension Gel Electrophoresis 22

3.6 Gel Staining 23

3.6.1 Silver Staining 23

3.6.2 Coomassie Brilliant Blue Staining 23

3.7 Spots Analysis 24

3.8 In-gel Digestion 24

3.9 ZipTip Desalting 25

3.10 MALDI-TOF/TOF Analysis 26

3.11 Protein Identification 27

CHAPTER 4 – RESULTS

4.1 Fish Observation 28

4.1.1 Sex Determination 28

4.1.2 Mouthbrooding 30

4.2 Protein Concentration 30

4.3 2-DE Gel Optimization 31

4.4 2-DE Gel Maps of Mouth Mucus Proteins 36

4.5 Significant Differences in the Regulation of the 25 Spots 44

4.6 MALDI-TOF/TOF Analysis and Protein Identification 51

CHAPTER 5 – DISCUSSION

5.1 Parental versus Non-parental 57

5.1.1 Fish 57

5.1.2 Mucus Protein Concentration 57

5.2 Keratin Contamination 58

5.3 Multiple Protein Expression Forms 59

5.4 Fish Epithelial Cells and Secretory Mechanism 59

5.5 Actin 60

5.6 Hemoglobin beta-A chain and Alpha-2 globin 61

5.7 Anterior gradient-2-like protein 2 61

5.8 Alpha-1-antitrypsin 63

5.9 Glycolytic Enzymes 64

5.10 Keratin type IIE 66

5.11 Natural killer enhancing factor 67

5.12 Heat shock cognate 70 68

5.13 Proteasome (prosome, macropain) subunit, alpha type, 4 69

CHAPTER 6 – SUMMARY AND CONCLUSION 72

REFERENCES 74

APPENDICES

Appendix A: Mass Spectrometry Results 84

Appendix B: MASCOT PMF Results 109

Appendix C: MASCOT MS/MS Results 134

LIST OF TABLES

		Page
Table 2.1.	Sources for MS-based protein identification tools.	17
Table 4.1.	Protein concentration.	30
Table 4.2.	Zoom-in of the 25 chosen and confidently identified protein spots.	39
Table 4.3.	Based on two-tailed *t*-test with 4 degrees of freedom, n= 3, and *p*= 0.05. The critical values are -2.776 and 2.776. The significant region are *t*< -2.776 and *t*> 2.776.	44
Table 4.4.	Peptide mass fingerprinting (PMF) results and MS/MS results of down-regulated proteins in parental mouth mucus by MALDI-TOF/TOF and MASCOT.	52
Table 4.5.	PMF results and MS/MS results of up-regulated proteins in parental mouth mucus by MALDI-TOF/TOF and MASCOT.	53

LIST OF FIGURES

		Page
Figure 2.1.	Female red tilapia carrying fertilized eggs in mouth (mouthbrooding).	4
Figure 2.2.	Red tilapia.	5
Figure 4.1.	Genital papilla of male red tilapia.	29
Figure 4.2.	Genital papilla of female red tilapia.	29
Figure 4.3.	Optimizations of 2-DE gel from small gel to big gel. Both gels were loaded with 60 µg of protein concentration and stained with conventional silver stain.	31
Figure 4.4.	Gel A was loaded with 60 µg of protein concentration. Gel B was loaded with 100 µg of protein concentration. Gel C was loaded with 200 µg of protein concentration. All 3 gels were loaded with non-parental mouth mucus samples and stained with conventional silver stain. The higher the protein concentration loaded, the more spots can be visualized but the more overlapping spots can be seen (circles), the darker and bigger area of background disturbance (upper-left of the gels), and also more horizontal and vertical streaks.	33
Figure 4.5.	Gel A was stained with conventional silver stain. Gel B was stained with Vorum silver stain. Both gels were loaded with 60 µg of protein concentration. Higher sensitivity of Vorum silver stain allowed more spots to be visualized with clearer background of gel.	35
Figure 4.6.	2-DE gel maps of female non-parental mouth mucus (A) and female parental mouth mucus (B). A total of 60 µg of protein was separated by 2-DE using 17 cm, pH 3-10 NL IPG strip and 15% SDS-PAGE. The 2-DE gels were stained with Vorum silver stain and scanned by GS-800 calibrated densitometer (Bio-Rad) and protein spots were analyzed using PDQuest version 7.3.1 (Bio-Rad).	37
Figure 4.7.	2-DE gel map of female parental mouth mucus. Arrows show the 25 chosen and confidently identified spots with 3 down-regulated spots (D1-D3), and 22 up-regulated spots (U1-U22).	38
Figure 5.1.	Exocytosis, apocrine and holocrine mechanisms. (Modified from http://medinfo.ufl.edu/pa/chuck/summer/ handouts/images/glands.gif)	60

LIST OF ABBREVIATIONS

2-D	two-dimensional
2-DE	two-dimensional electrophoresis
A	Ampere
AGR-2	anterior gradient-2
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate
CID	Collision-induced dissociation
Da	Dalton
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ESI	electrospry ionization
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid
HSC	heat shock cognate
HSP	heat ahock protein
Hz	Hertz
IEF	isoelectric focusing
IPG	immobilized pH gradient
kDa	kilodalton
kV	kilovolt
L	liter
m	meter
M	molar
MALDI	matrix-assisted laser desorption/ionization
mg	miligram
MHC	major histocompatibility complex
mL	mililiter
mM	milimolar
mRNA	messenger ribonucleic acid
MS	mass spectrometry
m/z	mass-to-charge ratio
NCBInr	National Centre for Biotechnology Information non-redundant
ng	nanogram
nm	nanometer
ns	nanosecond
NKEF	natural killer enhancing factor

PAGE	polyacrylamide gel electrophoresis
PMF	peptide mass fingerprinting
ppm	part per million
PSMA	proteasome (prosome, macropain) subunit, alpha type
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
S/N	signal-to-noise ratio
TAP	transporter for antigen processing
TOF	time of flight
Tris	tris(hydroxymethyl)aminomethane
V	Volt
Vhr	Volt x hours
v/v	volume per volume
w/v	weight per volume
xg	times gravity
µg	microgram
µL	microliter

PERUBAHAN MUKUS DALAM MULUT TILAPIA MERAH (*Oreochromis sp.*) SEMASA PENGERAMAN MULUT: SATU PENDEKATAN PROTEOMIKS

ABSTRAK

Tilapia merah (*Oreochromis sp.*) adalah pengeram mulut keibuan yang memaparkan cara penjagaan anak yang maju dengan menyimpan telur/anak ikan dalam mulut. Pendekatan proteomiks digunakan untuk menyelidik perubahan proteome dalam mukus mulut semasa pengeraman mulut. Proteome mukus mulut bukan induk digunakan sebagai kawalan. Dengan PMF dan MS/MS, sejumlah 25 tompok telah dikenalpasti dengan yakin. Protein-protein yang telah dikenalpastikan seperti enzim glikolitik, keratin jenis II, HSC70, proteasome, dan NKEF mencadangkan bahawa terdapat keperluan bagi produksi tenaga yang lebih tinggi, pengantaraan tekanan, pemeliharaan protein, dan mekanisme pertahanan khusus pada induk tilapia semasa pengeraman mulut. Rumusannya, pendekatan proteomiks telah mendedahkan bahawa terdapat perubahan dalam proteome mukus mulut tilapia semasa pengeraman mulut dan protein-protein yang telah dikenalpastikan membekalkan pandangan yang menarik dalam proteome mukus mulut.

CHANGES IN RED TILAPIA (*Oreochromis sp.*) MOUTH MUCUS DURING MOUTHBROODING: A PROTEOMICS APPROACH

ABSTRACT

Red tilapia (*Oreochromis sp.*) is a maternal mouthbrooder displaying an advanced form of parental care by keeping the eggs/fries inside the mouth. Proteomics approach was used to investigate the changes of the proteome in the mouth mucus during mouthbrooding. Non-parental mouth mucus proteome was used as control. With PMF and MS/MS, a total of 25 protein spots were confidently identified. The identified proteins such as glycolytic enzymes, type II keratin, HSC70, proteasome, and NKEF suggests that there is a need for higher energy production, stress mediation, protein maintenance, and specific defence mechanism in parental tilapia during mouthbrooding. In summary, proteomics approach has revealed that there are changes in the tilapia mouth mucus proteome during mouthbrooding and the identified proteins provide interesting insights in mouth mucus proteome.

CHAPTER 1

INTRODUCTION

Mouthbrooding is a very interesting behavior in fish as the mouthbrooder makes a very big sacrifice by facing starvation and hypoxia to ensure the survival of its offspring (Okuda 2001; Grüter and Taborsky 2004; Östlund-Nilsson and Nilsson 2004; Corrie *et al.* 2008). Besides protecting the offspring from the outside world, mouthbrooding must have played very important roles that some fish species willing to make such a big trade off to evolve into a mouthbrooder. However, these important roles are not observable as mouthbrooding happens inside the mouth.

With proteomics approach, previously, a senior of mine had successfully revealed the epidermal mucus proteome of discus fish and the changes of protein composition in the epidermal mucus of parental discus fish. He also identified some up-regulated and uniquely expressed proteins in parental discus fish which provide useful insights on various aspects of cellular and molecular mechanisms in discus fish during parental-care stage.

During mouthbrooding, the mouth mucus is the main component that separates the parent and the offspring, and proteins are the functional biological compounds that can be found in the mouth mucus. Therefore, proteomics approach was used in this research to investigate the tilapia mouth mucus proteome in order to

understand what actually happen to the mouth mucus proteome during mouthbrooding. The objectives of this project are:

1. To map out the 2-DE gel map of tilapia mouth mucus proteome.
2. To compare the mouth mucus proteome of parental and non-parental tilapia using proteomics approach.
3. To identify interesting proteins during mouthbrooding.

CHAPTER 2

LITERATURE REVIEW

2.1 Tilapia

The word 'tilapia' is the common name for a group of cichlid fishes. The group consists of three genera, namely *Tilapia*, *Sarotherodon* and *Oreochromis* (Popma and Messer 1999). *Tilapia* species are substrate guarding cichlids, where they build nest and guard the fertilized eggs in the nest. *Sarotherodon* and *Oreochromis* species are mouthbrooders, where they carry fertilized eggs in their mouth (Figure 2.1) instead of guarding the eggs in the nest. *Sarotherodon* species are either paternal mouthbrooders or biparental mouthbrooders, that is either male or both male and female are mouthbrooders. All *Oreochromis* species are maternal mouthbrooders which only females practice mouthbrooding.

Figure 2.1. Female red tilapia carrying fertilized eggs in mouth (mouthbrooding).

Tilapia or more specifically *Oreochromis niloticus*, was one of the first fish species being cultured, dated more than 3,000 years ago by the ancient Egyptian (Popma and Messer 1999). Tilapia was chosen to be cultured due to their positive aquacultural characteristics. Tilapia is more tolerant to high salinity, high water temperature, low dissolved oxygen, and high ammonia concentration compare to other commonly farmed freshwater fish. Besides that, as an omnivore fish, tilapia feeds on a wide variety of natural food as well as artificial feeds. Tilapia also more resistant to viral, bacterial and parasitic infection than other cultured fish. Along with their large size, rapid growth rate, and mild flavour, tilapia becomes the most widely cultured fish in tropical and subtropical countries.

Red tilapia is genetic mutant. The first red tilapia is the Taiwanese red tilapia. It was produced in Taiwan in the late 1960s by crossing mutant reddish-orange female *Oreochromis mossambicus* with normal male *O. niloticus* (Popma and Messer

1999). Another red tilapia strain is the Florida red tilapia. It was produced in Florida in the 1970s by crossing a normal female *O. hornorum* (a black colored species) with a mutant reddish-yellow male *O. mossambicus* (Watanabe *et al.* 1989). The third red tilapia strain was produced in Israel by crossing mutant pink *O. niloticus* with wild *O. aureus* (Popma and Messer 1999). Figure 2.2 shows the look of a red tilapia.

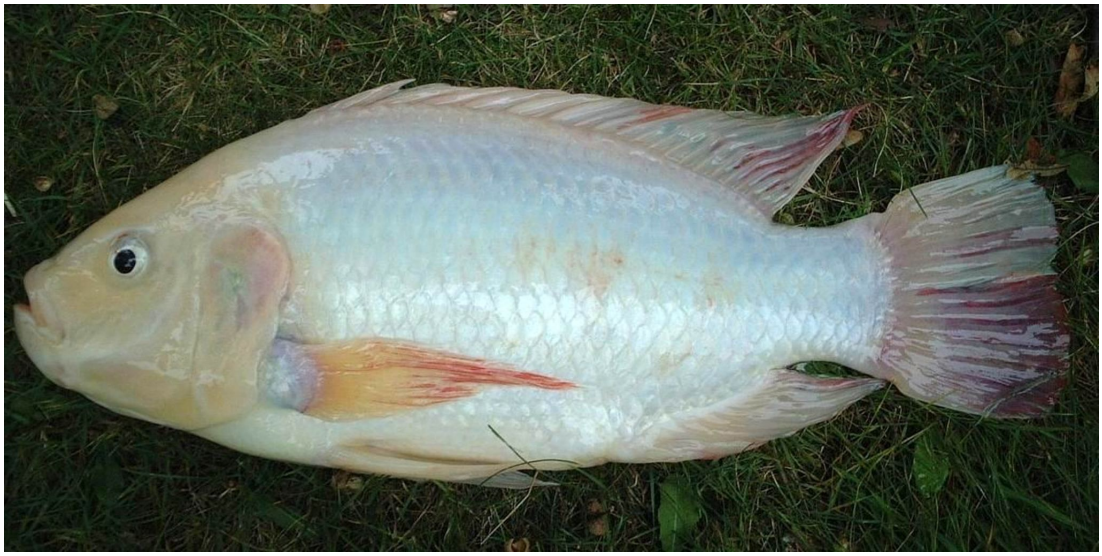

Figure 2.2. Red tilapia.

2.2 Mouthbrooding

Mouthbrooding is a form of parental care to ensure the survival of the young one by keeping the eggs/fries inside the mouth of the parents (Figure 2.1). Mouthbrooding is said to be evolved from ancestral substrate guarding, and uniparental mouthbrooding was arisen from biparental mouthbrooding (Goodwin 1998). Changes in the sex giving care from both male and female to only female allowed the male additional mating opportunities (Reynolds and Székely 1997).

During mouthbrooding, the egg clutch virtually fills the oral cavity of the mouthbrooder causing the mouthbrooder could not eat (Okuda 2001). Besides that, the mouth of a fish also constitutes an important part of the respiratory pump, forcing water over the gills. By having a mouth full of eggs, one would expect that this will severely limit the uptake of oxygen by the mouthbrooder during mouthbrooding (Okuda 2001; Grüter and Taborsky 2004; Östlund-Nilsson and Nilsson 2004; Corrie *et al.* 2008). Therefore, mouthbrooding must have played very important roles that some fish species willing to make such a big trade off to evolve into a mouthbrooder.

A research by Shaw and Aronson (1954) showed that embryos of the mouthbrooding *Sarotherodon macrocephala* cannot survive extra-orally in aquarium water taken from the tank where the spawning occurred (Shaw and Aronson 1954). They also found out that churning action of the mouth was one of the survival factors of embryos in mouthbrooding. Besides that, the research also showed that the fungus appeared on embryos raised extra-orally in aquarium was a secondary invader. In addition, they also noticed that there were special pharyngeal glands in the mouth of *S. macrocephala*. However, these glands are not present in immature *S. macrocephala* and other closely related species of non-mouthbrooding cichlids, such as *Tilapia sparrmani* and *T. zilli*.

All these findings show that the benefits of mouthbrooding not only provide protection to the eggs/fries from external danger but also as an important factor for the development of embryos. The disadvantages of mouthbrooding are the mouthbrooder has to suffer starvation and hypoxia. However, despite the disadvantages, mouthbrooding ensure the survival of the species.

2.3 Fish Mucus

Fish mucus is a slimy, slippery, clear fluid that consists of proteins, inorganic salts, and water. It covers every part of the fish, externally and internally. Fish mucus is known to be a highly multifunctional material by taking part in respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding, nest building, and protection (Shephard 1994). In mouthbrooding, the mouth mucus might play an important role as it is the main thing that separates eggs/fries and the parent. There is a high probability for the presence of some special proteins in the mouth mucus that aid the development of embryos and growth of fries. This is because not all types of fish perform mouthbrooding and mouthbrooding is an evolutionary advanced in fish world.

2.4 Biochemical Changes during Mouthbrooding

Changes in biochemical expression had been shown between non-parental and parental mouthbrooding tilapia. The plasma androgen and estradiol of black-chinned tilapia (*S. melanotheron*) were low during eggs mouthbrooding for both male and female mouthbrooders (Kishida and Specker 2000; Specker and Kishida 2000). When the eggs hatched, the levels of the sex steroids increased even though still mouthbrooding hatched embryos. The levels further increased until the fries were released. The elevated androgens on the male black-chinned tilapia on the day of releasing fries could reflect a very quick resumption of testicular activity so that they can mate again. Removal of eggs from mouthbrooding male black-chinned

tilapia eliminated the decrease of total androgen and estradiol (Kishida and Specker 2000). The females had significantly lower androgen and estradiol levels than pre-spawning females (Specker and Kishida 2000). *O. mossambicus* that exhibits maternal mouthbrooding also showed the similar changing pattern in sex steroid levels (Smith and Haley 1988). These studies showed that the presence of eggs inhibits the pituitary-gonadal axis in mouthbrooder and there might be a chemical signal from the eggs that delaying the initiation of the next brood.

Besides sex steroid, Kishida and Specker (2000) also showed significant changes in plasma vitellogenin levels during mouthbrooding of male black-chinned tilapia, with decreases occuring between eggs pickup and hatching of the embryo. However, the concentration of vitellogenin in the surface mucus during mouthbrooding peaked at the time of hatching (Kishida and Specker 2000). The amounts of vitellogenin in surface mucus of male *S. melanotheron* during and after mouthbrooding are strikingly similar to the amount measured in surface mucus of female *O. mossambicus* (Kishida and Specker 1994). The peak concentration of vitellogenin in the mucus of parent fish during mouthbrooding of hatched embryos raises the possibility of vitellogenin serve as nutrient for the fries. However, this is still an open question as the presence of vitallogenin in mucus might be contamination from burst eggs.

2.5 Extra-oral Survival of Mouthbrooding Embryos in Aquarium Water

Research conducted by Shaw and Aronson (1954) showed that there were no survival of embryos of *S. macrocephala* in aquarium water that removed from oral cavity prior to third day of development, which is about stage 19 (pectoral fin buds). Embryos that were removed from the mouth and placed in aquarium water during stage 1 (fertilized eggs) to stage 11 (neurula) did not develop beyond stage 19. When embryos were allowed to develop in the parent's mouth until stage 12 (head fold) to stage 15 (heart beat) before remove from the mouth and place in the aquarium water, the embryos survived until about stage 21 (iridiophores in eyes). Embryos removed from the mouth between stage 16 (closure of blastopore) and stage 19 sometimes survived through hatching (stage 24). Removal of embryos after stage 19 had higher extra-oral survival rates in aquarium water. This shows that mouthbrooding is necessary for the embryos of mouthbrooder to develop into fries.

2.6 **Fish Mucus and Proteomics**

In 2005, a series of research on discus fish epidermal mucus with proteomics approach had been conducted by Chong and co-workers. Discus fish demonstrating advanced parental-care behaviour by feeding fries with parental epidermal mucus. With 2-D electrophoresis, 2-DE gel map of epidermal mucus proteome of non-parental and parental discus fish were mapped out and compared (Chong *et al.* 2005). Spots of interest were excised for mass spectrometry analysis and these spots identified proteins such as fructose bisphosphate aldolase, nucleoside diphosphate

kinase, and heat shock proteins, which are essential to support energy provision, cell repair and proloferation, stress meditation, and defense mechanism in parental fish during parental-care period. Several antioxidant-related proteins such as thioredoxin peroxidase and hemopexin, were also been detected in discus fish epidermal mucus. The detection of antioxident-related proteins suggests a need to overcome oxidative stress during hypermucosal production in parental-care behaviour. C-type lectin was found to be uniquely express in parental epidermal mucus and could have important role in providing antimicrobial defense to both parental fish and fries. Besides that, they also discovered the presence of various free amino acids in discus fish epidermal mucus (Chong *et al.* 2005). Several essential amino acids such as lysin, isoleusine and phenylalanine were present at high levels in discus epidermal mucus. Besides as substrates for protein synthesis, free amino acids might provide a critical source of energy and nutrient to the developing fries with immature digestive system as compared to peptides and proteins but it is still remain to be studied.

The application of proteomics technologies onto discus fish epidermal mucus had showed that this approach can reveal an interesting insight on various aspects of cellular and molecular mechanisms in discus fish during parental-care stage. Therefore in this project, proteomics approach was used to investigate the tilapia mouth mucus proteome in order to understand the biological insight of mouthbrooding. The investigation was done onto the mouth mucus proteome because during mouthbrooding, the mouth mucus proteins are the functional biological compounds in between the parent and the offspring.

2.7 Proteomics

Proteomics is the study of proteome, in detailed, the qualitative and quantitative comparison of proteome under different conditions to further unravel biological processes. The genome of an organism is the same in every cell; is the same throughout a complete life cycle; and also the same under different environmental conditions. But the proteome of an organism is different in every cell; is different throughout a complete life cycle; and also different under different environmental conditions. Therefore, proteome analysis is required to determine which proteins have been conditionally express, how rapidly express, and whether any post-translational modifications are affected.

Even though the two-dimensional electrophoresis (2-DE) was invented in 1975 (O'Farrell 1975), but it is still the most commonly used technology to monitor changes in the expression of complex protein mixtures. Generally, after 2-DE, the 2-D gel will be analyzed by computer software to reveal the patterns of protein expression. Proteins of interest are then cut from the gel, enzymatically chopped into peptide fragments, and fed into mass spectrometer to generate mass fingerprint of the peptide fragments. Subsequently, the mass fingerprints will be compared to the information in a genomics database, such as NCBInr, to identify the corresponding DNA sequence in order to identify the proteins of interest.

2.8 Two-Dimensional Electrophoresis (2-DE)

With its high resolution and sensitivity, two-dimensional electrophoresis (2-DE) is a powerful tool for the analysis and detection of complex proteins mixtures (O'Farrell 1975). When first introduced in 1975, 2-DE had resolved 1100 proteins spots from *Escherichia coli* and should be capable of resolving a maximum of 5000 proteins (O'Farrell 1975). Even though the two-dimensional electrophoresis (2-DE) was invented in 1975 but it is still the most commonly used technology to monitor changes in the expression of complex protein mixtures. This technique separates protein according to isoelectric point of proteins by isoelectric focusing as first dimension and molecular weight of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as second dimension. With these two unrelated parameters, an almost uniform distribution of protein spots across a 2-D gel is possible to obtain. The reproducibility of the separation is sufficient to permit each spot on one separation to be matched with a spot on a different separation. By comparing the spot patterns on the 2-D gels from samples under different conditions, changes in individual proteins can be detected and quantified.

2.9 Spots Visualization

After electrophoresis, the protein spots and the gel are colourless. Therefore, the protein spots need to be stained to visualize the protein spot patterns. Among all the staining methods available, Coomassie Brilliant Blue (CBB) staining and silver staining are the two most widely used stains. CBB staining is the oldest and yet still the most popular method in protein visualization technique. CBB stain is low cost, easy to perform, shows good reproducibility, and most important, it is compatible with down-stream characterization method like mass spectrometry (Patton 2002). However, CBB stain is not very sensitive. The detection limit for conventional CBB stain is 30-100 ng (Simpson 2003). In order to increase the sensitivity, many modifications had been done onto the conventional CBB staining method, hence, form many types of new CBB staining methods like quick hot staining, fast colloidal Coomassie staining, sensitive stepwise colloidal staining, sensitive colloidal staining, "Blue Silver" staining, etc (Westermeier 2006).

Silver staining is up to 100 times more sensitive than CBB staining and has been used on 2-D gel. The detection limit for silver stain is 1-2 ng (Simpson 2003). Its high sensitivity is a big advantage when dealing with limited sample or sample with low protein concentration yield. However, its high sensitivity is also a disadvantage of silver staining that silver staining can stain contaminants, like keratin and chlorine, even in trace amount. Therefore, highly pure water should be used in every step from sample preparation until silver staining. The gel-to-gel reproducibility of silver staining is poor with certain proteins stain poorly, negatively or even not at all (Görg 2000). Variations of 20% in spot intensity have been

documented (Quadroni and James 1999). This is due to its complex, multiple-steps procedures that some steps are time critical. Another drawback of conventional silver staining is its incompatibility with mass spectrometry (Lilley *et al.* 2001; Mortz *et al.* 2001; Patton 2002). This is due to the used of high concentration of glutaraldehyde in conventional silver staining, which cross-links the proteins in the gel (Simpson 2003). Extensive cross-linking impairs protease activity during in-gel digestion, hence, impair mass spectrometry analysis. To make silver staining compatible with mass spectrometry, modifications had been done onto the conventional silver staining by either omitting glutaraldehyde or replacing glutaraldehyde with small amount of formaldehyde. In 2001, Mortz and co-worker had demonstrated three silver staining methods (EMBL silver staining, Blum silver staining, and Vorum silver staining) that are optimized for staining sensitivity and mass spectrometry compatibility.

2.10 Mass Spectrometry

Traditionally, protein analysis was done by *de novo* sequencing. Today, this technique tends to be replaced by mass spectrometry (MS). This is due to a 100-fold increase in sensitivity and 10-fold increase in speed in the latter technique. The availability of gene and genome sequence databases and technical and conceptual advances in many areas has made MS-based proteomics possible. A mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (m/z) of the ionized analytes, and a detector that registers the number of ions at each m/z value (Aebersold and Mann 2003). The most commonly used

techniques to volatize and ionize the proteins or peptides for mass spectrometric analysis are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). ESI ionizes the analytes out of a solution and is readily coupled to liquid-based separation tools (Fann *et al.* 1989), while MALDI sublimates and ionizes the analytes out of a dry, crystalline matrix via laser pulses (Karas *et al.* 1987; Karas and Hillenkamp 1988). ESI-MS is normally used for analysis of complex samples, whereas MALDI-MS is normally used to analyze simple peptide mixtures. There are four basic types of mass analyzer used in MS-based proteomics research. They are ion trap, time-of-flight (TOF), quadrupole, and Fourier transform ion cyclotron resonance mass spectrometer (Aebersold and Mann 2003). While having their own strength and weakness, these analyzers can be stand alone or put together in tandem to take advantage of the strength of each. Usually, MALDI is coupled to TOF analyzer and the MALDI-TOF is the most widely used technique in protein analysis.

2.11 MALDI-TOF and MALDI-TOF/TOF

The MALDI technique is based upon an ultraviolet absorbing matrix pioneered by Karas and coworkers in 1980s (Karas *et al.* 1987; Karas and Hillenkamp 1988). Usually, MALDI is coupled to TOF analyzer and due to its simplicity, excellent mass accuracy, high resolution and sensitivity, MALDI-TOF is the most widely used technique in protein analysis, especially to identify proteins by peptide mass fingerprinting (PMF). In PMF, proteins are identified by matching a list of experimental peptide masses (MS-generated) with the calculated list of all peptide

masses of each entry in a database. Table 2.1 shows the sources for MS-based protein identification tools. PMF requires an essentially purified target protein, therefore MALDI-TOF is commonly used in conjunction with prior protein fractionation using gel electrophoresis.

The sequencing capability of the MALDI method makes protein identification by MALDI-MS/MS more specific than those obtained by PMF from MALDI-MS. MALDI-TOF/TOF, a new configuration of ion source and mass analyzers has found wide application for protein analysis. The MALDI ion source is coupled to two TOF analyzers to allow the fragmentation of precursor ions. The two TOF analyzers are separated by a collision cell (Medzihradszky *et al.* 2000). MALDI-generated ions of a particular m/z are selected in the first TOF analyzer, fragmented in a collision cell and the fragment ion masses are 'read out' by the second TOF analyzer. MALDI-TOF/TOF has high sensitivity, resolution and mass accuracy. The resulting fragment ion spectra are often more extensive and informative.

Table 2.1. Sources for MS-based protein identification tools.

Sponsor (application)	Uniform Resource Locator (URL)
Matrix Science (Mascot)	http://www.matrixscience.com
Swiss Institute of Bioinformatics (ExPASy)	http://www.expasy.ch/tools
European Molecular Biology Laboratory (PeptideSearch)	http://www.mann.emblheidelberg.de
University of California (MS-Tag, MS-Fit, MS-Seq)	http://prospector.ucsf.edu
University of Washington (SEQUEST)	http://thompson.mbt.washington.edu/sequest
Institute for Systems Biology (COMET)	http://www.systemsbiology.org
Rockefeller University (PepFrag, ProFound)	http://prowl.rockefeller.edu
Eidgenossische Technische Hochschule (MassSearch)	http://cbrg.inf.ethz.ch
Human Genome Research Center (MOWSE)	http://www.seqnet.dl.ac.uk

CHAPTER 3

MATERIALS AND METHODS

3.1 Fish Rearing and Observation

Five red tilapias (four females and one male) were raised in a 1.5 m x 3 m x 0.7 m tank (W x D x H) containing ±1575 L of water at the Freshwater Aquaculture Research Complex, Universiti Sains Malaysia. Three tanks were used as replicates. The conditions were with flow-through fresh water at natural temperature (±32°C) under natural photoperiod. Fishes were fed with food pellet twice a day. All the fishes and food pellet were provided by the Fish Nutrition and Feeding Management team of University Sains Malaysia. Fish observation was done every morning during feeding time at around 10:00am. Female parental fish would not eat during mouthbrooding. The day when the female parental fish stop eating was counted as the first day of mouthbrooding.

3.2 Mouth Mucus Collection

Mouth mucus collection was done on the ninth day of mouthbrooding, where the yoke sack of the fries had fully absorbed. Mouth mucus of the female parental fish and female non-parental fish were collected using labeled pasture pipette and

inserted into labeled microcentrifuge tubes at 4°C. Female non-parental fish was randomly chosen from the same tank as of the female parental fish.

3.3 Sample Preparation

Collected mucus samples were centrifuged at 15,000 xg, 4°C for 20 minutes. The resulting supernatant was collected and proceeded with acetone precipitation. Briefly, ice-cold acetone (pre-chilled in -20°C for overnight) was added into samples with 4 acetone:1 sample (v/v). Then, the mixtures were incubated in -20°C for 2 hours. After that, the mixtures were centrifuged at 15,000 xg, 4°C for 10 minutes. The resulting pellet was dissolved in rehydration buffer [8 M urea, 50 mM DTT, 4% CHAPS, 0.2% ampholyte 3/10 (Bio-Rad, Hercules, CA, USA), 0.0002% bromophenol blue and deionized distilled water].

3.4 Protein Assay

Protein concentration was determined using RC DC protein assay kit (Bio-Rad). Briefly, 1 DC Reagent S:50 DC Reagent A (v/v) was prepared and labeled as Reagent A for later use. Each protein standard and samples assayed will require 127 µL of Reagent A. Three to five dilutions of a protein standard from 0.2 mg/mL to 1.5 mg/mL protein were prepared. After that, 25 µL of protein standards and samples were pipetted into separated labeled microcentrifuge tubes. Then, 125 µL of RC Reagent I was added into each tube and vortexed. The tubes were then incubated for

1 minute at room temperature. Subsequently, 125 µL RC Reagent II was added into each tube and vortexed. The tubes were then centrifuged at 15,000 xg for 5 minutes at 4°C. The supernatants were discarded and 125 µL RC Reagent I was added into each tube containing the pellet and vortexed. The tubes were then incubated for 1 minute at room temperature. After that, 40 µL RC Reagent II was added into each tube and vortexed. Then, the tubes were centrifuged at 15,000 xg for 5 minutes at 4°C. The supernatants were discarded and 127 µL Reagent A prepared earlier was added into each tube containing the pellet and vortexed. The tubes were then incubated at room temperature for 5 minutes. Subsequently, the tubes were vortexed and 1 mL of DC Reagent B was added into each tube and vortexed. The tubes were then incubated for 15 minutes at room temperature. After that, absorbance was read at 655 nm using Model 680 Microplate Reader (Bio-Rad). A standard curve was plotted from the absorbances of protein standards using Microsoft Excel. Protein concentrations of the samples were calculated from the standard curve.

3.5 Two-Dimensional Gel Electrophoresis

3.5.1 Rehydration

For analytical gels, 17 cm, pH 3-10 NL ReadyStrip IPG strip (Bio-Rad) was passive rehydrated with 300 µL of rehydration buffer containing 60 µg of protein in rehydration tray. IPG strip was overlaid with 3 mL of mineral oil to prevent evaporation during rehydration process. Then the rehydration tray was covered with the plastic lid provided and left sitting on a level bench for 16 hours. Whereas for

gels used for mass spectrometry, 3 mg of protein sample in 300 μL of rehydration buffer was loaded.

3.5.2 First-Dimensional Isoelectric Focusing

Precut electrode wicks (Bio-Rad) were placed on wire electrodes at both ends of the focusing tray. After rehydration, rehydrated IPG strip was held vertically with a forceps for about 10 seconds and the tip of the strip was blotted on a piece of filter paper to allow the mineral oil to drain. Then, the strip was transferred to the corresponding channel in the focusing tray. The IPG strip was overlaid with 3 mL of fresh mineral oil. Mineral oil was also applied to each wire electrode of unused lane to create a constant voltage supply to each lane. Isoelectric focusing (IEF) was carried out using PROTEAN IEF cell (Bio-Rad) at 250 V, 20 minutes, followed by 10,000 V, 2.5 hours and 10,000 V, 40,000 Vhr, 4 hours.

3.5.3 IPG Strip Equilibration

After IEF separation, focused IPG strip was removed from the focusing tray and placed into a clean equilibration tray with gel side facing upward. The focused IPG strip was then equilibrated with first equilibration solution [6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT] for 15 minutes with gentle shaking. After the first equilibration, the first equilibration solution was drained off. This was followed by second equilibration with second equilibration

solution [6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide] for 15 minutes with gentle shaking. Equilibrated strip was then washed with running buffer prior placing them onto 15% SDS-PAGE gel.

3.5.4 Second-Dimension Gel Electrophoresis

Prior 2-DE, 15% SDS-PAGE gel was cast and left standing overnight for better polymerization. After the strip was placed onto 15% SDS-PAGE, the strip was overlaid with 1% agarose gel with trace amount of bromophenol blue and left standing for 10 minutes for the agarose to solidify. The agarose gel was to hold the strips in place and the bromophenol blue in it acts as front dye to monitor the electrophoresis progress. Second-dimension was performed using PROTEAN II XL vertical electrophoresis system (Bio-Rad) with PowerPac Basic power supply (Bio-Rad) at constant ampere of 16 A per gel for 30 minutes followed by 24 A per gel until the tracking dye reached the end of the gel. Precision Plus Protein standards (Bio-Rad) was used as protein ladder.

3.6 Gel Staining

3.6.1 Silver Staining

Analytical gels were stained by Vorum silver stain (Mortz *et al.* 2001). Briefly, gels were fixed in fixing solution (50% methanol, 12% acetic acid, 0.05% formalin) for overnight. Fixed gels were washed 3 times in 35% ethanol for 20 minutes. Then, the gels were sensitized in sensitizing solution (0.02% sodium thiosulfate) for 2 minutes. Sensitized gels were washed 3 times in deionized distilled water for 3 minutes. Then, the gels were stained in staining solution (0.2% silver nitrate, 0.076% formalin) for 20 minutes. Stained gels were washed twice in deionized distilled water for 1 minute. Then the gels were developed in developing solution (6% sodium carbonate, 0.05% formalin, 0.0004% sodium thiosulfate) and subsequently stopped in sopping solution (50% methanol, 12% acetic acid) for 5 minutes.

3.6.2 Coomassie Brilliant Blue Staining

Gels used for mass spectrometry were stained by Coomassie Brilliant blue stain with slight modification. Briefly, gels were fixed in fixing solution (50% methanol, 10% acetic acid) for 2 hours. Fixed gels were stained in staining solution [0.1% (w/v) Coomassie Brilliant blue R-250, 10% acetic acid] for 4 hours. Then, the gels were destained in destaining solution (10% acetic acid) until the gels' background was clear.

3.7 Spots Analysis

Six sets of silver stained gels were scanned using GS-800 calibrated densitometer (Bio-Rad) and protein spots were analyzed using PDQuest version 7.3.1 (Bio-Rad). Briefly, each gel was analyzed for spot detection, background subtraction and protein spot OD intensity quantification. One of the non-parental gels was selected as a master gel and used for automatic alignment and matching of spots in other gels. The master and the aligned gels create a match set. To refine automatic spot matching, mismatched spots were corrected manually. Statistical significance was evaluated using the two-tailed t-test ($p < 0.05$) for the comparisons of the mean of the spot intensities ± standard error mean, where the spot intensities were obtained using the PDQuest software and the statistical analysis was done using Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA). Spots that are either up-regulated or down-regulated significantly were further process for protein identified.

3.8 In-gel Digestion

This protocol was provided by the Protein and Proteomics Center, National University of Singapore. Spots of interest were excised from Coomassie blue stained gels with new-clean scalpel blades and transferred to clean labeled 200 μL PCR tubes. Gel pieces were coarsely ground up using new-clean pipette tips. Ground gel pieces were destained 3 times with 100 μL of 50 mM ammonium bicarbonate/50% (v/v) acetonitrile for 5 minutes and subsequently dehydrated 3 times with 50 μL

acetonitrile for 5 minutes. Then, gel pieces were thoroughly dried using vacuum centrifuge.

The dried gel pieces were rehydrated with digestion solution [12.5 ng/µL trypsin (Promega, Medison, WI, USA) in 50 mM ammonium bicarbonate solution] at 4°C for 30 minutes. Then, all excess digestion solution were removed and 15 µL of 50 mM ammonium bicarbonate solution was added and incubated overnight (6-15 hours) in an oven at 37°C. After incubation, the gel pieces were allowed to cool to room temperature and then centrifuged at 3300 xg for 10 minutes to extract the peptides. Supernatant extracts were pipetted out and save in a new-clean labeled 200 µL PCR tubes. The pellets were treated with 15 µL of 20 mM ammonium bicarbonate and let stand for 10 minutes before centrifuged at 3300 xg for 10 minutes. The supernatant extracts were pipetted out and mixed with the previous supernatant extracts. The pellets were then treated with 15 µL of 5% formic acid in 50% aqueous acetonitrile and let stand for 10 minutes before centrifuged at 3300 xg for 10 minutes. Supernatant extracts were pipetted out and mixed with the previous extract mixtures. The extract mixtures were dried thoroughly using vacuum centrifuge.

3.9 ZipTip Desalting

This protocol was provided by the Protein and Proteomics Center, National University of Singapore. Dried extracts were re-dissolved in 10 µL of 0.5% formic acid and subsequently desalted using ZipTip C18 (Millipore, Bedford, MA, USA).